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Transfer of photosynthetic NADP⁺/NADPH recycling activity to a porous metal oxide for highly specific, electrochemically-driven organic synthesis†

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In a discovery of the transfer of chloroplast biosynthesis activity to an inorganic material, ferredoxin–NADP⁺ reductase (FNR), the pivotal redox flavoenzyme of photosynthetic CO₂ assimilation, binds tightly within the pores of indium tin oxide (ITO) to produce an electrode for direct studies of the redox chemistry of the FAD active site, and fast, reversible and diffusion-controlled interconversion of NADP⁺ and NADPH in solution. The dynamic electrochemical properties of FNR and NADP(H) are thus revealed in a special way that enables facile coupling of selective, enzyme-catalysed organic synthesis to a controllable power source, as demonstrated by efficient synthesis of L-glutamate from 2-oxoglutarate and NH₄⁺.

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Introduction

The small flavoenzyme (45 kDa) ferredoxin–NADP⁺ reductase (FNR) is the pivotal link between photosynthetic electron transfer and biosynthesis.^{1,2} Located in the stromal space of chloroplasts, FNR receives the energetic electrons dispensed from photosystem I *via* ferredoxin, a strongly reducing one-electron carrier protein, and catalyses the two-electron reduction of NADP⁺ to NADPH, thereby recycling the unique reducing agent for the Calvin cycle that assimilates CO₂. Many other biosynthetic processes depend on NADP⁺/NADPH recycling, for which there is considerable technological interest in achieving a wide range of stereo- and regio-specific organic reactions using enzymes – ease, efficiency and kinetic performance being key considerations.

As shown in Fig. 1A, electron transfer between ferredoxin and FNR to produce NADPH is essentially an electrochemical process, the essence of which might be realized at a suitable electrode material, thereby creating an ‘electrochemical leaf’. Fig. 1B and C show the structure of FNR in its complex with ferredoxin: NADP⁺ has been incorporated using data from another structure of FNR containing bound NADP⁺, thus forming the ternary complex implicated by kinetic studies.³ The interaction between FNR and ferredoxin arises mainly from

electrostatic attractions: ferredoxin is a negatively charged protein with a large excess of acidic amino acids, and the contact surface of FNR is dominated by basic residues. The shortest distance between the [2Fe–2S] cluster of ferredoxin and the FAD group of FNR is approximately 6 Å, which allows rapid, direct electron transfer.³

Guided by the natural interaction with ferredoxin, we investigated porous conducting metal oxides as candidates for the incorporation of FNR to reveal, directly, the electrocatalytic properties of the FAD cofactor, and yield a robust material for rapid cofactor recycling. Indium tin oxide (ITO), manufactured on a large scale for electronic displays, has been extensively used as an electrode for protein electrochemistry,^{6–8} and with an isoelectric point around pH 6,⁹ its surface should be negatively charged in the range pH 6–9 useful for enzyme-catalysed organic synthesis. In this article, we describe the remarkable electrocatalytic activity of FNR when immobilized on and within porous ITO: the discovery leads to the reversible electrochemistry of NADP⁺/NADPH and potentially limitless opportunities, either for selective synthesis or sensors, through coupling to enzyme-catalysed organic reactions that use this cofactor system.

Results and discussion

FNR under non-turnover conditions

Fig. 2A shows a scanning electron microscope (SEM) image of an ITO electrode, constructed by electrophoretically depositing ITO nanoparticles onto an ITO glass slide (see Experimental methods). The individual ITO particle sizes were estimated to

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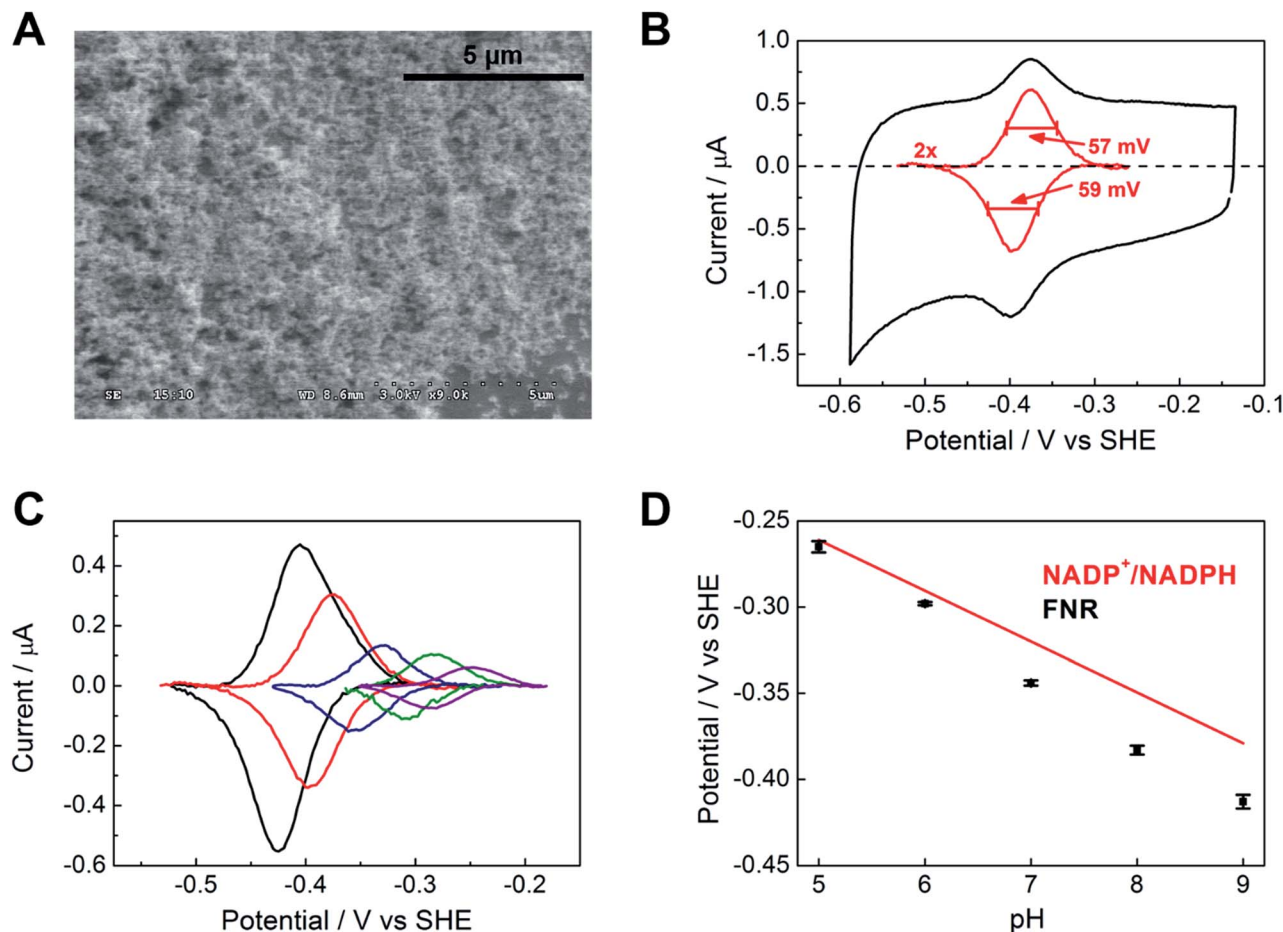


Fig. 2 Electrochemistry of ferredoxin-NADP⁺ reductase (FNR) immobilized at an ITO electrode, demonstrating rapid and reversible inter-conversion of oxidized and reduced forms. (A) SEM image of an ITO electrode made by electrophoretic deposition of ITO particles onto an ITO glass slide at 9000 \times magnification. Scale bar is indicated as a black line. (B) Cyclic voltammogram of FNR (black line) and background-subtracted trace (red line, magnified 2 \times) at pH 8.0. The label indicates the peak width at half maximum height. (C) Background-subtracted signals from pH 5.0 (right) to 9.0 (left). (D) pH dependence of the reduction potential of FNR compared to the pH dependence of the reduction potential of the NADP⁺/NADPH couple (red). Other conditions: 25 mM MES (2-(*N*-morpholino)ethane-sulfonic acid), 25 mM TAPS (tris(hydroxymethyl)methylaminopropanesulfonic acid), 20 $^{\circ}$ C, scan rate 5 mV s⁻¹, stationary electrode, solution purged with Ar before measurements.

The diameter of FNR is approximately 6 nm, therefore an ideal monolayer coverage assuming a planar surface would be approximately 5 pmol cm⁻². The high coverage of FNR, more than 100 monolayer equivalents, must be due to the porous nature of the ITO electrode,^{13,14} as confirmed by electron microscopy (see Fig. 2A). When the electrode was transferred to a solution at lower pH, the coverage decreased but was largely restored upon returning to pH 9. In contrast, when FNR was initially applied at pH 5, the low coverage did not increase when the electrode was transferred to a solution of higher pH. The electrochemical homogeneity is particularly remarkable, recalling that 45 mV is the minimum half-height peak value were the FAD to undergo a fully cooperative 2-electron reaction ($E_2 \gg E_1$).

From the results of the pH-step experiment, we conclude that binding of FNR within the pores involves: (i) general permeation that is more favourable at high pH, (ii) localized binding in an electroactive configuration that is also stronger at high pH. Lowering the pH therefore causes some enzyme molecules to become more loosely bound, but most remain buried in the

pores, so that restoring the high pH condition causes the still-entrapped enzyme molecules to re-bind.

Fig. 2D compares the average (two-electron) reduction potential of FNR, obtained from cyclic voltammograms recorded in the pH range 5–9, with the reduction potential of the NADP⁺/NADPH couple. The latter value is -0.32 V vs. SHE at pH 7, with a slope of -30 mV per pH unit.¹⁵ In this pH range, the potential of FNR is always more negative than that of NADP⁺, the difference increasing with pH; therefore FNR is biased slightly to favour NADP⁺ reduction, especially in the chloroplast stroma where the pH is approximately 8. As long known, the two-electron potential of FAD in the enzyme is much more negative than in the free state (-0.22 V at pH 7.0);¹⁶ accordingly, we also verified that free FAD has a reduction potential of -0.25 V at pH 8.0.

Reversible, diffusion-controlled NADP⁺/NADPH conversion by FNR@ITO

Fig. 3A shows the electrochemical activity of a stationary FNR@ITO/PGE electrode in the presence of NADP⁺ at pH 8.0.



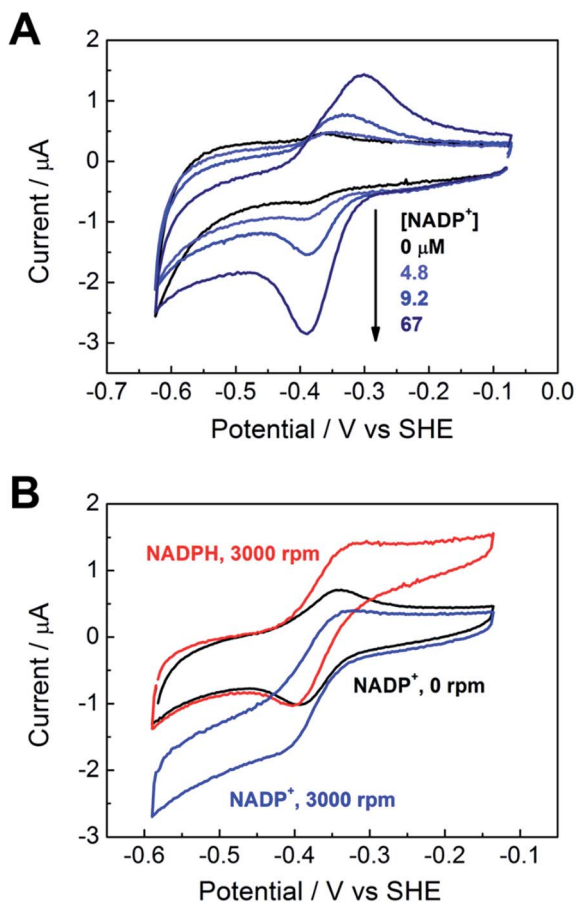


Fig. 3 Cyclic voltammetry of NADP⁺/NADPH. (A) Diffusion-controlled, quasi-reversible interconversion of NADP⁺ and NADPH at a stationary FNR@ITO/PGE electrode, with increasing amounts of NADP⁺, scan rate 5 mV s⁻¹. (B) Effect of electrode rotation at scan rate 1 mV s⁻¹: stationary electrode with 50 μM NADP⁺ (black) and under 3000 rpm rotation with 50 μM NADP⁺ (blue) or 50 μM NADPH (red). Other conditions: buffer solution containing 25 mM MES, 25 mM TAPS, pH 8.0, 20 °C, purged with Ar before measurement.

Upon adding NADP⁺ to the cell, an enhanced reduction current is observed, and on the return scan, the resulting NADPH is oxidized. The result proves that FNR adsorbed at the ITO electrode is highly active for cofactor regeneration in both directions. Scan rate dependence measurements (ESI[†]) showed that the peak current varies linearly with the square root of scan rate, which is diagnostic for diffusion-controlled interconversion of NADP⁺ and NADPH. A diffusion coefficient of $4.2 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ at 20 °C was calculated for NADP⁺, well within the range reported^{17–20} for NADH and NADPH in aqueous solution, which is $2.0\text{--}6.7 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. The diffusion coefficients for NADH and NADPH in water should be similar.¹⁸ The Michaelis-Menten constant (K_m) for NADP⁺, calculated from the reduction current at each NADP⁺ concentration, was $39 \pm 15 \text{ μM}$ at -0.375 V vs. SHE , close to the range ($7\text{--}30 \text{ μM}$)^{5,21} obtained by solution measurements using reduced ferredoxin.

A rotating disc FNR@ITO/PGE electrode was used to measure NADP⁺/NADPH interconversion under hydrodynamic control. In Fig. 3B, a fully sigmoidal curve for reduction of 50

μM NADP⁺ was only obtained at a high rotation rate (3000 rpm) and a slow scan rate (1 mV s⁻¹). At slower rotation and/or faster scan rates, NADP⁺ reduction showed a small peak-like feature. Likewise, if the cell solution contained only NADPH (also 50 μM), a sigmoidal oxidation wave became clear at 3000 rpm and 1 mV s⁻¹. Interconversion between NADP⁺ and NADPH is clearly fast and reversible. Currents for NADP⁺ reduction over the range -0.51 to -0.61 V vs. SHE gave linear Levich plots over the rotation rate range 400–3000 rpm (*i.e.* the current was proportional to the square root of the scan rate). The diffusion coefficient was always lower than obtained from cyclic voltammetry, and the origins of this difference, which may relate to surface geometry and uniformity of enzyme coverage, are under investigation.

The turnover frequency (TOF) of FNR, defined as moles of NADPH produced per mole of total electroactive FNR per second, is obtainable from the steady-state catalytic reduction current if the number of electroactive enzyme molecules is known (*e.g.* from the area of a non-turnover peak).¹⁰ An immediate observation was that the TOF with 50 μM NADP⁺ was typically 0.4 s^{-1} at pH = 8.0, which is three orders of magnitude lower than expected from steady state experiments.^{5,22} The discrepancy could be explained as follows, using the fact that the electrochemical reduction of NADP⁺ is controlled by diffusion through the bulk aqueous solution. In this situation, only those FNR molecules bound closest to the solution interface will contribute to catalysis, and buried FNR molecules could be completely redundant. Assuming, instead, that the amount of FNR accessible to free nicotinamide is equivalent to (at most) one monolayer, this leads to a revised TOF of at least 40 s^{-1} . In support of this model, a shoulder is sometimes clearly visible on the oxidative scan, located negative of the oxidation peak for NADPH (ESI[†]). From its position, the shoulder coincides with the FNR oxidative non-turnover peak, and it may be assigned to FNR molecules that are buried and thus redundant in regard to catalytic activity. The shoulder fades in comparison to the main peak as the scan rate is decreased (since slow conditions favour oxidation of diffusing NADPH) and is not visible on the reductive scan because its position is more negative than the NADP⁺ reduction potential, thus it is masked under the NADP⁺ reduction peak. Redundant FNR likewise accounts, at least in part, for the faint, peak features appearing during electrode rotation. Ferredoxin–NADP⁺ reductase thus joins an increasing number of enzymes displaying *reversible* electrocatalysis.²³ Most notably, the large, soluble domain of mitochondrial complex I attached to a PGE electrode shows reversible electrocatalysis of NAD⁺/NADH interconversion, but the current is too small and short-lived to be useful.²⁴

Coupling FNR@ITO to stereo-/regio-selective organic synthesis

We proceeded to exploit the parallel between the activity of FNR in the chloroplast, and at the FNR@MO/support electrode, aptly named the “Electrochemical Leaf”, as shown in Fig. 1A. In the biological leaf, FNR receives electrons from the light harvesting photosensitizer apparatus *via* ferredoxin to reduce NADP⁺ to



NADPH, which is used by downstream enzymes for CO₂ assimilation. In the electrochemical leaf, NADP⁺ reduction is driven by a small overpotential, and the electrode can be coupled to an NADPH-dependent enzyme of choice. The system can also be driven in reverse, *i.e.* to oxidize NADPH and regenerate NADP⁺, in contrast to the natural case.

As a test case, we coupled FNR@ITO to glutamate dehydrogenase (GLDH), which catalyses the conversion of 2-oxoglutarate (also known as α -ketoglutarate) to L-glutamate in the

presence of NH₄⁺ and NADPH.²⁵ Strong coupling of GLDH to NADPH regeneration is directly observable by voltammetry as shown in Fig. 4A.

First, an FNR@ITO/PGE working electrode (area 0.09 cm²) was prepared as described in the previous section, then NADP⁺ was added to the cell solution (final concentration 50 μ M) to obtain the expected NADP⁺ reduction and NADPH oxidation currents. The substrates for GLDH were already present (10 mM (NH₄)₂SO₄, 10 mM 2-oxoglutarate). Additions of GLDH (0.4–6.0

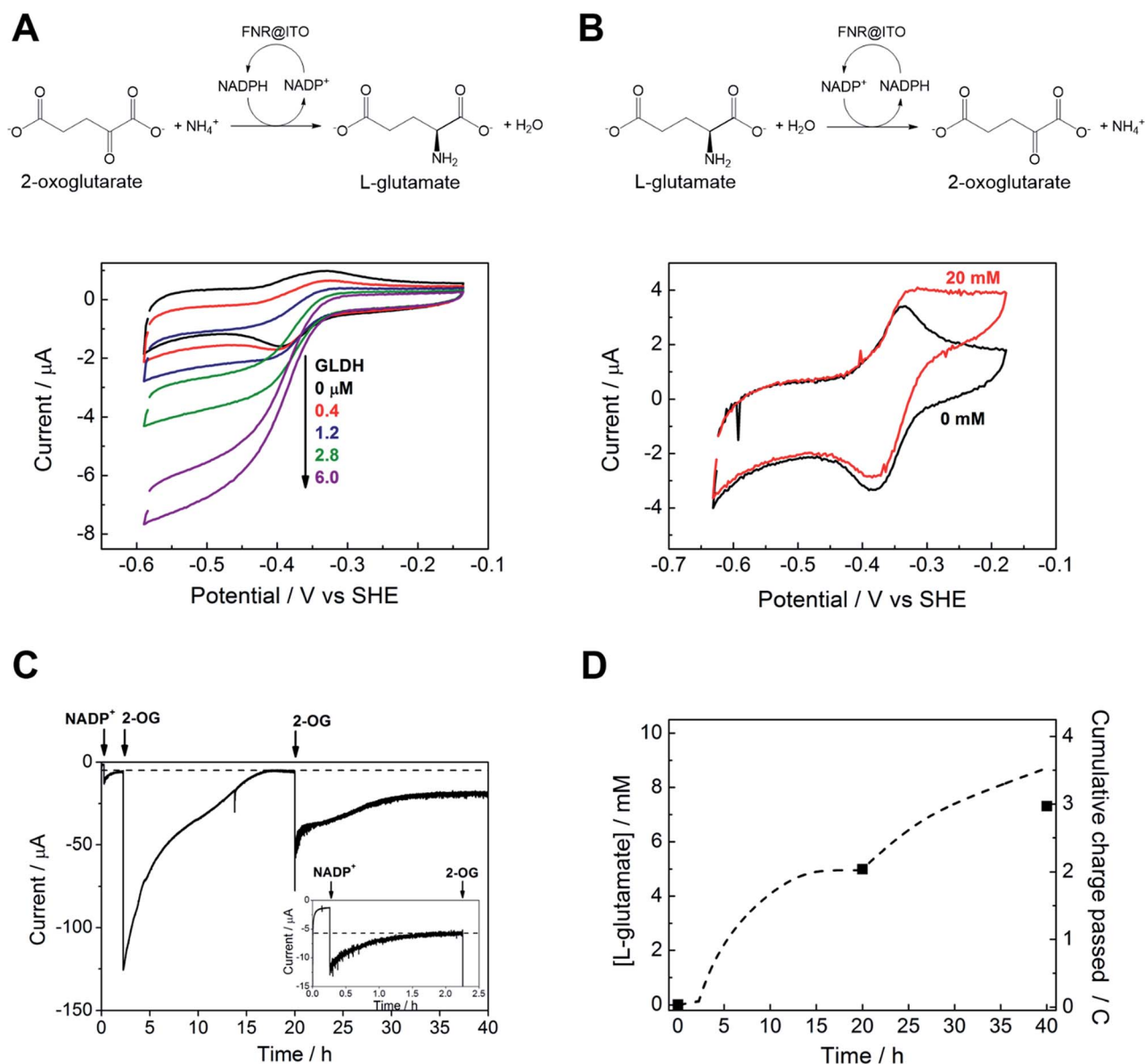


Fig. 4 Coupling FNR@ITO to enzyme-catalysed organic synthesis. (A) Reductive amination of 2-oxoglutarate by glutamate dehydrogenase contained in the cell solution. (NH₄⁺ 20 mM, 2-oxoglutarate 10 mM, NADP⁺ 50 μ M, scan rate 5 mV s⁻¹). (B) Oxidative deamination of L-glutamate by glutamate dehydrogenase co-loaded on to FNR@ITO electrode (see text for details), NADPH 50 μ M, L-glutamate 20 mM, scan rate 1 mV s⁻¹. Other conditions: 25 mM MES, 25 mM TAPS buffer solution at pH 8.0, 20 °C, stationary electrode, solution purged with Ar before measurement. (C) Electrochemical monitoring of L-glutamate synthesis coupled to FNR@ITO. Electrode: FNR@ITO/ITO glass (3 cm²) conditions: electrode potential -0.46 V vs. SHE, 50 mM borate (pH 8.0), cell volume 2.1 mL, NH₄⁺ 20 mM, glutamate dehydrogenase 4 μ M, room temperature, constant Ar bubbling. Injections of NADP⁺ 20 μ M and 2-oxoglutarate 5 mM (twice) are indicated by arrows. Inset shows magnification of the initial 2.5 h. (D) Background-subtracted cumulative charge passed (dashed line) from (C). Black symbols dots refer to direct measurement of L-glutamate concentration by NMR spectroscopy.



μM final concentration) caused the oxidation and reduction peaks to convert to sigmoidal reduction waves, the intensity of which increased with the amount of GLDH added. The coupled catalytic reaction is directly observable by cyclic voltammetry because it is sufficiently fast to regenerate the initial state of the cofactor within the diffusion layer.

Fig. 4B shows the same reaction operated in reverse, where FNR@ITO/ITO glass (geometric area 1.5 cm^2) is used as the electrode for regenerating NADP^+ , which is then used to oxidize L-glutamate to 2-oxoglutarate with release of NH_4^+ . To observe the voltammetric response in this less favourable direction,²⁵ it was necessary to co-immobilize GLDH directly onto the larger FNR@ITO/ITO glass electrode and use a slower scan rate (1 mV s^{-1}). From the beginning, FNR ($6\text{ }\mu\text{L}$ of $200\text{ }\mu\text{M}$) and GLDH ($8\text{ }\mu\text{L}$ of $200\text{ }\mu\text{M}$) were loaded at the same time onto the electrode and incubated for 10 minutes at room temperature before recording the cyclic voltammetry of NADPH ($50\text{ }\mu\text{M}$). Upon injecting L-glutamate (final concentration 20 mM), the oxidation peak converted to a more sigmoidal shape, showing that NADPH is no longer depleted at the electrode surface, and confirming that the FNR@ITO electrode also behaves as a NADP^+ regeneration system.

The GLDH-catalysed production of L-glutamate provided an excellent reporting tool for probing cofactor regeneration dynamics. Small-scale bulk conversion experiments were therefore conducted to obtain L-glutamate product for direct detection *via* NMR analysis (see ESI Fig. S5†), along with simultaneous monitoring of the faradaic charge passed. All experiments were carried out 'on the bench' with the solution agitated by bubbling Ar without any further steps being taken to improve anaerobicity. The results of several trials are shown in Table 1. In all cases, the Pt counter electrode was separated in a side arm linked to the main compartment by a glass frit. Fig. 4C corresponds to entry A of Table 1. An FNR@ITO electrode was constructed on an ITO glass support (total active area 3 cm^2) and the experiment was initiated with only GLDH ($4\text{ }\mu\text{M}$)

and NH_4^+ (20 mM) in solution, with the potential held at -0.46 V vs. SHE (*i.e.* approximately 0.11 V of overpotential for NADP^+ reduction at pH 8.0). Upon injection of NADP^+ ($20\text{ }\mu\text{M}$) a reduction current was observed, which decreased exponentially to a steady level after 1.5 h, indicating exhaustive consumption of NADP^+ (see below). At this point, 2-oxoglutarate was injected to give a final concentration of 5 mM , which immediately generated a large current that was monitored until it dropped to a similar low background level. After 20 h, an aliquot of the cell solution was removed for NMR analysis. The charge consumed was also determined (Fig. 4D) taking into account the small residual background current that was observed in all chronoamperometric experiments. We attribute the background current to traces of dissolved O_2 entering the cell from the atmosphere or through the glass frit, despite continuous bubbling with Ar.

For Fig. 4C, the amount of charge passed before adding 2-oxoglutarate was 8.9 mC , equivalent to 4.6×10^{-8} moles of NADPH (concentration $22\text{ }\mu\text{M}$, in 2.1 mL of solution). The charge passed after adding 2-oxoglutarate, corrected for background, was 2.01 C , equivalent to 1.04×10^{-5} moles of L-glutamate, giving a concentration of 4.97 mM in 2.1 mL of solution. From NMR, the L-glutamate concentration after 20 h was 5.2 mM , the increase above the expected concentration (5.0 mM) indicating that some concentration had occurred. An important metric for evaluating a NADPH regeneration system is the total turnover number (TTN), defined as the number of moles of product of the coupled enzyme per mole of starting NADP^+ , *i.e.* the average number of times NADP^+ has been recycled in the system. Based on the coulometric data, the TTN was 226 after 20 h: this compares with 261 using the expected quantity of NADP and the NMR-derived L-glutamate concentration. Errors in coulometric calculations arise from the uncertainty in subtracting background current, whereas errors in chemical quantities arise from the difficulty in accurately weighing small amounts of nicotinamide cofactor, and

Table 1 Extended data for electrosynthesis of L-glutamate by reductive animation of 2-oxoglutarate catalysed by glutamate dehydrogenase coupled to FNR@ITO/support electrodes^a

	Electrode area (cm^2)	Support	Average FNR coverage (pmol cm^{-2})	Duration (h)	L-Glutamate		TTN ^c (product/ NADP^+)		
					mM	μmoles	By charge (continuous)	By NMR	Rate ($\mu\text{moles h}^{-1}\text{ cm}^{-2}$)
A	3	ITO glass	163	37.7	7.31	15.4	436	366	0.14
B	2.5	Ti foil	19.5	16.7	4.74	9.5	207	237	0.23
C ^b	10	Ti foil	90	41.7	13.3	33.4	813	667	0.08
D	17	Ti foil and wound wire	115	16.4	17.5	43.7	908	873	0.16
E	20	Ti foil and wound wire	60	19.6	20.1	50.2	1137	1004	0.13
F	6	Ti wire (crumpled)	136	37.8	20.5	51.2	1108	1025	0.22

^a Experiment conditions: electrode potential held at -0.46 V vs. SHE ; 50 mM borate, pH 8; $20\text{ }\mu\text{M}$ NADP^+ ; 40 mM NH_4^+ (for C, D, E and F); 30 mM NH_4^+ (for A and B); 30 mM 2-oxoglutarate (for, D, E and F); 20 mM 2-oxoglutarate (for A, B and C); $3.2\text{ }\mu\text{M}$ glutamate dehydrogenase (for C, D, E and F); $4\text{ }\mu\text{M}$ glutamate dehydrogenase (for A and B); $20\text{ }^\circ\text{C}$; bubbled with Ar throughout, unless otherwise indicated. The duration is counted from the addition of 2-oxoglutarate to the end of chronoamperometry. ^b No Ar bubbling up to 22 h (*i.e.* stationary solution); Ar bubbling from 22 h onwards.

^c TTN: total turnover number defined as number of moles of L-glutamate made per initial number of moles of NADP^+ (TTN = (moles L-glutamate)/(moles of NADP^+)); moles L-glutamate estimated from charge passed in chronoamperometry and also as measured by NMR.



electrochemical leaf – has numerous options for development, particularly electrode and reactor design (ultimately achieving the advantages of dimensionality so obvious in a chloroplast) and exploration of myriad coupling reactions, including monooxygenases. It seems likely that total turnover numbers (product/NADP) of several thousand for a day-long batch will be easily achievable. A large range of complex regio- and stereo-specific organic reactions thus becomes accessible to simple electrochemical procedures and continuous monitoring.

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