NADPH performs mediated electron transfer in cyanobacterial-driven bio-photoelectrochemical cells

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Summary

Previous studies have shown that live cyanobacteria can produce photocurrent in bio-photoelectrochemical cells (BPEC) that can be exploited for clean renewable energy production. Electron transfer from cyanobacteria to the electrochemical cell was proposed to be facilitated by small molecule(s) mediator(s) whose identity (or identities) remain unknown. Here, we elucidate the mechanism of electron transfer in the BPEC by identifying the major electron mediator as NADPH in three cyanobacterial species. We show that an increase in the concentration of NADPH secreted into the extracellular medium (ECM) is obtained by both illumination and activation of the BPEC. Elimination of NADPH in the ECM abrogates the photocurrent while addition of exogenous NADP⁺ significantly increases and prolongs the photocurrent production. NADP⁺ is thus the first non-toxic, water soluble electron mediator that can functionally link photosynthetic cells to an energy conversion system and may serve to improve the performance of future BPECs.

Keywords

Photosynthesis, solar energy conversion, cyanobacteria, electron transfer, fluorescence, Ferrodoxin-NADP reductase; chronoamperometry;

Introduction

World energy consumption continues to increase each year. To meet the increasing demand, use of fossil fuels continues at ever growing amounts, and as a result, more pollution is released into the environment. Estimation of the energy consumption between the years 2008 and 2035 has predicted
an increase of \(>50\%\) that will reach an annual average of \(\sim 26\text{TW}\). The increase in pollution has recently become a major concern because it might cause severe climate change which will be globally detrimental. Therefore, there is an urgent need for replacement polluting energy production technologies with non-polluting, renewable, green energy technologies.

One such technology, which is believed to be a good base for production of green energy, is microbial fuel cells (MFCs). The main principle of MFCs is to use bacteria in electrochemical cells as electron donors\(^2\) at the anode or acceptors\(^3,4\) at the cathode. The electron transfer between the inner membrane of live bacteria and the anode of the electrochemical cell was first demonstrated by Potter in 1910.\(^5\) Since the membranes of most bacterial species have low conductivity, MFCs utilize small molecules which can mediate electrons between the inner membrane and the electrodes. Over the years, the use of various bacterial species has been reported.\(^1,3-6,25\) Some of these species were able to produce endogenous electron mediators.\(^14,15,21,23-25\) Addition of different exogenous electron mediators were reported to significantly increase the produced current including cystine, neutral red, thionin, sulphides, ferric chelated complexes, quinones, phenazines, and humic acids.\(^5,12,13,26,27\) As most of these compounds are considered to be toxic, their use must be tightly regulated. Current production can also be performed by direct electron transfer (DET) mechanisms. Such a mechanism was reported for Geobacter sulfurreducens biofilms which use conductive pili to reduce the anode.\(^28-30\) Shewanella oneidensis MR-1 biofilms were reported to conduct direct electron transfer through Mtr complexes\(^31,32\) and an indirect electron transfer which is mediated by flavin molecules.

Recent studies have shown that photosynthetic microorganisms can be used as the source of electrons that can be collected in bio-photo electrochemical cells (BPEC) for solar energy conversion to electrical current and/or clean fuels (such as hydrogen)\(^33\). Therefore, it has been suggested that their use could serve as the main photo-active component in the development of innovative renewable energy applications.\(^34-47\) In some systems, light energy was converted utilizing reaction centre/light harvesting proteins\(^48\) or isolated photosystems conductively attached to electrodes in BPECs.\(^47,49-54\) Some of these systems require the addition of an exogenous mediator and the photosystems must be attached to the anode which can be done by utilizing an immobilized electron mediator such as redox polymer. Use of PSI or reaction centers from non-oxygenic photosynthetic organisms requires addition of an electron donor\(^46,47,50,53\), while in systems based on PSII, water serves as the initial electron donor.\(^54\) Use of isolated complexes, especially when PSII is utilized, is hampered by their relative instability, leading to loss of activity. Utilization of thylakoid membranes, whose isolation process is much easier and cheaper than isolating complexes\(^45\) makes the instability problem less of an issue in applications. A potentially more stable system can be achieved by utilizing living photosynthetic microorganisms, which have internal repair mechanisms for replacing photodamaged components. Use of intact cells in BPECs leads to a new problem however: how to enable external
electrodes to acquire electrons from internal membrane components that are sequestered behind multiple layers of membranes that make up the external barrier of the cells.

This problem can be solved by performing mediated electron transfer (MET) with mediators that are able to cross these barriers and efficiently interact with either inorganic or metallic electrodes and photosynthetic biological components. Previous studies have used exogenously added mediators such as ferricyanide, 2,6-dichloro-1,4 benzoquinone (DCBQ), 2-hydroxy-1,4-naphthoquinone (HNQ) or 1,4-benzoquinone. However, all bacteria, and especially cyanobacteria, have outer membranes that limit the ability of such molecules to enter and exit the cells. Some of the mediators are toxic and most have a low solubility in water-based media. The use of exogenous mediators might be avoided using a semi-dry cyanobacterial monolayer which can produce current by a DET mechanism, however, such system might damage the survival of the cyanobacteria and limit its ability to multiply which might be very important for construction of useful systems. One of the possible mechanisms for DET with cyanobacteria is through type IV pili. However, in a recent study, no significant difference was found between the photocurrent production of wt Synechocystis and the pili deficient mutant ΔpilD. A different option for cyanobacteria is MET that could be conducted by an endogenous mediator which can be secreted by the cells in similar with other small inorganic molecules that can pass the outer membrane through porin channels. It has recently been shown that live cyanobacteria can also produce photocurrent without the addition of any exogenous mediator. It was suggested that the electron source originates from both the natural photosynthetic and respiratory pathways which can combine to reduce plastoquinone molecules which then continue to transfer electrons between a sequence of molecules until they reach the final step of the photosynthetic pathway, which is the reduction of NADP⁺ to NADPH. Efforts were made to identify the endogenous mediators using cyclic voltammetry and metabolomic measurements of the cyanobacterial external cell medium (ECM). However, the identity of the mediator(s) remained elusive.

In the present work, we use two-dimensional fluorescence map (2D-FM) fingerprinting to identify NADPH as a major component that is exported from cyanobacteria and accumulates in the BPEC medium upon the application of light and electric potential. NADH can also mediate between the cells and the BPEC but it is not accumulated in the external medium upon illumination. Therefore, NADPH serves as the endogenous electron mediator of cyanobacteria and since its addition to the system increased the produced electrical current, this finding would assist the generation of bio-photoelectrochemical devices that use living photosynthetic organisms to produce electricity and hydrogen.
Results and Discussion

Light dependent generation of electricity by live Synechocystis in a miniBPEC system

We have previously shown that photocurrent could be produced by live Synechocystis sp. PCC 6803 (Syn) cells in a BPEC.\(^4\) This BPEC was composed of 3 bulk electrodes: a graphite disk anode, a platinum rod cathode and an Ag/AgCl/3M NaCl reference electrode, with direct contact between the cells and the anode in 50 ml of buffered solution. The large ECM volume made chemical identification of the internal mediator, released from the cyanobacteria, difficult.\(^4\) In order to chemically analyse the BPEC solution and identify the mediator, while measuring current, we utilized commercial screen-printed electrode (SPE) system that formed the basis of a miniBPEC, where a minimal ECM volume would be sufficient. The new miniBPEC consisted of SPE with carbon anode, platinum cathode and Ag coated with AgCl reference electrode enabling recording a current of about 3 µA cm\(^{-2}\) using 50 µl live Syn cells (5 µg of chlorophyll, about 1.4x10\(^8\) cells) (Fig. 1A). In agreement with the previous results that were obtained with the large BPEC\(^4\), live Syn cells produced appreciable light-dependent electrical current without the addition of an exogenous electron mediator. In addition, as previously reported for the large BPEC, the current was significantly enhanced by the addition of the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and remained constant for a longer period of time when the cells were pre-incubated with glucose (Glu, Fig. 1B).\(^4,7\) Therefore, the electron source from the cyanobacteria is the respiratory pathway from which the electrons are transferred to PSI and from this light-driven reaction to an internal mediator(s) that is/are secreted from the cell and reduce(s) the graphite anode.\(^4\) PSII is not the major source of electrons in this pathway since the enhancing effect of DCMU happens also with Syn mutant lacking PSII.\(^4\) When the

![Fig. 1 Light dependent generation of electricity by live Syn. Syn cells (5µg chlorophyll) were placed in a 50µl drop on minielectrodes based BPEC and the light dependent chronoamperometry (CA) measurement was performed applying a voltage bias of 0.5V on the anode. a. Schematic drawing of the miniBPEC setup. b. CA of the light dependent produced current of live Syn (black), with the addition of 100 µM of the herbicide DCMU (+DCMU, red) and after a pre-incubation in}
dark for 1 h with 5 mM glucose (+Glu, blue). A picture of the minielectrodes BEPC is shown in the inset. C. Calculation of the total current that was accumulated during 10 min in CA measurements of Syn (black), +DCMU (red) and +Glucose (blue). The error bars represent the standard deviation over 3 independent measurements.

ECM was collected following operation of the miniBPEC and filtered through a 3 kDa filter, the filtrate was able to reduce oxidized cytochrome c (Fig. S1).

In our previous work using the 50 ml BPEC, we have found that mechanical treating the live Syn by low pressure microfluidizer or by osmotic shock significantly enhanced the amount of current produced in the BPEC. To simplify our system, in the following experiments the cells were not pre-treated, other than centrifugation from their growth medium (see Experimental for additional details).

**Identification of NADPH and/or NADH in the ECM.**

Production of photocurrent from live Syn without addition of an exogenous electron mediator has been suggested to occur by the secretion of endogenous electron mediator(s) to the ECM. We previously performed metabolomic assays on whole cells used in the BPEC, however, no significant change in any of the known metabolites could be observed. We further performed analysis of the ECM from the BPEC (following removal of the cells), using HPLC separations (with different column chemistries) and mass spectrometric detection. These measurements indicated that the ECM contains a very large number of molecules or fragments of sensitive molecules that decompose in the BPEC. While over 1700 peaks with different m/z ratios could be measured, we were unsuccessful in identifying unique redox active molecules that could serve as the mediator, perhaps because of low stability of such molecules during the purification and characterization procedures.

A less harmful approach to screen the ECM for known redox components utilizing spectroscopic methods was thus sought. Absorption spectra, FTIR and NMR measurements did not result in the disclosure of a clear redox active molecule, especially due to the challenge of the presence of hundreds of molecules in the ECM mixture. Another method that can identify molecule(s) in the ECM is 2D fluorescence maps (2D-FM), as many redox active molecules contain moieties that absorb and fluoresce energy at specific wavelengths. When the ECMs, taken following the current production experiment in the BEPC, were analyzed by this method, a strong signal that matched the fluorescence fingerprint of NADPH and/or NADH (NAD(P)H) was observed. The signal in the ECM was compared to commercial samples of NAD(P)H used as standards and to previously published measurements of these molecules (Fig. 2).
Accumulation of NAD(P)H in the ECM is dependent on light and the connection between the cells and the electrochemical system.

We suggest that the presence of NAD(P)H in the ECM is the result of the secretion of NADH, NADPH or both to the ECM by the live Syn. Therefore, we next ask if this secretion is light dependent and whether or not it depends on physical linkage between the cells and the electrochemical system in the miniBPEC. To this end, we performed 2D-FM spectra on ECM from Syn cells, which were kept in the dark or exposed to light in either small transparent test tubes or in the miniBPEC on the electrodes during 100 sec of CA measurement. No significant difference was observed between the fluorescence of the ECMs which were illuminated or not illuminated without any connection to the electrochemical system (in tubes). The NAD(P)H fluorescence intensity of Syn ECMs after CA measurement was significantly higher and further enhanced about upon illumination (Fig. 3).
In order to determine the NAD(P)H concentration in the *Syn* ECM, a calibration curve of NADH concentration vs. fluorescence intensity was prepared based on NADH fluorescence (Fig. S2). This evaluation relied on the presumption that the 2D-FM of NADH and NADPH are spectrally identical, as has been seen by others.⁸²

![Fig. 3](image)

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**Fig. 3** Accumulation of NAD(P)H in the ECM is dependent on light and the connection between the cells and the electrochemical system. 2D-FM spectra of the ECM of *Syn* after incubation in test tubes or during CA measurements on the electrodes in dark or under illumination. **a.** In test tubes in the dark. **b.** In test tubes under illumination. **c.** On the electrodes after 100 sec of CA measurement in the dark. **d.** On the electrodes after 100 sec of CA measurement under illumination. The inset in panel **b** displays NAD(P)H concentrations which were calculated based on the fluorescence intensities at \( \lambda_{(ex)} = 350 \text{ nm} \) and \( \lambda_{(em)} = 450 \text{ nm} \). The error bars in all panels represent the standard deviation over 6 independent measurements. The fluorescence intensities in the colour bar are displayed as normalized fluorescence (N.F).

NAD(P)H concentrations were calculated for ECM of *Syn* solutions which were illuminated or not – illuminated in test tubes or on electrodes during CA measurements as shown in the inset in Fig. 3b. As can be seen, illumination in the BPEC results with the accumulation of about \(~2 \mu\text{M}\) NAD(P)H in the ECM. This concentration is about ten times greater than the amount of NAD(P)H that accumulates without any connection to the electrochemical system and about four times greater than accumulated
without illumination. These results showed that the connection between the active electrochemical system and live Syn cells enhances secretion and accumulation of NAD(P)H in the ECM that can reduce the anode and produce electric current. Under illumination, the concentration of NAD(P)H in the cells is increased further, there is a concomitant increase in NAD(P)H release to the ECM and more current is produced. In the absence of connection to the electrochemical system, the release of NAD(P)H is low, and no significant difference can be observed between cells which were illuminated or not illuminated. These results show that in the absence of the electrochemical system, NAD(P)H release is not favoured by the cells.

**NADPH accumulates in the Syn ECM more than NADH**

Enzymatic kits were used for quantification and differentiation between NADH and NADPH in the Syn ECM (see Experimental for details). Both enzymatic assays were applied to the ECM of Syn after 100 sec of CA measurements in either dark or under illumination. The presence of both NADH and NADPH was detected. No significant difference was found between NADH concentrations in the ECM of cells which were illuminated and not illuminated and was about 0.6 µM (Fig. 4). However, the concentration of NADPH in the ECM, following illumination, was higher, increasing from about 0.4 to 1.6 µM (Fig. 4) The sum of NADH + NADPH concentrations is about 2 µM which is in agreement with the NAD(P)H concentration which was calculated based on the 2D-FM maps. This result indicates that NADPH, rather than NADH, accumulates in the ECM in the light and suggests that this molecule may serve as the primary mediator.

![Fig. 4 NADPH accumulates in the Syn ECM more than NADH](image_url)

**Fig. 4 NADPH accumulates in the Syn ECM more than NADH.** Quantification of NADH and NADPH concentrations in the ECM of Syn, after 100 sec of CA measurement with or without illumination, was done using a chemical enzymatic assay. The error bars represent the standard deviation over 9 independent measurements.
Addition of FNR eliminates both the light dependent electric current and the NAD(P)H accumulation in the ECM

Ferredoxin NADP⁺ reductase (FNR) binds both NAD(P)⁺ and NAD(P)H. In order to further support our identification of NAD(P)H as the main endogenous electron mediator, increasing concentrations of the enzyme FNR were added to Syn cells and CA was measured. We observed that the current was reduced as function of the increasing FNR concentrations and was almost totally abrogated at FNR concentration of (30 µM).

Fig. 5 Addition of FNR that binds NAD(P)H eliminates both the light dependent electric current and the NAD(P)H accumulation in the ECM. a. CA of Syn (black) and Syn + BSA (red) and Syn + 3.75, 7.5, 15 and 30 µM FNR (light blue, magenta, green and dark blue, respectively). b. Calculations of the current sum that was accumulated during 3 min in CA measurements of Syn (black), + Syn + BSA (red) and Syn + 3.75, 7.5, 15 and 30 µM FNR (light blue, magenta, green and dark blue, respectively). The error bars represent the standard deviation over 3 independent measurements. c. 2D-FM of Syn filtrate. d. 2D-FM of Syn + FNR filtrate. The ECMs of Syn, with and without the addition of FNR at the concentration of 1 mg/ml, were filtrated through a 3 kD filter and measured with 2D-FM. The fluorescence intensities in the colour bar is displayed as normalized fluorescence (N.F).
In order to make sure that the current was abrogated because of the specific binding activity of FNR and not by generic protein binding or changes in the conductance and rigidity of the electrolyte, the CA of Syn was measured with 1 mg/ml of bovine serum albumin (BSA). Addition of BSA did not significantly affect the photocurrent (Fig. 5a). In addition, the experiment was repeated with 15µM of a recombinant FNR enzyme whose origin is the green algae C. reinardii. Addition of the CrFNR also abrogated the photocurrent (Fig. S4).

In order to assess further whether the reduction in current was caused by FNR binding of NADPH and preventing it from being the mediator, Syn cells were incubated for 30 min with or without 1 mg/ml FNR which is a ~10 fold molar excess to that of the calculated NADP(H) concentration. The samples were then centrifuged in ultrafiltration devices with a 3kD filter. 2D-FM (λ(ex) = 260 – 400 nm, λ(em) = 400 – 520 nm) of the filtrates were measured. The spectral fingerprint NAD(P)H was observed only in the filtrates of samples which did not contain FNR, indicating that NAD(P)H molecules were bound to FNR and therefore did not appear in the filtrate (Fig. 5c and 5d). Since the addition of FNR almost completely abolished both photocurrent and the 2D-FM signal of NAD(P)H, these results imply that NADPH is the primary mediator of the electrons from Syn PSI to the graphite anode when generating the light dependent electric current in the BPEC.

**Addition of an exogenous NADP⁺ to the ECM enhances photocurrent production.**

There are two scenarios whereby NADPH can serve as the mediator in MET-type current production. In the first, NADPH is secreted but following electron transfer is not uptaken by the cells for additional cycles. In the second scenario, following oxidation, NADP⁺ is uptaken for further cycles. To differentiate between these two scenarios, we performed a pre-incubation of Syn with exogenously added NADP⁺ to observe whether indeed more NADPH would be produced under illumination resulting in an elevated electric current. ⁷⁴,⁸⁴ We measured the BPEC photocurrent with 5 mM NADP⁺ in the absence of cells or in the presence of Syn at different pre-incubation times (0 – 2 h). In the absence of cells, addition of NADP⁺ did not produce any significant photocurrent. In the presence of Syn cells, addition of NADP⁺ significantly increased the photocurrent. The longevity, maximum and total current all increased in correlation with the pre-incubation time (Fig. 6a).

This result implies a mechanism whereby NADP⁺ can enter and exit the cells and mediate electrons from the photosynthetic system (PSI) and the BPEC anode. Therefore, the addition of NADP⁺ at a concentration higher than what is released from the cells can significantly increase the photocurrent.
**Fig. 6** Addition of NADP⁺ enhances photocurrent production. **a.** CA measurement of Syn cells with or without pre-incubation with 5 mM NADP⁺ for 0, 1 and 2 h under continuous illumination. NADP⁺ without cells (-Syn, black), Syn without NADP⁺ (red), Syn after 0, 1 and 2 h incubation with NADP⁺ (blue, magenta and green respectively). **b.** Calculations of the current sum that was accumulated during 10 min in CA measurements of NADP⁺ without cells (-Syn, black), Syn without NADP⁺ (red), Syn after 0, 1 and 2 h incubation with NADP⁺ (blue, magenta and green respectively). The error bars represent the standard deviation over 3 independent measurements.

**Two additional cyanobacterial species export NAD(P)H and generate photocurrent.**

Our previous studies which showed that photocurrent can be produced without addition of an external electron mediator were focused on Syn. We wished to screen additional species in our miniBPEC to assess whether the ability to produce photocurrent by secretion of an endogenous electron mediator is specific to Syn or a more general phenomenon in cyanobacteria. In order to address this question, two additional cyanobacterial species were studied: *Acaryochloris marina MBIC 11017 (Am)* and *Synechococcus elongatus PCC 7942 (Se)*.

Similar to Syn, the native habitat of Se is fresh water. However, Se has an elongated filament shape which is different than the round cells of Syn. The native habitat of Am is the oceanic salt water. Moreover, Am contains chl d which enables it to absorb wavelength beyond 700 nm for efficient photosynthesis.

2D-FM of the ECs of Am and Se after a 100 sec of CA measurement in dark or under illumination were measured. NAD(P)H concentrations were calculated based on the fluorescence intensities and compared to standards of known concentrations. The results revealed that both strains exhibit light induced accumulation of NAD(P)H in their ECM (Fig. 7). In addition, the difference between light and dark incubation of both strains was similar to that shown for Syn.
Fig. 7 *Am* and *Se* also exhibit light-driven current and export NADP(H) into the BPEC ECM. CA of *Am* and *Se* cells were measured for 100 sec in dark and under illumination. The cells were filtrated immediately following the measurement and 2D-FM spectra of their ECMs were measured. **a.** CA of *Am* cells in dark (black) and under illumination (red). **b.** 2D-FM spectra of the ECM of *Am* after measurements in dark. **c.** 2D-FM spectra of the ECM of *Am* after measurements under illumination. **d.** CA of *Se* cells in dark (black) and under illumination (red). **e.** 2D-FM spectra of the ECM of *Se* after measurements in dark. **f.** 2D-FM spectra of the ECM of *Se* after measurements under illumination. The inset in panels c and f displays NAD(P)H concentrations which were calculated based on the fluorescence intensities at (λ(ex) = 340 nm, λ(em) = 450 nm) after incubation in dark and under illumination. The error bars represent the standard deviation over 6 independent measurements. The fluorescence intensities in the colour bar is displayed as normalized fluorescence (N.F).

Based on our findings and in agreement with our previous work, we suggest a model for the mechanism of the electron transport in the BPEC in which the electron source starts from the respiratory pathway and continue through the plastoquinone pool to PSI which reduce NADP⁺ to NADPH. The link to the electrochemical component of the BPEC induces the release of NADPH, which reduces the anode and re-enter the cyanobacterial cell to accept additional electrons from PSI. (Fig. 8). Another minor electron donor is NADH which was also found to exist the cell and functions similar to NADPH.
Fig. 8 Schematic depiction of the main electron transport pathway in cyanobacterial BPECs. The electron transport originates from the respiratory pathway which reduces the PQ pool and continues downstream to PSI which under illumination reduces NADP⁺ to NADPH. NADPH exits the stroma (cytoplasm) and outer membrane to the ECM and reduces the graphite anode. It then re-enters the cyanobacterial cells to accept more electrons from PSI. Yellow flash shapes indicate the light illumination which is absorbed in the photosystems. Green arrows represent the directionality of the electron transport pathway. Black arrows indicate the internal and outer cells membranes. A dashed line represents the electron blockage between PSII and the plastoquinone pull (PQ) in the presence of DCMU. Red arrows represent the binding of NADP⁺ and NADPH to exogenous FNR that prevent them from reducing the anode or re-entering the cell and in this manner abrogate the current production.

The actual capacity of photosynthetic organisms to perform efficient solar energy conversion has been a subject of debate for the past 20 years since the first studies of attempts to utilize photosynthesis (in the form of live cells, membranes or isolated complexes) were reported. Comparisons with solid-state or dye-sensitized solar cells are seldom performed using the same metrics. Overall photosynthetic efficiency is typically seen as low since much of the chemical output (in the form of sugars and ATP) goes towards cell growth and control. Comparison of PV-driven electrolysis vs. biomass production is for instance greatly in favour of PV. This of course does not take into account the energy expended in mining the raw materials (or used in their synthesis), or the carbon footprint of moving raw materials to sites of synthesis/assembly and transportation of completed panels to their site of use. When comparing pure quantum efficiency (photons absorbed to electrons transferred), PSII and PSI have been shown to be highly efficient comparable to PV/DSSC devices. Translating the maximal electron flux through PSII (with water as the donor), or PSI (with a sacrificial electron donor) one can obtain sustained currents of 10-3000 mA mg chl⁻¹ (depending on organism source and level of isolation) for saturating illumination in the range of 400-700 nm. Rates of PSI reduction of NADP⁺ are comparable. We estimate that a two-dimensional 1 cm² solar panel based on photosynthetic material would require ~ 100 µg chl (in cells or thylakoid membranes) or about 10 µg chl (in isolated PSII/PSI) to operate.
This is about the amount of material that can be isolated from a few spinach leaves or 1 gram (dry weight) of cyanobacterial cells. This current is on the same order of current that is obtained from commercial DSSC solar cells. Use of isolated components (membranes or complexes) requires systematic replacement due to irreparable damage due to photoinhibition but can provide elevated rates of electron transfer. Cells continually self-repair damaged systems, however if dependent on the release of endogenous electron mediators, the currents will be an order of magnitude smaller. For this reason, we have identified the mediator, to see if we can improve our BPEC that utilizes cells. Our identification of NADPH as a major mediating molecule is thus important, since this natural molecule has many of the characteristics one would hope to use in a BPEC device: it is non-toxic, water soluble, redox active and relatively stable. Developing methods where a single addition of NADP⁺ to the ECM of a system based on live cells that can operate during a single day would be a tremendous advancement towards the future use of such a BPEC in the future for small-scale local solar energy conversion.

Conclusions

We show here that live cyanobacterial cells use endogenous NADPH as an electron mediator in a BPEC. NADPH is a redox active metabolite that is found in these cells at relatively high concentration (>15nmol/mg chl) and is continually produced by the coupled respiration and photosynthesis processes in active cells. Taken with our previous studies, these observations indicate that use of easily grown cells can provide current with the potential for either fuel (H₂) production or power storage for the entire length of a day without addition of polluting chemicals. In the future, we may see the production of sufficient energy to run a small local agricultural device using such a clean-energy system.

Experimental Procedures

Materials

All chemicals were purchased from Merck unless mentioned otherwise.

Two different Ferredoxin NADP⁺ Reductase (FNR) proteins were used in this work. The first (mcFNR) was isolated from thermophilic cyanobacteria Mastigocladus laminosus and kindly obtained from Prof. Rachel Nechushtai, the Hebrew University of Jerusalem. The second (crFNR) was produced in E. coli using an expression plasmid harbouring the Chlamydomonas reinhardtii FNR cDNA, kindly provided by Prof. Iftach Yacoby at Tel Aviv University. crFNR was purified as described by P. Marco et al.

Cells cultivation
Synechocystis sp. PCC6803 (Syn) and Synechococcus elongatus PCC 7942 (Se) were cultivated in BG11 medium in growth chamber at light intensity of about 100 μE / m² s, shaking at 100 rpm and at 29°C. Acaryochloris marina MBIC 11017 (Am) was cultivated in MBG11 media (a BG11 medium containing 30 g/L sea salts (Instant Ocean)).

**Samples preparation**

O.D measurements of the cells was done at 750 nm (Nanodrop 2000 UV-Vis spectrophotometer, Thermo Fisher Scientific). Cell count was done under the microscope using a haemocytometer grid. For the measurements in the BEPC, log phase grown cells (O.D750nm = 0.6 – 1.0) were centrifuged (Multifuge X1R, Thermoscientific) for 10 min at 7500 rpm. The supernatant was removed, and the pellet was resuspended in a solution of MES buffer (MES 50mM (pH=6), 10 mM NaCl, 5 mM MgCl. Chlorophyll concentration was determined by 90% methanol extraction followed by absorption measurements at 665 nm for chl a and 696 nm for chl d. Calculation of Chl concentration was performed as previously described. 78,79 For use in the BPEC, cyanobacterial cells were diluted in MES buffer solution to have a final concentration of 0.1 mg/ml total chl.

**Chronoamperometry measurements**

Chronoamperometry measurements were done using Plamsens3 potentiostat (Palmsens) connected to screen printed electrodes (AC1.W4.R1, BVT technologies). Illumination of 1.5 SUN (calibrated at the electrode surface height without samples) was done using a solar simulator (Abet). In all measurements, a bias potential of 0.5 was applied on the graphite anode. Total current calculations were done by summing all current values of the measurement which were acquired in time steps of 0.5 s. The volume of all measured samples was 50 µl. As preparation for fluorescence measurements, 50 µl of each sample was diluted with buffer to a final volume of 2 ml.

**Fluorescence measurements**

All fluorescence measurements, unless mentioned otherwise indicated, were done using a Fluorolog 3 fluorimeter (Horiba) with excitation and emission slits bands of 4 nm. Quantification of NAD(P)H concentrations were calculated based on NADH calibration curve which was based on increasing concentrations measured at (λ(ex) = 350 nm, λ(em) = 450 nm).

**Selective quantification of NADH and NADPH.**

Selective quantification of NADH was done using NAD+/NADH Quantification Kit (Cat. #MAK037, Sigma). Quantification of NADPH was done using a NADP+/NADPH Assay Kit (Cat. #MAK312, Sigma). Briefly, in both kits 50 µl of Syn ECM was incubated with 50 µl enzymatic probes for specific detection of NADH or NADPH. Each sample was analysed in 9 replicates (each in a different well) in 96 wells plates. Triplicates of 5 different known NADH and NADPH increasing
concentrations were incubated in the same plates for the construction of calibration curves. Absorption for NADH quantification (450 nm) or fluorescence for NADPH quantification (ex = 530 nm / em = 585 nm) were measured using Spectramax M2 plate reader (Molecular Devices). Concentrations were determined based on the calibration curves which were plotted based on the known concentration of standards.

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Author contributions Yaniv Shlosberg, Gadi Schuster and Noam Adir conceived the idea. Yaniv Shlosberg, Gadi Schuster and Noam Adir designed the experiments. Yaniv Shlosberg performed the main experiments. Ben Eichenbaum, Tünde N. Tóth and Guy Levin assisted in performing the experiments. Yaniv Shlosberg, Gadi Schuster and Noam Adir wrote the paper. Gadi Schuster and Noam Adir supervised the entire research.

Declaration of interests. The authors declare no competing interests.

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NADPH performs mediated electron transfer in cyanobacterial-driven bio-photoelectrochemical cells

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Supplementary Information

Fig. S1 - Following incubation of Syn in the BPEC, the ECM reduces Cytochrome C. In order to show that the ECM of Syn contains reducing agents, Absorption spectra of the ECM (black), oxidized Cytochrome C (red) and a mixture of ECM + Cytochrome C (blue) were measured after 20 min incubation. The observed spectral split from a single peak into 2 peaks in the mixture indicates the reduction of Cytochrome C by the ECM solution.
Fig. S2 - Calibration curve of NADH concentration vs. fluorescence intensity. Fluorescence intensities was plotted against several NADH concentrations ($\lambda$(ex) = 350, $\lambda$(em) = 450 nm) (black). Linear fitting analysis (red). ECM samples with a volume of 50µl after or not after CA measurements were diluted to a final volume of 2ml (in order to have enough volume for the measurement) and their original concentrations were determined based on the curve and the dilution factor.
Fig. S3 - Addition of exogenous NADPH to the BPEC with or without Syn cells. In order to estimate the ability of exogenous NADPH to produce photocurrent in the BPEC, 1µM NADPH was added with or without Syn cells. In both measurements a fast increase of about 4 µA was obtained followed by a fast decrease of the current to the same level as it was prior to the addition within a few seconds. The fast current decay suggests that in order to maintain the current cyanobacteria needs to continuously secrete NADPH to the ECM. 1µM NADPH was added to the BPEC in the absence of Syn cells (black) and in the presence of Syn measurement after the current reaches it maxima at 1.6 min (red). Arrows indicate the time point of NADPH addition.
Fig. S4 - CA of Syn was measured with or without addition of 15µM recombinant FNR enzyme whose plasmid origin is *Chlamydomonas*. To strengthen the previous results that showed that addition of FNR isolated from thermophilic cyanobacteria *Mastigocladus laminosus* to Syn abrogates the photocurrent, we wished to repeat the same experiment with FNR from additional origin. For this purpose, CA of Syn with or without 15µM of the recombinant FNR enzyme whose plasmid origin is from the green algae *Chlamydomonas reinhardtii* was measured. Addition of the recombinant FNR has also abrogated the photocurrent. Syn (black), Syn + 15µM FNR (red).
**Fig. S5** - Addition of FNR in the presence of NADP⁺ with a significantly higher concentration do not abrogate the current. CA of Syn + 5 mM NADP⁺ (after 2 h incubation) was measured in with or without 30 μM FNR (from thermophilic cyanobacteria *M. laminosus*). No significant difference was observed between the 2 measurements. This finding indicates that in the presence of such NADP⁺ concentration which is 3 magnitudes higher than FNR, all the FNR is bound. Therefore, it cannot bind to NADPH molecules that the cells secrete and do not abrogate the photocurrent production. Syn + 5 mM NADP⁺ (black). Syn + 5 mM NADP⁺ + 30 μM FNR (red).
Fig. S6 - *Am* and *Se* also increase their photocurrent production in the presence of exogenous glucose (Glu), DCMU, and NADP⁺ and do not produce current in the presence of FNR. CA of *Am* and *Se* without and with DCMU, Glu, NADP⁺ and FNR were measured. Both species showed a similar pattern to *Syn* in which addition of DCMU, Glu and NADP⁺ has increased the photocurrent and FNR has almost totally abrogated the current. Based on this finding, we suggest that the same mechanisms for photocurrent production which exist in *Syn* are probably general for many other cyanobacterial species. 

**a** CA of *Am* (black), *Am* + DCMU (red), *Am* + Glu (blue), *Am* + NADP⁺ (magenta) and *Am* + FNR (green). 

**b** CA of *Se* (black), *Se* + DCMU (red), *Se* + Glu (blue), *Se* + NADP⁺ (magenta) and *Se* + FNR (green). Addition of Glucose and NADP⁺ were done 1 and 2 h before the beginning of each measurement, respectively, and the incubation was done in the dark. Addition of DCMU and FNR was done right before the beginning of the measurement.