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A system consisting of a [NiFeSe]-hydrogenase (H\textsubscript{2}ase) grafted on the surface of a TiO\textsubscript{2} nanoparticle modified with polyheptazine carbon nitride polymer, melon (CN\textsubscript{x}) is reported. This semi-biological assembly shows a turnover number (TON) of more than 5.8 × 10\textsuperscript{3} mol H\textsubscript{2} (mol H\textsubscript{2}ase)\textsuperscript{-1} after 72 h in a sacrificial electron donor solution at pH 6 during solar AM 1.5G irradiation. An external quantum efficiency up to 4.8 % for photon-to-hydrogen conversion was achieved. The CN\textsubscript{x}-TiO\textsubscript{2}-H\textsubscript{2}ase construct was also active under UV-free solar light irradiation (λ > 420 nm), where it showed a substantially higher activity than TiO\textsubscript{2}-H\textsubscript{2}ase and CN\textsubscript{x}-H\textsubscript{2}ase due to the formation of a CN\textsubscript{x}-TiO\textsubscript{2} charge transfer complex and highly productive electron transfer to the H\textsubscript{2}ase. The CN\textsubscript{x}-TiO\textsubscript{2}-H\textsubscript{2}ase system sets a new benchmark for photocatalytic H\textsubscript{2} production with a H\textsubscript{2}ase immobilised on a noble- and toxic-metal free light absorber in terms of visible light utilisation and stability.

The use of efficient electrocatalysts in artificial photocatalytic schemes has been an area of recent interest for the conversion of protons to hydrogen using sunlight. Specifically, the use of redox enzymes in photocatalytic schemes highlights the importance of investigating the compatibility of biological systems with light harvesting materials and testing the stability of the resultant bio-hybrid assemblies.\textsuperscript{1} Hydrogenases (H\textsubscript{2}ases) are the most efficient noble-metal free electrocatalysts for H\textsubscript{2} production and achieve a turnover frequency (TOF) of more than 1000 s\textsuperscript{-1} with a small overpotential.\textsuperscript{2} H\textsubscript{2}ases also show impressive H\textsubscript{2} production rates and yields in sacrificial photocatalytic schemes in pH neutral aqueous solution.\textsuperscript{3} In these systems, a photoexcited light absorber provides electrons to the protein via an interlayer wire, the iron-sulfur electron relay, to the active site where proton reduction occurs. Examples are the immobilization of a H\textsubscript{2}ase or Ru-sensitised TiO\textsubscript{2},\textsuperscript{3} on CdS quantum dots\textsuperscript{4} as well as homogeneous systems using the H\textsubscript{2}ase with a covalently linked Photosystem I\textsuperscript{5} or in combination with an organic dye,\textsuperscript{6} and multi-component systems with a dye and a soluble redox mediator.\textsuperscript{7}

Polymeric carbon nitride (polyheptazine or melon, herein CN\textsubscript{x}) is a promising visible-light absorber for the photocatalytic generation of H\textsubscript{2}.\textsuperscript{8} We have recently reported the use of CN\textsubscript{x} as a light harvesting material in combination with a H\textsubscript{2}ase and a H\textsubscript{2}ase-inspired synthetic Ni catalyst for solar H\textsubscript{2} generation.\textsuperscript{9} The CN\textsubscript{x}-H\textsubscript{2}ase system showed sustained catalysis with a turnover number (TON) of more than 50,000 after 70 h solar light irradiation. However, this hybrid system suffered from a weak interaction between the H\textsubscript{2}ase and the CN\textsubscript{x} surface, and consequently, poor electron transfer from CN\textsubscript{x} to the H\textsubscript{2}ase. Furthermore, CN\textsubscript{x}-H\textsubscript{2}ase only showed efficient H\textsubscript{2} production up to wavelengths of approximately 420 nm and therefore only limited visible light harvesting capabilities.

Here, we selected a hybrid material consisting of TiO\textsubscript{2} (Hombikat UV 100, anatase, BET surface area: 300 m\textsuperscript{2} g\textsuperscript{-1}, crystallite size < 10 nm) surface-modified with CN\textsubscript{x} polymer as a light absorbing hybrid material for the photocatalytic system with a H\textsubscript{2}ase for three main reasons (Figure 1; see ESI and Figure S1 for synthesis and characterisation). Firstly, CN\textsubscript{x}-TiO\textsubscript{2} can be readily prepared on a gram scale by heating TiO\textsubscript{2} nanoparticles in the presence of urea, an inexpensive and sustainable material.\textsuperscript{10}

Secondly, CN\textsubscript{x}-TiO\textsubscript{2} provides us with substantially improved solar light harvesting performance compared to individual CN\textsubscript{x} and TiO\textsubscript{2}. Band gap excitation of TiO\textsubscript{2} (pathway 1) efficiently utilises the UV spectrum (band-gap of 3.2 eV for anatase TiO\textsubscript{2} with CB\textsubscript{TiO\textsubscript{2}} at approximately −0.6 V vs. NHE at pH 6).\textsuperscript{11} A significant portion of the visible spectrum is utilised with CN\textsubscript{x}-TiO\textsubscript{2} as it can, upon photo-excitation of CN\textsubscript{x}, perform photoinduced electron transfer from the LUMO\textsubscript{CN\textsubscript{x}} to CB\textsubscript{TiO\textsubscript{2}} (pathway 2). In addition, direct optical electron transfer can occur from the HOMO\textsubscript{CN\textsubscript{x}} (with contributions of molecular
Orbitals formed upon interaction of CN₄ with TiO₂ directed to the CB_{TOx} (pathway 3), extending the absorption even further into the visible region (up to 540 nm). This absorption pathway 3 is based on strong coupling between CN and TiO₂, resulting in strong charge-transfer absorption. Conclusive evidence of this charge-transfer includes previously reported spectroscopic, photoelectrochemical, and theoretical investigations. The generated CB_{TOx} electrons provide the H²ase with an overpotential of approximately 0.2 V for proton reduction.

![Chemical Science Accepted Manuscript](image)

**Figure 1.** (A) Schematic representation of photo-H₂ production with Dmb NiFeSe-H₂ase (PDB ID: 1CC1) on CN₄-TiO₂ suspended in water containing EDTA as a hole scavenger. (B) Irradiation of CN₄-TiO₂ can result in photo-induced electron transfer by three distinct pathways: 1. TiO₂ band gap excitation. 2. Excitation of CN (HOMO_{CN}–LUMO_{CN}) followed by electron transfer from LUMO_{CN} to the conduction band of TiO₂ (CB_{TOx}). 3. Charge transfer excitation with direct optical electron transfer from HOMO_{CN} to CB_{TOx}. The CB_{TOx} electrons generated through pathways 1 to 3 are then transferred via the [Fe₅S₆] clusters to the [NiFeSe] H₂ase active site.

Thirdly, the H₂ evolution catalyst employed in this study, Desulfomicrobium baculatum (Dmb) [NiFeSe]-hydrogenase is not only known for its high H₂ evolution activity, lack of H₂ inhibition and O₂-tolerance, but also for its titaniophilicity. This high affinity of the enzyme to adsorb strongly to TiO₂ stems from a protein surface rich in glutamatic and aspartic acid residues close to the distal [Fe₅S₆] cluster, which act as anchor sites to TiO₂ and allow for stable binding and efficient electron flow into the hydrogenase active site (Figure 1A). Thus, the CN₄-TiO₂ hybrid is expected to support a more robust H₂ase-particle interaction than with CN₄ alone, which would result in improved charge transfer and ultimately increased catalytic turnover for H₂ production.

Photocatalytic systems were assembled by dispersing CN₄-TiO₂ particles in an aqueous electron donor solution (0.1 M; 2.98 mL) in a photoreactor vessel (headspace volume: 4.74 mL; see ESI for experimental details). The vessel was sonicated under air (15 min) before sealing and purging with an inert gas (2 % CH₄ in N₂). The H₂ase (16.5 μL, 3 mM) was then added and the photo-reactor purged again to ensure anaerobic conditions. The stirred suspension was irradiated at 25 °C with a solar light simulator (air mass 1.5 global filter, I = 100 mW cm⁻²) and the headspace H₂ was quantified at regular time intervals by gas chromatography against the internal CH₄ standard. The conditions were optimised for maximum turnover frequency (TOF_{H₂ase}) by varying the electron donor and pH of the solution (Table S1; Figures S2 and S3). Optimised conditions consisted of ethylenediamine tetraacetic acid (EDTA; 0.1 M) as the electron donor at pH 6. A ratio of semiconductor (5 mg unless otherwise noted) to H₂ase (50 pmol) was used for ease of comparison to previously reported photosystems with Dmb NiFeSe-H₂ase.

Solar (UV-visible) irradiation (λ > 300 nm) of CN₄-TiO₂-H₂ase under standard conditions generated an initial TOF_{H₂ase} of (2.8 ± 0.3) × 10⁻² s⁻¹ with the production of 5.85 ± 0.59 μmol H₂ after 4 h and 28 ± 3 μmol H₂ with an overall TOF_{H₂ase} > (5.8 ± 0.6) × 10⁻² after 72 h (Figures 2 and S4). Negligible amounts of H₂ were detected in the absence of H₂ase, CN₄-TiO₂ or EDTA. UV band-gap excitation of TiO₂ did not result in the formation of O₂, which suggests that holes generated upon UV band-gap excitation of TiO₂ are either efficiently quenched by EDTA either directly or scavenged after being trapped by CN₄.

To qualitatively determine the contributions from the three excitation pathways in Figure 1B, irradiation was also performed with different long-pass filters. The CN₄-TiO₂-H₂ase system was studied under visible light irradiation at λ > 420 nm to study the contribution of CN₄ to light absorption (pathways 2 & 3) without the contribution of intrinsic absorption by TiO₂ (pathway 1). A photoactivity with an initial TOF_{H₂ase} of 6353 ± 635 h⁻¹ was observed, which results in the generation of 1.31 ± 0.13 μmol H₂ after 4 h. After 72 h, 13 ± 1 μmol of H₂ were generated with a TOF_{H₂ase} of more than (2.6 ± 0.3) × 10⁵ (Figure 2).
Subsequently, irradiation was carried out at $\lambda > 455$ nm to investigate the contribution of the direct charge-transfer from the HOMO$_{\text{CN}}$ to CB$_{\text{TiO}_2}$ to the photocatalytic activity. A TOF$_{\text{H}_2\text{ase}}$ of $1096 \pm 175$ h$^{-1}$ with the evolution of $0.26 \pm 0.06$ $\mu$mol H$_2$ after 4 h and $2.9 \pm 1.6$ $\mu$mol H$_2$ after 72 h was observed, which corresponds to 18% of the visible light activity. This suggests that all three pathways in Figure 18 contribute to the UV-vis photoactivity, whereas pathways 2 and 3 are responsible for the visible-light response of CN$_x$-TiO$_2$-H$_2$ase. Previous investigations of CN$_x$-TiO$_2$ hybrids have shown that their activity is limited by the strong electronic coupling between CN$_x$ and TiO$_2$ leading not only to intense visible light absorption but also to fast back electron transfer (primary recombination) $^{13,16}$

In order to study the role of TiO$_2$ as heterogeneous electron relay in CN$_x$-TiO$_2$-H$_2$ase in more detail, a sample of CN$_x$-ZrO$_2$ (15 mg) was also tested with the H$_2$ase. The negative CB$_{\text{ZrO}_2}$ at approximately $-1.35$ V vs. NHE at pH 6, prevents electron injection from LUMO$_{\text{CN}}$ (approximately $-1.25$ V vs. NHE at pH 6).$^{27}$ This band level mismatch allowed us to demonstrate that spatial proximity of surface-bound H$_2$ase to CN$_x$ alone cannot promote productive electron transfer as no H$_2$ was observed with CN$_x$-ZrO$_2$-H$_2$ase ($\lambda > 300$ nm; Figure S4). Thus, charge transfer from the HOMO$_{\text{CN}}$ into CB$_{\text{ZrO}_2}$ is not possible, nor is the direct electron transfer from LUMO$_{\text{CN}}$ to CB$_{\text{ZrO}_2}$, which are crucial to the formation of H$_2$ with the hybrid material.

For comparison, H$_2$ production was also tested with CN$_x$ (5 mg) and H$_2$ase (50 pmol) in the absence of metal oxide under standard conditions. A TOF$_{\text{H}_2\text{ase}}$ of 14852 $\pm$ 1485 was obtained after 4 h with an initial TOF of 6288 $\pm$ 649 h$^{-1}$ when irradiated with UV-visible light ($\lambda > 300$ nm, Table S1). Under visible light irradiation ($\lambda > 420$ nm), a TOF$_{\text{H}_2\text{ase}}$ of 2375 $\pm$ 267 was observed after 4 h and no H$_2$ was produced at $\lambda > 455$ nm, demonstrating the substantially enhanced activity with CN$_x$-TiO$_2$-H$_2$ase compared to CN$_x$-H$_2$ase at all wavelengths (Figure S4).

Experiments were also performed with TiO$_2$-H$_2$ase. While the system showed comparable activity under UV-visible irradiation due to efficient band gap excitation of TiO$_2$ (pathway 1), it showed significantly reduced activity under visible only irradiation at $\lambda > 420$ nm and displayed negligible H$_2$ yields at $\lambda > 455$ nm compared to CN$_x$-TiO$_2$-H$_2$ase (Figure S4).$^9$ Thus, UV-band gap excitation of TiO$_2$ dominates the absorption of the CN$_x$-TiO$_2$-H$_2$ase hybrid material under UV-light irradiation, which becomes less significant under visible irradiation.

The effect of light intensity on the photocatalytic activity ($\lambda > 300$ nm) was studied by employing neutral density filters. A photoactivity of approximately 90% remained when employing a 50% absorbance filter (50 mW cm$^{-2}$) and 44% of activity remained with an 80% filter (20 mW cm$^{-2}$; Figure S5). The initial non-linear decrease in activity implies that the system is not limited by light at 1 sun intensity as has been observed previously with synthetic H$_2$ evolution catalyst-modified Ru dye-sensitised TiO$_2$ systems.$^{18}$

The CN$_x$-TiO$_2$-H$_2$ase system sets a new benchmark for visible light driven and prolonged H$_2$ production with a heterogenised H$_2$ase without the need for expensive or toxic materials.$^{14,15,23}$ A part of this improvement can be attributed to the direct optical electron transfer (pathway 3) within CN$_x$-TiO$_2$, which draws the absorption of solar light significantly into the visible spectrum.

The enzyme loading onto CN$_x$-TiO$_2$ was calculated based on the BET surface area of 111 m$^2$ g$^{-1}$, a crystalline surface area of $\sim 314$ nm$^2$ per particle and an estimation that approximately one-quarter of the surface area of TiO$_2$ is accessible for the enzyme to adsorb. This equates to $\sim 0.7$ H$_2$ase per particle of CN$_x$-TiO$_2$. The approximate 1:1 enzyme:particle ratio allows the H$_2$ase to function at the maximum rate (i.e., TOF) as the maximum electron flux of conduction band electrons is directed towards a single enzyme. To qualitatively determine the amounts of surface-bound and solubilised H$_2$ase in the optimised system, H$_2$ase (50 pmol) was loaded onto CN$_x$-TiO$_2$ (5 mg) in aqueous EDTA solution by stirring under N$_2$ for 15 min. The suspension was centrifuged and the supernatant decanted (see ESI for experimental details). The CN$_x$-TiO$_2$-H$_2$ase pellet was re-dispersed in fresh EDTA solution (3 mL, 0.1 M, pH 6) and the photocatalytic vessel purged with 2% CH$_4$ in N$_2$. The suspension was then irradiated ($\lambda > 420$ nm) and H$_2$ production monitored (Figure 3). The H$_2$ production activity was nearly identical to a sample that was not centrifuged, both in the presence and absence of methyl viologen (MV$^2+$, see below), indicating that attachment of H$_2$ase to CN$_x$-TiO$_2$ is essentially quantitative. The substantially improved adsorption of the enzyme on the TiO$_2$ surface compared to the inert CN$_x$ polymer therefore also contributes to the increased activity of CN$_x$-TiO$_2$-H$_2$ase compared to CN$_x$-H$_2$ase. Previously an 88% decrease in photoactivity was observed with the poorly interacting CN$_x$-H$_2$ase after centrifugation and re-dispersion in fresh electron donor buffer.$^9$

The external quantum efficiency (EQE) of the CN$_x$-TiO$_2$-H$_2$ase system was measured by applying narrow band
pass filters (λ = 360 ± 10 nm; I = 2.49 mW cm⁻² and 400 ± 10 nm; I = 4.34 mW cm⁻²; see ESI for experimental details). UV-irradiation gave an EOE of approximately 4.8 % and under visible irradiation an EOE of 0.5 % was obtained. These values are more than a 10-fold improvement over the UV and visible EOE for the CN₄-H₂ase system, which can be attributed to the improved light absorption (Figure S6) and increased electron transfer rate due to adsorption of the H₂ase onto the particle surface.

We previously showed that a significantly increased photoactivity was observed under standard conditions using CN₄-H₂ase upon addition of an excess of the redox mediator MV²⁺, producing up to 77 μmol H₂ after 69 h of UV-visible irradiation. A long-term experiment with H₂ase (50 pmol), CN₄-TiO₂ (5 mg) and added MV²⁺ (5 μmol) in aqueous EDTA (0.1 M) at pH 6 was performed with both λ > 300 nm light and with visible light only (λ > 420 nm). Under UV-visible irradiation after 72 h, the CN₄-TiO₂-MV₂-H₂ase system produced 193 μmol H₂ with a TONₜₙ₉ₐ₉ of > 3.8 × 10⁷ and an initial TOFₜₙ₉ₐ₉ of 35 s⁻¹ (Figure S7). Under visible-light only, 66 μmol H₂ was produced with a TONₜₙ₉ₐ₉ of 1.3 × 10⁶ and an initial TOFₜₙ₉ₐ₉ of 9 s⁻¹ (Figure S8). The ratio of the amount of hydrogen produced in the presence and absence of MV²⁺ can be used to estimate the relative efficiency of the charge transfer from material to H₂ase. Under full spectrum irradiation (λ > 300 nm) with CN₄-H₂ase the ratio was found to be 22, whereas for both TiO₂-H₂ase and CN₄-TiO₂-H₂ase systems the ratio was 5. This strongly supports the fact that there is a significant improvement in the charge transfer from material to H₂ase. In addition, this ratio remains constant when the wavelength of light used is restricted to the visible region (λ > 420 nm).

The H₂ production rates in the presence of MV²⁺ are significantly higher than those obtained in the absence of MV²⁺. The blue colour of the vials containing MV²⁺ is indicative of the formation of reduced MV⁺ in solution (Figure S9). By comparison, addition of MV²⁺ to the previously reported Ru dye-sensitised TiO₂-H₂ase system caused a slight decrease in activity, which was attributed to the decreased availability of electrons for the H₂ase and the absorption of incident photons by MV⁺. Here, solubilised MV⁺ does not limit light absorption by CN₄-TiO₂ significantly and is able to efficiently donate electrons to surface-bound H₂ase, resulting in increased H₂ production. This result implies that interfacial electron transfer from CN₄-TiO₂ to H₂ase is still not fully optimised in this system, where the orientation of the H₂ase is not fully ‘directed’. Ideally, the distance from the CN₄-TiO₂ surface to the [Fe₃S₅] electron transport chain should be minimised and an improved orientation of the enzyme would allow trapping of CB₃TiO₂ electrons more efficiently for maximised turnover.²⁹

Favourable electron transfer kinetics at the CN₄-TiO₂-H₂ase interface can be assumed based on previous reports. Electron transfer in the order of 10⁻¹ s⁻¹ was reported from CdS nanorods to an [FeFe]-H₂ase isolated from Clostridium acetobutylicum. In addition, a long lived photo-excited state lifetime of τ₂/3 ~ 0.8 s was previously reported for TiO₂ conduction band electrons in a photocatalytic system with Ru dye-sensitised TiO₂ and electron transfer to co-immobilised molecular cobaloxime catalysts occurred with τ₂/3 ~ 5 to 50 μs.²⁰ Based on these previous reports, we can assume that a reasonably long-lived TiO₂ conduction band electron is generated and that H₂ase is capable of collecting these electrons readily.

In summary, solar light driven H₂ production with a semi-biological system consisting of TiO₂ modified with polymeric CN₄ and immobilised H₂ase has been demonstrated. We have shown that by improving the surface interaction of the enzyme with the light harvesting CN₄ material, specifically by adsorption of the enzyme onto the TiO₂ surface, H₂ generation is drastically improved. Another important factor is the improved visible light absorption by direct CN₄ excitation (pathway 2) and CN₄-TiO₂ charge transfer (pathway 3), which enables high photoactivity. The CN₄-TiO₂-H₂ase assembly achieved a TOF of 8 s⁻¹ and a TON of > 5.8 × 10⁷ after 72 h in the absence of an external soluble redox mediator, thereby setting a new benchmark for photochemical architectures based on abundant and non-toxic materials and a heterogenous H₂ase. The additional use of the redox mediator MV²⁺ allowed for the photo-generation of H₂ with a TOF of 35 s⁻¹ and a TON of > 3.8 × 10⁶. This work advances the use of hybrid photocatalytic schemes by integrating highly active electrocatalysts with advanced light absorbing materials such as CN₄-TiO₂, which is shown to be compatible with H₂ases in aqueous solution.

Notes and references
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Solar light driven hydrogen production with a heterogenised hydrogenase on a carbon nitride-TiO$_2$ hybrid is reported that sets a new benchmark for photo-H$_2$ production.