Hydrogen Production

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Silicification-Induced Cell Aggregation for the Sustainable Production of H_2 under Aerobic Conditions

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Abstract: *Photobiological hydrogen production is of great importance because of its promise for generating clean renewable energy. In nature, green algae cannot produce hydrogen as a result of the extreme sensitivity of hydrogenase to oxygen. However, we find that silicification-induced green algae aggregates can achieve sustainable photobiological hydrogen production even under natural aerobic conditions. The core–shell structure of the green algae aggregates creates a balance between photosynthetic electron generation and hydrogenase activity, thus allowing the production of hydrogen. This finding provides a viable pathway for the solar-driven splitting of water into hydrogen and oxygen to develop green energy alternatives by using rationally designed cell–material complexes.*

Hydrogen (H_2) is considered to be a promising substitute for fossil fuels owing to its superb conversion efficiency, environmental friendliness, and high energy capacity.[1] H_2 production today is predominately dependent on steam reformation from hydrocarbons, coal gasification, and nuclear-powered water electrolysis, which are energy-intensive and unsustainable.[2] Biological H_2 production from solar power offers the possibility of generating H_2 that is renewable and carbon-neutral, as it directly uses inexhaustible resources: sunlight energy and electrons from H_2O.[3] In nature, photosynthetic microorganisms, particularly green algae, can photolyze water to produce H_2 using hydrogenase (an enzyme that catalyzes the reversible oxidation of molecular hydrogen) coupled to the photosynthetic machinery.[4] However, this is a transient process that occurs within a few minutes during dark–light transition.[5] This is because hydrogenase loses its function in the presence of oxygen.[6] Under darkness, cellular respiration creates anaerobic conditions that activate hydrogenase.[7] At the moment of transition from darkness to light, photosynthetic electrons originating from the water photooxidation reaction ($H_2O \rightarrow 2H^+ + 1/2\,O_2 + 2e^-$) in the photosystem II (PSII) reaction center can be delivered to hydrogenase to produce H_2 ($2H^+ + 2e^- \rightarrow H_2$). However, the oxygen generated from the photosynthetic reaction quickly inactivates hydrogenase.[8] To achieve an improved photobiological H_2 production, scientists have screened for[9a,b] and constructed mutants with improved oxygen tolerances and decreased intrinsic hydrogenase oxygen sensitivities through various strategies.[9c] Unfortunately, limited progress has been made. Currently, sulfur deprivation is the most common method used to generate an anaerobic environment for green algae.[7a] However, this metabolic treatment can simultaneously suppress PSII activity and gradually terminate subsequent H_2 production.[7a,10] As a consequence of the unfavorable results, large-scale application of green algae in H_2 production is still not realistic.

In nature, many living organisms, such as diatoms, coccoliths, and magnetotactic bacteria, have developed specific mineral structures from biomineralization processes to provide extensive protection and unique functions.[11] Recent achievements suggest that biomimetic mineralization can be developed as a useful tool to modify cells and viruses, and the resulting cell–material complexes always have different biological characteristics from the native ones.[12] Herein we report a biomimetic silicification that can confer green alga with a new capacity for sustainable photobiological H_2 production under natural atmospheric conditions.

Chlorella pyrenoidosa (*C. pyrenoidosa*) is a unicellular green alga with a prominent pyrenoid within its chloroplast, and is also one of the few commercialized microalgae species that has been cultured on a large scale. Typically, the cells are in a unicellular state, 3–4 μm in diameter, without tendency to self-aggregate (Figure 1a). It has been well-established that poly(diallyldimethylammonium chloride) (PDADMAC) molecules can simulate silicification proteins to induce in situ silica deposition onto cell surfaces,[12b,e] which could also be applied to green algae. After the biomimetic modification, the silicified *C. pyrenoidosa* cells could be self-aggregated (Figure 1b). For this reason, the silicified cell had the reduced zeta potential (about −3 mV) in comparison with the native cell (about −15 mV), which was favorable to particle agglomeration (Supporting Information, Figure S1). We also confirmed that the cells cohered with each other via amorphous SiO_2 (Supporting Information, Figure S2) to form the cell–material aggregates during the silicification. The aggregate sizes could be controlled to 10–500 μm by the

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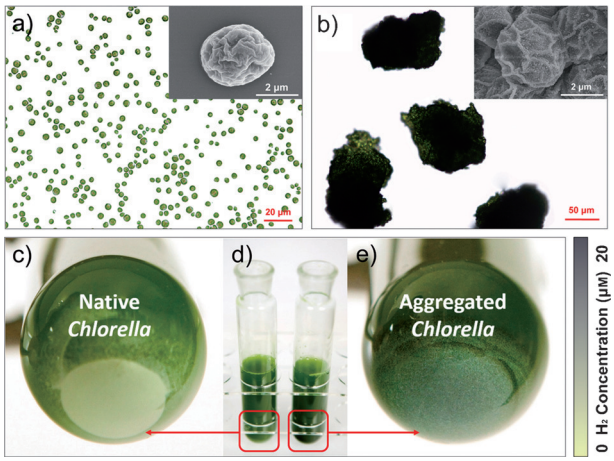


Figure 1. *Chlorella pyrenoidosa* cells and their aggregates. a) Native cells viewed using an optical microscope and a scanning electron microscope (SEM). Scale bars: 20 μm (inset: 2 μm). b) Aggregated cells viewed using an optical microscope and an SEM. Scale bars: 50 μm (inset: 2 μm). c) Native *Chlorella* culture medium with WO_3 powders (base of the tube). d) Native and aggregated *Chlorella* culture medium with WO_3 powders in the tubes. e) Aggregated *Chlorella* culture medium with WO_3 powders (base of the tube). The bar indicates the standard color changes of WO_3 in the presence of H_2 in solutions.

regulation of *C. pyrenoidosa* density in the cell silicification medium (Supporting Information, Figures S3 and S4).

The property of color change of some metal oxides with their oxidation state has been exploited for convenient optical H_2 detection.[13] Tungsten oxide (WO_3) is commonly used as it reacts with H_2 to produce tungsten bronze, which is accompanied by a color change from light yellow to blue gray.[13] Here WO_3 powder was added to the cell cultures as an indicator of H_2 production under ambient atmosphere. The H_2 production ability of the native and aggregated *C. pyrenoidosa* (ca. 100 μm) is shown in Figure 1 c–e. In this experiment, *C. pyrenoidosa* (the total cell density in the culture medium was 3.0×10^7 cells mL^{-1}) was illuminated under a light intensity of 100 μE $m^{-2}s^{-1}$ for 12 h. In native *Chlorella*, the unchanged color of WO_3 powder indicated no detectable H_2 in the system (Figure 1 c), consistent with the conventional opinion that green algae cannot produce H_2 under natural aerobic conditions. However, in aggregated *Chlorella*, the color of the WO_3 powder turned blue–gray (Figure 1 e), following the production of H_2 at a solution concentration of about 10 μmol L^{-1} (Supporting Information, Figure S5). It should be noted that the culture medium was exposed to air, and consequently the H_2 generated by the aggregated cells occurred under natural atmospheric conditions.

To quantitatively investigate the properties of H_2 production in aggregated *Chlorella* cells, we monitored the amounts of H_2 and O_2 in the headspace of airtight glass tubes using gas chromatography (Figure 2 a). Culture medium (30 mL) containing *Chlorella* (1.2×10^8 cells mL^{-1}) was added into 60 mL airtight glass tubes under illumination (100 μE $m^{-2}s^{-1}$). For the native *Chlorella*, H_2 in the headspace cannot be detected at any measuring points, while the O_2 content in the headspace increased from an initial 21 % to 23 %. The results

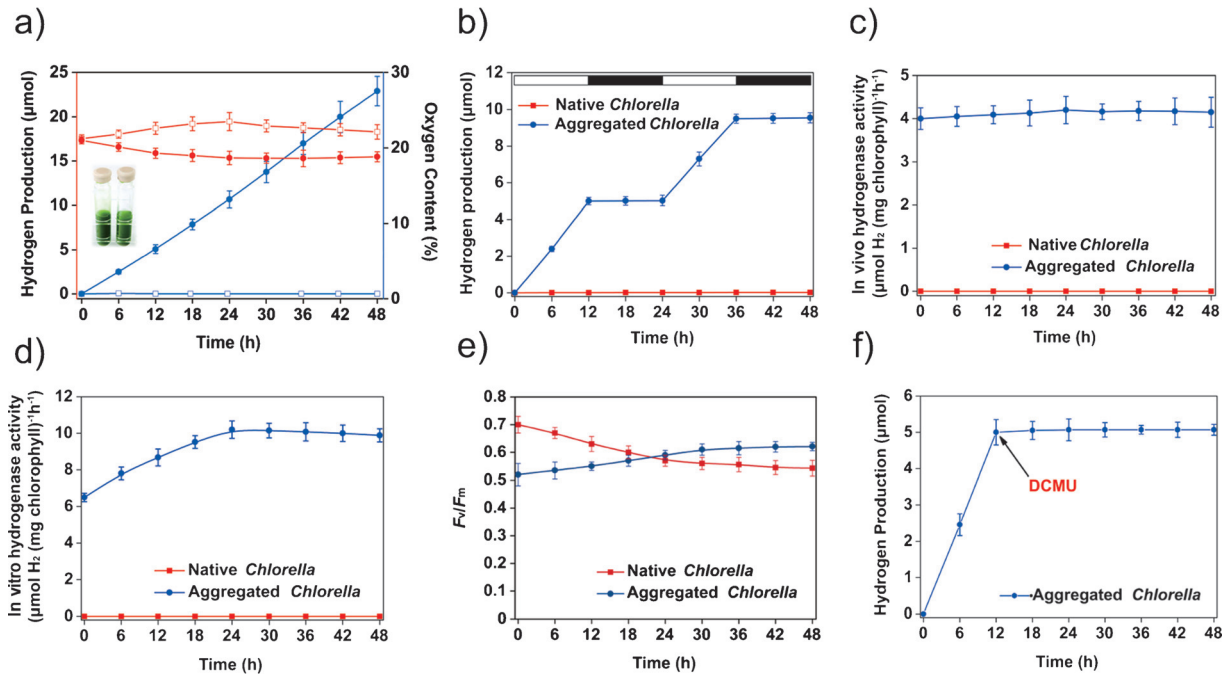


Figure 2. Photobiological hydrogen production by aggregated *Chlorella* under aerobic conditions. a) The amount of H_2 and the content of O_2 in the headspace of sealed tubes under a light intensity of 100 μE $m^{-2}s^{-1}$ at different time periods ($n=6$). Red lines: O_2%. Blue lines: H_2amount. Open squares: native *Chlorella*. Filled circles: aggregated *Chlorella*. b) Cumulative H_2 accumulation in sealed tubes with aggregated *Chlorella* during light-dark transitions ($n=5$). c) In vivo hydrogenase activity in native and aggregated *Chlorella* ($n=5$). d) In vitro hydrogenase activity in native and aggregated *Chlorella* ($n=5$). e) Maximum quantum yield of PSII (F_v/F_m) of native and aggregated *Chlorella* under a light intensity of 100 μE $m^{-2}s^{-1}$ at different time periods ($n=3$). f) The effect of DCMU on H_2 production by aggregated *Chlorella* ($n=3$).

indicated that the native *Chlorella* cells performed photosynthetic O_2 evolution but not H_2 production, owing to inactivation of hydrogenase under aerobic conditions. Interestingly, the 100 μm aggregates were observed to continuously release H_2 at a constant rate of about 0.35 μmol H_2 h^{-1} (mg chlorophyll)$^{-1}$ (Figure 2a; Supporting Information, Figure S6). This rate was 1.75 times the instantaneous biomass-to-fuel yield in nature (0.20 μmol H_2 h^{-1} (mg chlorophyll)$^{-1}$).[14] More importantly, this H_2 production was not limited to a transient period of a few minutes; rather, this production was detected over a period of at least 48 h. Subsequently, the rate of H_2 production started to decrease (Supporting Information, Figure S6), which is likely due to compromised aggregation structures resulting from cell proliferation within the aggregates (Supporting Information, Figure S7). The production of H_2 could be sustained for at least 60 h prior to the aggregate structure decomposition (Supporting Information, Figures S6 and S7). In contrast to native *Chlorella*, we noted that the O_2 content in the headspace of the aggregates decreased from 21% to 19% during the experiment. This decrease was likely attributed to cell respiration that consumed the O_2 molecules present in the closed system. In the aggregated cells, O_2 evolution decreased and consequently H_2 production increased. Furthermore, during the alternating periods of light and dark (Figure 2b), we found that the aggregates only generated H_2 during light periods; no H_2 was detected under dark conditions. This light–dark switch effect implied that the H_2 production was directly related to photosynthesis.

In green algae, photosynthetic water splitting is functionally linked to H_2 production by the activated hydrogenases. In the native *Chlorella*, neither in vivo nor in vitro hydrogenase activity could be detected (Figure 2c and d), because hydrogenase is inactivated in the presence of environmental oxygen.[6] In the *Chlorella* aggregates, in vivo hydrogenase activity slightly increased in the first 24 h, and then remained at a relatively stable level of 4 μmol H_2 h^{-1} (mg chlorophyll)$^{-1}$ (Figure 2c), which is also higher than the rate of H_2 production from atmospheric argon (Supporting Information). This result showed that the hydrogenase in the aggregates was still active at atmospheric oxygen levels. In vitro examination confirmed the presence of hydrogenase activity in the aggregates, and the values even increased from 6.5 to 10.3 μmol H_2 h^{-1} (mg chlorophyll)$^{-1}$ within the first 24 h (Figure 2d). These results indicated that the aggregation treatment may favor the expression of the hydrogenase to enhance the H_2 production potential.

Apart from hydrogenase, another important factor affecting the sustained photobiological H_2 production is its catalytic substrates, that is, photosynthetic electrons, that originate from the water oxidation reaction in the PSII reaction center.[15] PSII is a large multisubunit membrane protein complex that can capture photon energy, separate charge, and drive the electron transfer in the thylakoid membrane.[16] The maximum quantum yield of PSII (F_v/F_m) has been extensively used to evaluate the activity of PSII. The value of F_v/F_m in native *Chlorella* was 0.7 at the beginning of the experiment, and decreased to about 0.5 in the end (Figure 2e). This decrease was most probably the result of stress caused by the sealed environment. The F_v/F_m parameter in the aggregated *Chlorella* decreased to approximately 0.5 at the beginning of the experiment (Figure 2e), possibly caused by the aggregated treatment. However, the maximum quantum yield of PSII in the aggregated *Chlorella* increased from an initial value of 0.52 to a final value of 0.62 (Figure 2e). The results indicated that an appropriate aggregation treatment almost did not affect the activity of PSII in the enclosed cells, unlike the effects of the sulfur deprivation treatment.[17] The compound 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is a specific inhibitor of PSII that disrupts the transfer of electrons from Q_A to Q_B,[17] and its addition to the aggregates completely terminated hydrogen production even under light (Figure 2f). This phenomenon confirmed the necessity of the coupling between hydrogenase and the photosynthetic machinery for the production of H_2 in the aggregated cells.

Microsensors are a reliable tool to study biological microenvironments in the aggregates (Figure 3a; Supporting Information, Figure S8). Here we used H_2 and O_2 microsensors to examine the interior environment in our aggregates. In the 100 μm aggregates, H_2 production was not detected on the aggregate surface. The H_2 concentration increased as the depth of the probe increased, and it reached a maximum (ca. 1.7 μmol L^{-1}) at the aggregate core (Figure 3b). This profile implied that H_2 was produced by the core cells rather than the surface cells. In contrast, the O_2 concentration decreased as the probe depth increased. The core microenvironment was oxygen-deficient, and the O_2 concentration was nearly zero at the core center, where hydrogenase was activated and consequently H_2 was produced. The spatial H_2 and O_2 distribution in aggregated *Chlorella* cells implied the presence of spatial–functional differentiation (SFD) between cells.

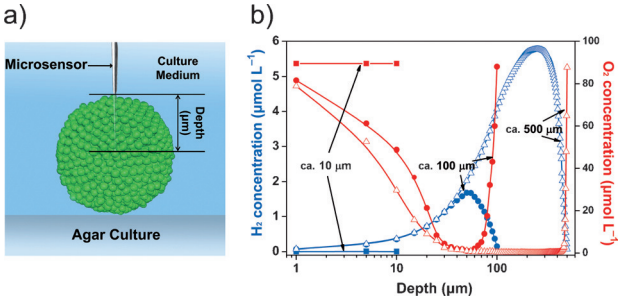
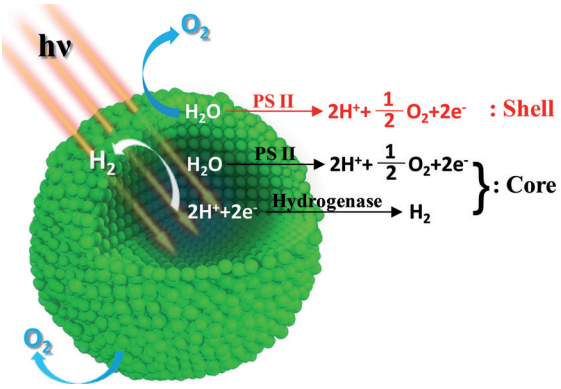


Figure 3. Spatial functional differentiation of aggregated *Chlorella*. a) Diagram of microsensor measurement. b) H_2 (blue) and O_2 (red) microprofiles of aggregated *Chlorella* with different sizes ($n = 5$).

The aggregate can be considered to be an autogenously structured core–shell complex with differentiated cell functions. The surface cells were exposed to the open environment, and their functions were similar to those of the native algae. However, the cells on the exterior also acted as a shell to prevent the penetration of ambient O_2 into the aggregate core. The respiration reaction of the inner cells consumed any diffused O_2 or photosynthetically produced O_2 to create a hypoxic domain. This anaerobic condition was a result of the dynamic balance between O_2 diffusion, O_2 generation by

PSII, and cellular respiration in the isolated system defined by the enclosed shell space. The hydrogenase and PSII activities could be maintained in this domain, and their coupling guaranteed a sustainable photobiological H_2 production. The functional transformation from the individual green alga to aggregated green algae can be understood by the SFD in the core–shell structure: the core cells gained H_2 production capabilities because of the isolated anaerobic microenvironment; the shell cells retained the native algae functions and isolated the core cells from the open environment (Scheme 1).



Scheme 1. Spatial–functional differentiation in aggregated *Chlorella* cells.

The core–shell-based SFD effect on the capacity for photobiological H_2 production could be adjusted by controlling the size of the aggregates. For small aggregates with diameters of about 10 μm, the H_2 concentration in both the internal and external cells could be ignored (Figure 3b). The O_2 concentration remained at the same level (ca. 90 μmol L^{-1}) from the outside to the core (Figure 3b). The results indicated that there were no SFD in small aggregates with diameters of 10 μm. As the aggregate sizes increased, however, more core cells were enclosed and isolated from the open environment. The microsensor measurement showed that large aggregates (500 μm) exhibited a better H_2 production capability than the 100 μm aggregates. The H_2 concentration reached 5.3 μmol L^{-1} at the core of the large aggregate, while the O_2 concentration decreased to zero as the depth of the probe increased to nearly 50 μm (Figure 3b). Therefore, an optimal SFD for photobiological H_2 production is highly dependent on the aggregate size.

The experiments in the airtight glass tubes showed that overall, relative to the 10 μm and the 500 μm aggregates, the 100 μm aggregates (with the same initial total chlorophyll content) exhibited a superior capability for H_2 production (Supporting Information, Figures S9 and Figure S10). The 10 μm aggregates could hardly produce H_2 or perform hydrogenase activity. That the amounts of headspace H_2 produced by the 500 μm aggregates were less than those produced by the 100 μm aggregates at different time periods (Supporting Information, Figure S9). Although the in vitro hydrogenase activity in the 500 μm aggregates was higher, the in vivo hydrogenase activity of the 500 μm aggregates displayed lower than that in the 100 μm aggregates (Figure 2c and Supporting Information, Figure S11). This was likely because of an unfavorable limit for internal photosynthetic electron generation by PSII, that was demonstrated by measuring the PSII activity (Supporting Information, Figure S12). This indicates that the coupling of hydrogenase and PSII was key to guaranteeing photobiological H_2 production. An examination of the PSII efficiency showed that the F_v/F_m value for the 500 μm aggregates was only 0.1–0.3. Without sufficient photosynthetic electrons, the core cells in the large aggregates could not efficiently produce H_2 by hydrogenase despite the high activity of the hydrogenase.

It is acknowledged that the use of oxygenic photosynthetic microorganisms that can produce H_2 under aerobic conditions would be an important step forward for biological H_2 production.[18] However, it is still a great challenge to screen for natural species with significant oxygen tolerance. Recently, Hwang et al. identified a novel microalgal strain (*Chlorella vulgaris* YSL01) and this strain can continuously produce up to 1.9 mL H_2 (L culture)$^{-1}$ with 5% O_2 at 10% CO_2.[9b] However, this H_2 production only occurs in the artificial rather than the natural aerobic environment. In our study, the aggregated green algae (*C. pyrenoidosa*) can continuously produce about 0.5 mL H_2 (30 mL culture)$^{-1}$, which is equivalent to about 17 mL H_2 (L culture)$^{-1}$. This value under natural condition is about 9-fold more than *Chlorella vulgaris* YSL01 under modified conditions.[9b] Our study demonstrates that the green algae aggregates are capable of photoautotrophic H_2 production during exposure to continuous illumination, which provides a first case of sustained photobiological H_2 production under natural aerobic conditions. This achievement is essential to promoting green energy development.

In our attempt, the in situ silification can induce cell aggregation of *C. Pyrenoidosa*, because the cells can be cohered by silica materials, which is dependent upon a biomimetic chemical modification of cell surface. This chemical–material cell engineering can result in a new function of H_2 production through the novel cell-material complexes that is feasible, inexpensive, and effective. More generally, the similar chemical–material-based modification of cells may be extended to other microorganisms to induce a designed functional transformation. It further follows a biomineralization-inspired strategy for organism evolution via chemical and material pathways.

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