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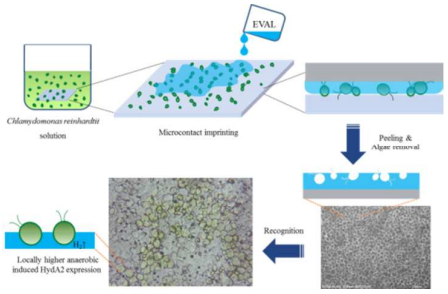

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ARTICLE TYPE

Recognition of Algae to Microcontact-imprinted Polymers Modulates Hydrogenase Expression

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Because extracellular matrix is known to affect animal cell metabolism, there is good reason to explore whether physical and chemical environment might similarly affect algal cell metabolism and, consequently, hydrogen production, which can be used for electricity generation in miniature biofuel cells. In this work, green algae were microcontact-imprinted onto a poly(ethylene-co-vinyl alcohol) (EVAL) film. Scanning electron microscopy was used to characterize the surface morphology, and Raman spectroscopy was used to assay for algal cytochrome species involved in electron transfer and hydrogen production. The production of hydrogen by algae was measured electrochemically. The power and current density of algal biofuel cells that use algae-imprinted, or non-imprinted EVAL-coated and control electrodes were measured and compared. Finally, hydrogenase mRNA levels in algae on various substrates were measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR), providing direct evidence that culturing algae on the imprinted substrate leads to increased hydrogenase gene expression.

Introduction

Cellular imprinting is attractive for biosensing,[1-5] bioseparation[6-8] and modulating cellular gene expression.[9, 10] The two basic approaches to synthesize cell-imprinted substrates are (1) microcontact imprinting using soft lithography, and (2) cell replication or mold casting with polydimethylsiloxane (PDMS). In the former, cells or microorganisms are adsorbed on either a glass slide[7] or PDMS,[5] which are used as stamps and pressed on spin- or drop-coated pre-polymers[1, 3, 11] or sol-gel materials.[2, 4, 12] In the latter, cells are treated with fixation materials, such as formaldehyde and/or glutaraldehyde[8] and then PDMS[6, 8-10, 13] is utilized as a shaping material because its micro/nanostructure is easily molded. It is not clear whether these methods give rise to good *molecular* recognition of cell surface biomolecules by the imprinted polymer, as they may not optimize the molecular interactions between complementary functional groups. However, in many cases they still provide the ability to recognize and bind imprinted cells, possibly more by shape recognition of the cell[14] than by cellular surface biomolecule recognition.

Recently, the interactions between cells and imprinted polymers were studied by selective separation. A study of imprinting of osteosarcoma cells showed that cells that were cultured longer (14 days vs. 4 hours) prior to imprinting gave rougher and deeper cavities, on average; furthermore, cells grown on the more deeply imprinted surface exhibited higher cellular viability and activity (in, for example, bone mineralization by the human osteosarcoma cell line (MG-63)).[9] A culture of adipose-derived mesenchymal stem cells (ADSCs) grown on PDMS imprinted with mature or dedifferentiated chondrocytes for a week exhibits a change in gene expression (e.g. type I and II collagen and aggrecan, which are important for chondrogenesis).[10]

In Chlamydomonas reinhardtii (C. reinhardtii) (a soil-dwelling microalga), hydrogen is efficiently produced by the reduction of free protons by hydrogenase.[15] Mutants of C. reinhardtii exhibited enhanced hydrogen production by a factor or up to ten, by targeting hydrogenase, sulfate permease, ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), water-plastoquinone oxidoreductase (photosystem II, PSII), starch reserves or respiration genes. However, some *C. reinhardtii* mutant strains may exhibit deficient hydrogen production.

In our previous work, *C. reinhardtii*, was used as the template organism, and microcontact imprinting was performed on poly(ethylene-*co*-vinyl alcohol) via solvent evaporation.[16] A fuel cell was then assembled using an algae-immobilized algae-imprinted EVAL anode and its voltage output was measured to evaluate the hydrogen production performance. Surprisingly, the power and current density of the algal biofuel cell with the algae-imprinted EVAL-coated electrode were measured to be approximately double those of the cell with the electrode that comprised platinum sputtered on poly(ethylene terephthalate).

In this work, the surface morphology of, and recognition of algae by, the microcontact imprinted poly(ethylene-*co*-vinyl alcohol) films were characterized by scanning electron microscopy. The power and current density of the algal biofuel cell with the algae-, non-imprinted EVAL-coated and control electrodes were also determined. The levels of β-carotene in the algae were assayed with Raman spectrometry. Finally, the hydrogenase mRNA levels in algae on various substrates was

examined with quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Experimental Section

Reagents

Poly(ethylene-*co*-vinyl alcohol)s (EVALs) containing ethylene of 38 mol% was purchased from Scientific Polymer Products (Ontario, NY). Dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS) and potassium hydrogen phosphate were from J. T. Baker (ACS grade, NJ). Potassium dihydrogen phosphate and sodium chloride were from Riedel-deHaën Co. ((Seelze, Germany) and Sigma-Aldrich Co. (St. Louis, MO), respectively. *Chlamydomonas reinhardtii* was a generous gift by Professor Chung-Kuang Lu at National Museum of Marine Biology and Aquarium (Pingtung, Taiwan). The Tris-Acetate-Phosphate (TAP) medium[17] for *Chlamydomonas reinhardtii* was purchased from *phyto*technology laboratories (Shawnee Mission, KS). The KingFisher Total RNA Kit (Cat. #: 97020196, Thermo Scientific, Vantaa, Finland) includes KingFisher magnetic beads 3.1 mL, rDNase 3vials, rDNase buffer 35 mL, reducing agent (tris(2-carboxyethyl)phosphine, TCEP) 1 vial, lysis buffer 40 mL, binding buffer 75 mL, wash buffer I 65 mL, wash buffer II 200 mL, elution buffer 20 mL and RNase-free water 120 mL. The Deoxy+ real-time 2x SYBR green RT-PCR kit contains (1) RealStart Taq DNA polymerase; (2) Reverse transcriptases; (3) dATP , dCTP , dGTP , dTTP mix; (4) 5 mM $MgCl_2$; (5) SYBR® Green I and (6) ROX was purchased from Yeastern Biotech Co., Ltd, Taiwan. Diethylpyrocarbonate (DEPC) water was purchased from Protech Technology Enterprise Co, Ltd., Taiwan. All chemicals were used as received unless otherwise mentioned.

Preparation of Alga-imprinted Polymer Coated Electrodes

The preparation of alga-imprinted polymer coated electrodes followed a published protocol[16] with the following changes: An indium tin oxide (ITO) coated poly(ethylene terephthalate) (PET) thin film was employed, cleaned and sputtered with platinum to achieve a higher power output of the biofuel cells systems than was obtained in our previous work.[16] Then, a glass slide with the adsorbed algae was cast with the EVAL solution (7 wt% in DMSO) and covered with the Pt/ITO/PET electrode as shown in Scheme 1. Finally, the microcontact-imprinted algae were completely removed by washing with deionized water for 3 hrs on an orbital shaker (OSR201- 01, GenePure Tec., Taiwan) at 30 rpm.

Cultivation of Algae and Characterization of Alga-imprinted Polymeric Thin Films

Bottles and medium for algae cultivation were sterilized in an autoclave. The chlorophyll a concentration (or the Chl a + pheophytin a concentration) is routinely measured and often is the only available index used to quantify the biogenic content.[18] Typically, the algae concentration is taken to be proportional to the chlorophyll a fluorescence[19] with excitation wavelength of 485 nm and emission wavelength of 685 nm, using a fluorescence spectrophotometer (F-7000, Hitachi Co., Japan). The algae cell numbers can be calibrated with fluorescence intensity in the *Electronic Supplementary Information* Fig. S-1. Various algae concentrations were seeded and cultivated under 15000 Lumen white light illumination for 14 hrs/day. Alga-imprinted polymers before washing, after washing and rebinding were freeze-dried and then examined examination by a scanning electron microscope (Hitachi S4700, Hitachi High-Technologies Co., Tokyo, Japan). The adsorption of algae to the alga-imprinted polymer film was done by immersion into 2 mL algae solution (10^7 cells/mL) for 60 minutes; films were then examined by a Raman microscope (NTEGRA Spectra, NT-MDT Co., Moscow, Russia) equipped with a 532 nm laser, with output power is up to 22±2 mW. The instrument's proprietary "Nova" software was used to control the system.

The Performance Measurement of the Algae Fuel Cell

The power output of the algal biofuel cells was measured with the protocols reported previously.[16] In this work, algae were imprinted onto EVAL on the Pt sputtered indium tin oxide (ITO)-coated poly(ethylene terephthalate) (PET) thin films, rather than the PET thin films used in our previous studies. A fuel cell was assembled using the algae-imprinted coated Pt/ITO/PET electrode (2.5x 4.5 cm^2) (in culture medium – magnesium suphate heptahydrate), platinum wire (5 cm), and Nafion 117 film (2.0x 2.0 cm^2, DuPont Fuel Cells, Wilmington, DE) as the proton exchange membrane. The polarization curves were plotted by measuring the resulting cell current under fixed cell potential (V, potentiostatic control)[20] from the maximum voltage output by -0.05 V/step by a potentiostat (model 608-1A, CH instruments Inc., Austin, TX). Power density (P = VI/A) was then calculated from the measured current (I) and surface area (A) of the anode electrode.[21]

Gene Expression of Algae on Algae-imprinted EVAL Thin Films

The sequence (5'- 3') of primers for HydA2[22] and 16S rRNA genes: HydA2, Forward: CCCGACTCAGCCTACTTGTT; Reverse: CGCCTCCCACTACCACCATA. 16S rRNA, Forward: CAGCTCGTGTCGTGAGATGT; Reverse: TAGCACGTGTGT AGCCCAAC. The total RNA from the algae from the algae cultured for one day on the substrates was purified using the KingFisher. otal RNA Kit and the KingFisher mL magnetic particle processors, both from Thermo Scientific. (Vantaa, Finland) RNA extraction was done following the KingFisher kit protocol. The concentration of cellular RNA was quantified by determining the absorbance maximum at the wavelength of 260 and 280 nm to give the optimum OD between 1.6 to 2.0 in a UV/Vis spectrometer (Lambda 40, PerkinElmer, Wellesley MA). Complementary DNA was obtained by mixing 1 μL of total RNA and 19 μL of reaction mixture including 10 μL of Deoxy+ real-time 2x SYBR green RT-PCR premix, 7 μL of diethylpyrocarbonate (DEPC) water and 1 μL of forward and reverse primers (Yao-Hong Biotechnology Inc., HPLC grade, New Taipei City, Taiwan) in Table 1. Finally, the mixture was kept at 48 °C for 30 min and then incubated at 90 °C for 10 min. The real-time PCR was performed in a PikoReal real-time PCR system (Thermo Scientific, Vantaa, Finland). Relative gene expression was determined using a $\Delta\Delta$Cq method[23] and normalized to a reference gene (16S rRNA) and to a treatment control (free algae).

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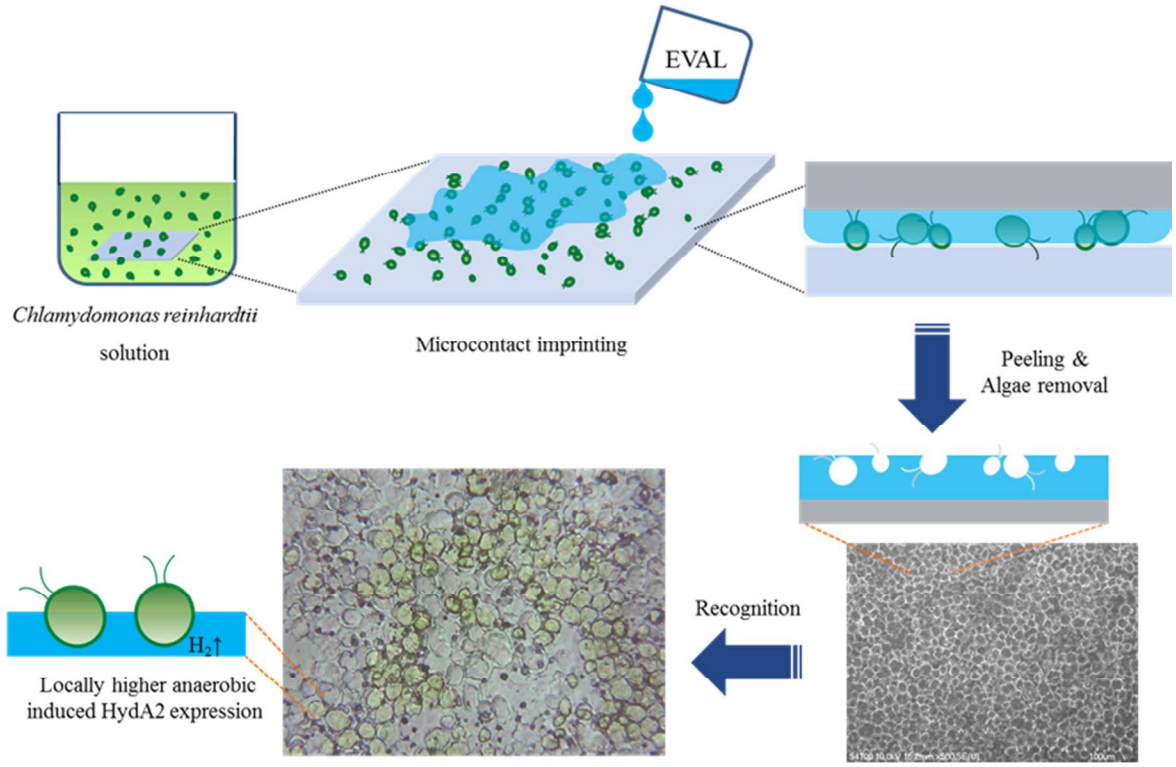

Scheme 1. The positive modulation of hydrogen production from algae adsorbed on the microcontact algae-imprinted polymers.

Results and Discussion

Figure 1 plots the growth curves of *Chlamydomonas reinhardtii*. In Fig. 1(a), the saturated algae concentrations were 1.2~1.4 x 10^7 cells/mL. As the inoculated concentration varied, the saturation time varied from one to nearly two weeks, as presented in Fig. 1(b). Therefore, a centrifuge was required at higher algae concentrations to prepare the algae stamps using a 3 mL 1.5x10^7 cell/mL algae solution for 45 mins. An algae concentration of 1x 10^7 cells/mL was used in subsequent adsorption and biofuel experiments. Figure 2 displays the surface morphology of the microcontact-imprinted algae. The peeling of stamps from the EVALs in Fig. 2(a) reveals that some template algae had also been removed; however, algae were retained in the darker area in the figure. The diameter of the imprint by an alga was approximately 10 μm. Figure 2(b) shows the algae-imprinted cavities after the algae had been carefully removed; the figure shows the surface structures that are complementary to algae. Owing to the flagellar motility of the algae, the binding of alga by an imprinted cavity may be transient, with a duration of less than 60s on average (c.f. the multimedia file in the reference 16),[16] thus reducing the amount of algae that can be re-adsorbed, Figure 2(c). Nonetheless, imprinting does increase overall algal binding and biofuel cell performance (compared with non-imprinted films.)

Figure 3 presents the micro-Raman spectra of an alga bound to an AIP (alga-imprinted polymer region), an apparently vacant alga-imprinted cavity, and a NIP (non-imprinted polymer region). For the alga, three major characteristic peaks of β-carotene were obtained at wavenumbers 1004, 1155 and 1522 cm^{-1},[24] corresponding to the methyl rocking, C-C and C=C stretching,[25] respectively. Three unique prosthetic chlorophyll α, β-carotene, and heme c_n (also known as heme x) are present in cytochrome b_6f.[26] The cytochrome b_6f complex transfers electrons between the two reaction complexes from PS II to PS I in photosynthesis, and the produced electrons are involved in the hydrogenase pathway leading to H_2 production.[27] Recently, Raman spectroscopy of algae[28] was reviewed by Parab and Tomar and the scattering peaks were employed for the identification of algae species. Although carotenoids may share the contribution of those peaks, β-carotene is the most common form of carotene in plants. In Figs. 3(c), the relative peak intensity for β-carotene, for algae on imprinted cavities and on glass, was normalized to that of stock algae in TAP. Interestingly, the concentrations of

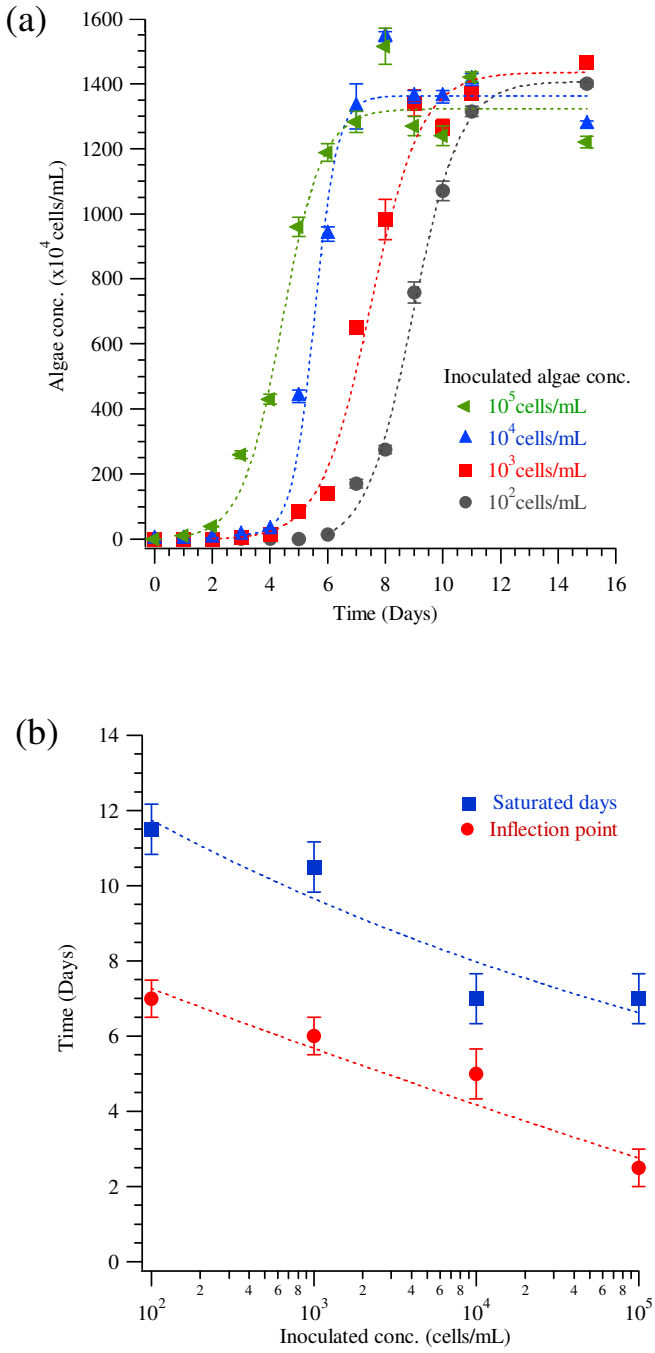

Figure 1. (a) The growth curves of algae and (b) the inflection point and saturation day with various inoculated concentrations.

β–carotene in the algae that were adsorbed on AIPs exceeded those of the algae on the glass slides by about a factor of three, although it was slightly lower after two days of hydrogen production.

Figure 4 plots the power output of the algal biofuel cells. The open circuit voltages (OCV) and polarization behavior of the algae fuel cells with various algal concentrations in Fig. 4(a) demonstrate that the maximum OCV and power intensities were

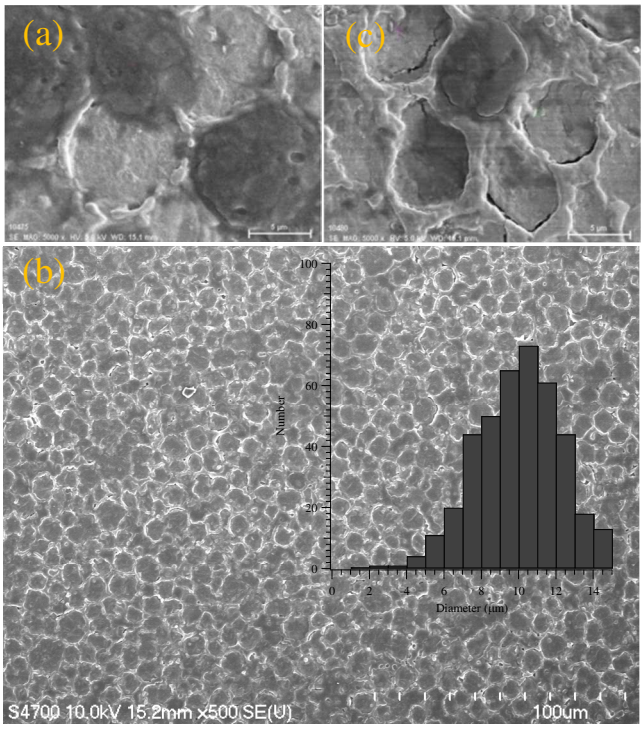

Figure 2. The SEM images of the algae-imprinted EVAL (containing 38 mole % of ethylene) thin films (a) with template algae still present, (b) after algae removal and (c) after rebinding. Inset: the size distribution of imprinted cavities.

0.60-0.65 V and 0.5 mW/m^2, respectively. An algal concentration of over 1x10^6 cells/mL yielded an enhancement of both OCV and power intensity of only approximately 10%. Since the saturated culture concentration is around 1.2~1.5 x10^7 cells/mL, the algal concentration that was used to test the algal biofuel cells with an algae-imprinted electrode was 1 x10^7 cells/mL. The output voltage obtained when algae- and non-imprinted EVAL and a Pt/ITO/PET electrode were used reveals initial output open circuit voltages of 0.60 to 0.65 V. The use of ITO-coated PET considerably increased current compared with "our previous work on algal biofuel cells".[16] When the algae-imprinted EVAL-coated Pt/ITO/PET electrode was used, the output power density was around 1.55 mW/m^2 when the current density was 6.21 mA/m^2, which is about ten times that obtained in our previous work,[16] and about two and three times the values obtained using bare and NIP-coated Pt/ITO/PET electrodes.

The nuclear genome sequence of *C. reinhardtii* was completed in 2007.[29] A few primers for the hydrogenase expression (e.g. HdyA2)[22] of *C. reinhardtii* were tried and 16S rRNA was selected as the reference gene. Real-time PCR is rapid and sensitive and it has a large dynamic range. It has multiplexing capacity, involves no radioactivity, no post-run manipulations and generally uses less starting material than other methods for elucidating gene expression. Threshold crossing values (Cq) are determined for each sample after a real-time PCR run is completed. The point of RFUs (relative fluorescent units) at

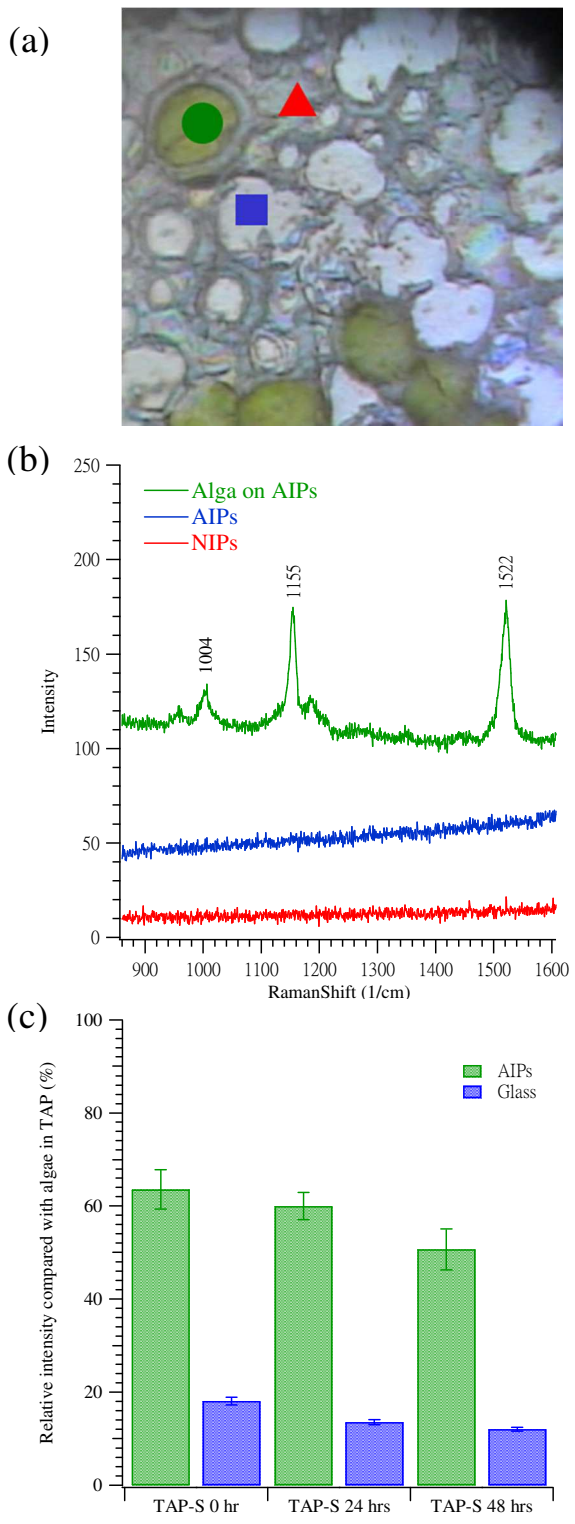

Figure 3. (a) The optical image of the algae-imprinted EVAL (containing 38 mole % of ethylene) thin films. (b) The Raman spectrum of three locations in Fig. 3(a): an alga on AIPs (green spot); NIPs (red spot); AIPs (deep blue spot). (c) The comparison of relative peak intensity of alga adsorbed on AIPs or glass slides for 0, 24 and 48 hours.

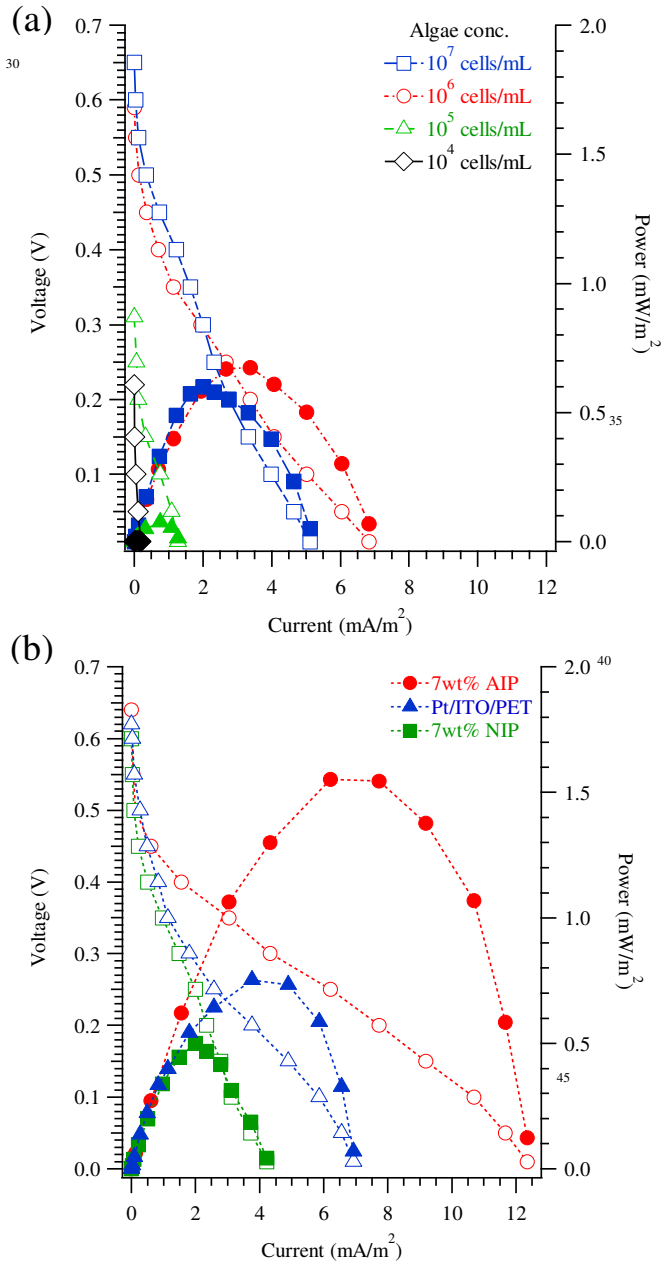

Figure 4. The polarization behavior of the algae fuel cells using (a) various algae concentrations on bare Pt/ITO/PET electrodes and (b) algae-, non-imprinted EVAL-coated and bare Pt/ITO/PET electrodes as the anode.

which the fluorescence increases significantly above the background fluorescence is indicated.

In Fig. 5, the HydA2 mRNA expression by algae on AIPs is approximately double that of free algae, algae on glass and even algae on NIPs. Accordingly, the adsorption of algae onto the microcontact-imprinted EVAL modulates (upward) their expression of hydrogenase. This is likely a contributing factor in the higher performance of the biofuel cell that uses the imprinted film. The HydA2 protein in C. reinhardtii was previously found to be expressed during the anaerobic induction by depriving the

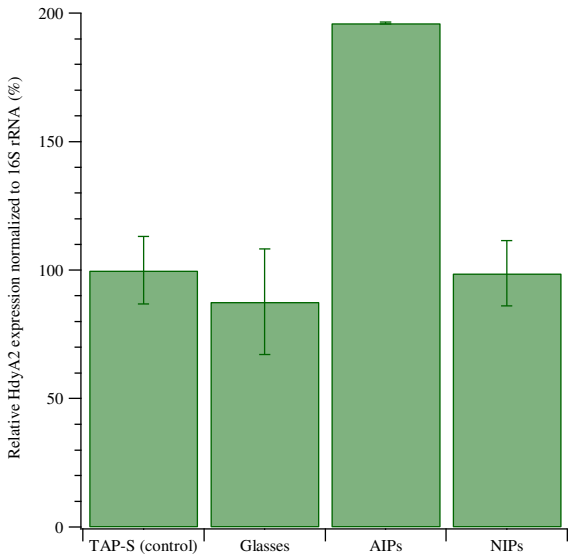

Figure 5. Relative HydA2 expression was determined using a ΔΔCq method and normalized to a reference gene (16S rRNA) and to a treatment control (free algae).

cultures of sulfur. The adsorption of algae on the algae-imprinted surface may induce partially anaerobic conditions and thus promote the expression of hydrogenase, increasing the production of hydrogen.

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Conclusions

The microenvironment for culturing microorganisms not only provides structural support but also regulates their gene expression. This study showed that the β-carotene concentration was affected by substrate and correlated with biofuel cell performance. We also found the open circuit voltages (OCV) of algal biofuel cells decreased by 10~20% on the second and third days of electricity extraction. Most importantly, the measurement of hydrogenase gene expression showed increases for AIP-absorbed algae, consistent with a locally more anaerobic environment. The higher expression of hydrogenase when algae bound on the AIPs may be responsible for the ability of these algae to generate approximately twice as much power as on conventional electrodes.

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