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Pyrococcus furiosus-immobilized anodized tubular titania cathode in a hydrogen production system

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ABSTRACT

Anodized tubular titania (TiO_2) electrodes (ATTEs) are prepared and used as both the photoanode and the cathode substrate in a photoelectrochemical system designed to split water into hydrogen with the assistance of an enzyme and an external bias of 1.5 V. In particular, the ATTE used as the cathode substrate for the immobilization of the enzyme is prepared by two methods—adsorption and crosslinking. Results show that the optimized amount of enzyme is 10.98 units for the slurried enzyme, 3.66 units for the adsorbed one and 7.32 units for the crosslinked one, and the corresponding hydrogen evolution rates are 33.04, 148.58 and 234.88 $\mu mol\,h^{-1}$, respectively. The immobilized enzyme, specifically the chemically crosslinked one, seems to be much superior to the slurried enzyme, due to the enhanced charge-transfer process that is caused by the lower electrical resistance between the enzyme and the ATTE. This results in a greater number of accepted electrons and a larger amount of enzymes able to deal with the electrons.

1. Introduction

The conversion of chemicals such as water, harmful organics and toxic metal ions by photocatalysis has been an attractive issue since early in the 1970s when Fujishima and Honda [1] first made pioneering efforts to split water using solar light concentrated on UV-absorbing materials such as titania (TiO_2). Recently, there has been increasing interest in hydrogen, which has the potential to supplement and ultimately replace fossil fuels for the production of energy. In addition, the emission of greenhouse gases also attracts much attention, due to the problem of climate change. Therefore, the photoelectrochemical (PEC) splitting of water into hydrogen and oxygen is a promising subject, because the light-driven electrolysis of water using a semiconductor photoanode is a non-polluting, waste free, sustainable method.

The efficiency of the photoelectrochemical production of hydrogen is determined by a combination of factors, such as imperfections in the crystalline structure of the photocatalytic material, the bulk and surface properties of the semiconductor photoanode, its resistance to corrosion in electrolytes, and its ability to drive the water-splitting reaction [2]. Generally speaking, the photocatalytic process has been unfortunately criticized as being uneconomical compared with other hydrogen production systems, due to its inherently low efficiency and the resulting high overall energy cost [3]. Hence, to improve the conversion efficiency obtained with solar energy, it is essential to design an energetically coordinated, stably operated, and economically feasible cell configuration. For this purpose, many recent publications and reviews have addressed the photoelectrochemical production of hydrogen [4,5], semiconductor particulate systems [3], TiO_2 photocatalysts [6], and other material-related issues [4,7]. Compared with other photocatalytic materials, TiO_2 is much more promising and its functional properties for solar hydrogen production have been thoroughly summarized in a previous report [8].

Over the past decade, photoanodes covered with TiO_2-based films have been prepared using techniques such as anodization [9–14] and sputtering [15]. Meanwhile, the former TiO_2/Ti nanotube array has attracted the authors' particular interest as a stable, light-sensitizing photoanode and a cathodic substrate for the enzymatic production of hydrogen driven by light [16–20]. The light-sensitized enzymatic (LSE) system is a way of producing hydrogen by coupling an inorganic semiconductor with enzymes in a photoelectrochemical configuration. This system uses the intrinsic proton reduction ability of the hydrogenase enzyme immobilized on a cathode in tandem with an anodic compartment in which electron donors, such as water, undergo an oxidative reaction on a light-sensitized photoanode. The generated electrons are separated and moved to the cathodic compartment through an external circuit powered by a

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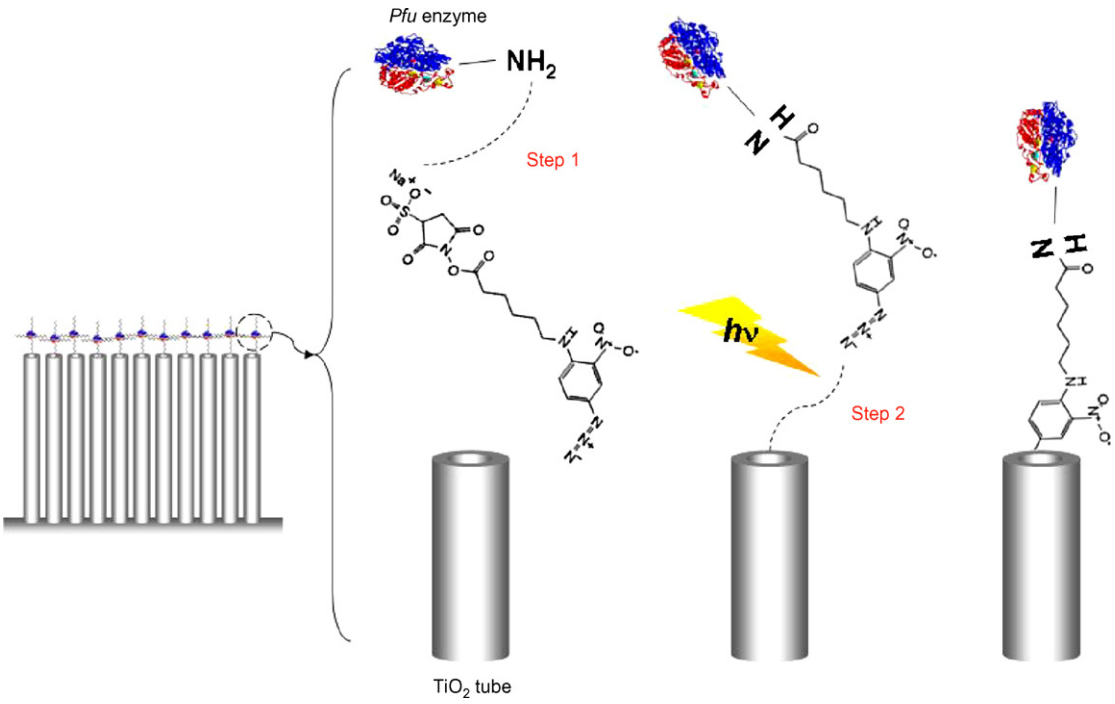

Fig. 1. Schematic view of two-step process for immobilization of enzyme by crosslinker (method 2).

solar cell and used to reduce protons to hydrogen on the active sites of the enzymes. The oxidized ions move to the cathodic compartment through a nanofiltration (NF) membrane. There are two major issues arising in LSE systems. One is the absorbtion of visible-light by TiO_2. Since the widespread use of TiO_2 is inhibited by its response being limited to UV light with wavelengths of less than 387 nm, further research to extend its photoresponse to the visible-light region must be conducted through approaches such as the co-doping of F and P or B into the TiO_2 matrix [21,22]. The other issue is the use of an enzyme-immobilized cathode with a low electrical resistance to improve the electron transfer, thereby eliminating the methyl viologen used as an electron relay and buffer solution in the cathodic compartment. For the latter issue, this study presents two ways to immobilize the enzyme and compares them in terms of the surface morphology and the rate of hydrogen evolution.

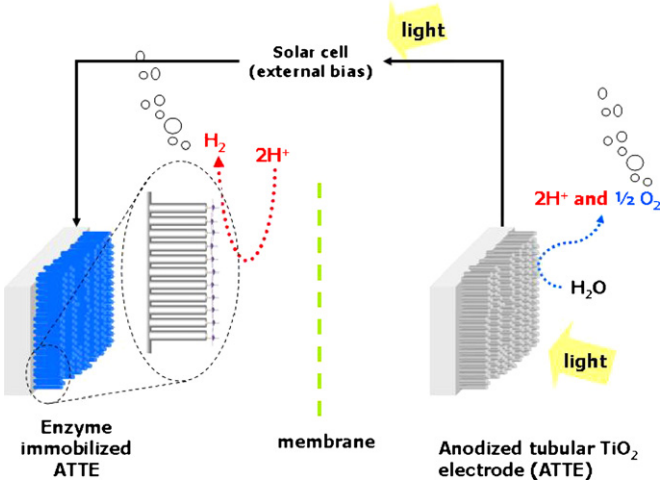

Fig. 2. Schematic view of system for light-sensitized enzymatic production of hydrogen with anodized tubular TiO_2 as both photoanode (ATTE) and cathodic substrate, a solar cell, and NF membrane.

2. Experimental

2.1. Materials and sample preparation

Titanium foil (Ti, 99.6% purity, thickness 0.25 mm, Goodfellow, England) was cut into pieces (2 × 2 cm) and subjected to potentiostatic anodization at 20 V for 45 min in a two-electrode electrochemical cell connected to a dc power supply using a platinum counter electrode with magnetic agitation in 0.5 vol.% hydrofluoric (HF) acid at 5 °C, after which the samples were annealed in an oxygen atmosphere (400 ml min^{-1}) at 650 °C for 5 h. The resulting electrode is called an "anodized tubular TiO_2 electrode" or "ATTE". A detailed explanation of the role of each component of the anodizing solution is available in the literature [9–13]. The area of the illuminated working electrode (photoanode) and the cathode for enzyme immobilization was 1 × 1 cm. Potassium hydroxide solution (1.0 M) was prepared from KOH pellets (99.99%, Sigma–Aldrich, USA) and used as the electrolyte in both compartments.

Purified hydrogenase (from *Pyrococcus furiosus*, '*Pfu*' hereafter) was supplied by Prof. Adams at the University of Georgia, by whom the *Pfu* was first isolated. This enzyme with a molar mass of 150 kDa is known to be remarkably resistant to inactivation by heat and chemical reagents [23]. The activity of *Pfu* was 21 834 unit ml^{-1}, as determined with Tris–HCl (50 mM, pH 8.5, 50 °C, absorbance at 570 nm). One unit of hydrogenase activity catalyzes the production of 1 μmol of H_2 per minute. Enzyme purification is sometimes expensive and the cost of the enzyme is based on its activity [24]. This study is mainly focused on utilization of the enzyme, assuming the affordable cost of enzyme.

For the immobilization of the enzyme, the ATTE was immersed for 12 h in 50 mM Tris–HCl buffer solution (pH 7.5 at 4 °C, Trizma base, minimum 99.9% titration and 2 M HCl, Sigma) containing dithiothreitol (DTT), $MgCl_2$ and 3.6–18.3 units of the enzyme (method 1, *Pfu*-adsorbed ATTE). Dithiothreitol (99% Aldrich) and $MgCl_2$ (99.99%, Aldrich) were used as stabilizers before the enzyme was adsorbed; DTT is effective in sample buffers for reducing protein disulfide bonds and can also be used for reducing the disulfide

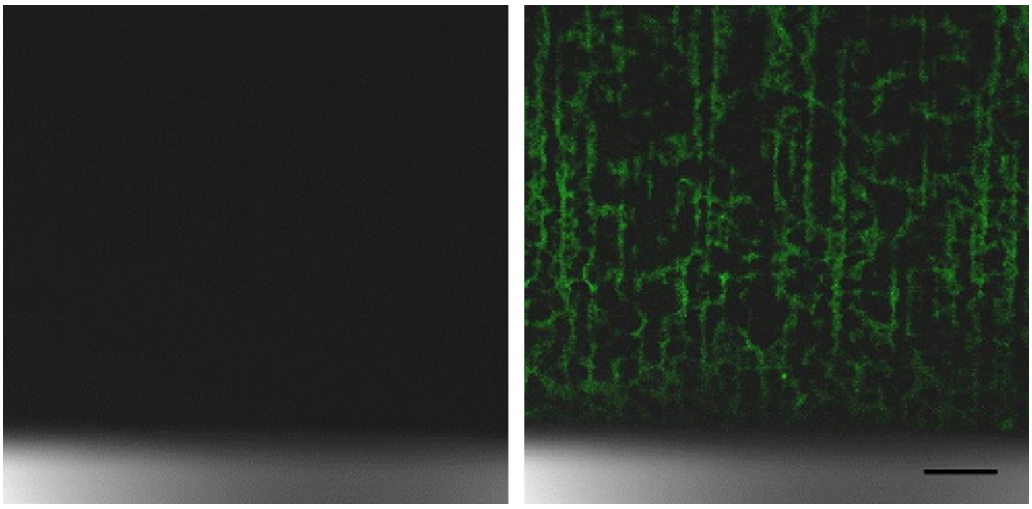

Fig. 3. Confocal images of bare ATTE (left) and *Pyrococcus furiosus*-immobilized ATTE (right) (ATTE at 20 V for 45 min in 0.5% HF at 5 °C and annealed at 650 °C for 5 h. Scale bars on images represent 100 μm).

bridges of the crosslinker. It has been reported that disulfide bonds, crosslinked using thiolation reagents, can be cleaved by reduction with DTT in order to introduce sulfhydryl groups [25]. Next, to improve the strength of the interfacial connection between the enzymes and the ATTE, the electropolymerization of pyrrole was carried out on the ATTE with 2.0 mM of pyrrole monomer (purity >98%, Sigma) at 0–0.8 V vs. a Pt counter electrode for 20 min.

A second method uses one of the aryl azide crosslinking reagents (sulfo-SANPAH, MW 492.40, PIERCE Biotechnology, USA) that are photoreactive (method 2, *Pfu*-crosslinked ATTE). Sulfo-SANPAH is a heterobifunctional crosslinker that contains an amine-reactive *N*-hydroxysuccinimide (NHS) ester (stored at 4 °C in a dark container until use) and a nitrophenyl azide that can be activated. The NHS esters react efficiently with primary amino groups ($-NH_2$) in buffers at pH 7–9 (50 mM borate buffer for 1 h with 4.9 mg of crosslinker to 5 ml buffer and chosen amount of *Pfu* enzyme) to form stable amide bonds (step 1 in Fig. 1). After removing the hydrolyzed and non-reacted crosslinker by dialysis, the nitrophenyl azides form a nitrene group that can initiate addition reactions with double bonds, insertion into C—H and N—H sites, or subsequent ring expansion to react with a nucleophile (step 2 in Fig. 1) when exposed to UV light (under 300 W solar simulator (Oriel, USA) with a 309 nm cut-on filter), finally linking the enzyme. Then, following this procedure, the ATTE is subjected to electropolymerization.

2.2. Apparatus and analysis

The experiments were conducted in a reactor composed of (anode and cathode compartments separated by a nanofiltration (NF) membrane and connected to a solar cell), as shown in Fig. 2. A detailed explanation of the NF membrane and solar panel is given in previous works [17–20]. Prior to the reaction, the mixture was deaerated with argon gas for 30 min to remove the oxygen in the water and the headspace, unless otherwise noted. Confocal laser scanning microscopy with an Ar laser (LSM 510, Carlzeiss, FRG) was used to study the sample used in 'method 1' after dyeing the *Pfu* enzyme by the NHS (excitation and emission wavelengths of 491 and 518 nm, respectively)-fluorescein method. In order to protect the fluorescent dye from the UV irradiation, all of the fluorescein labelling experiments were performed under dark conditions. The Tris–HCl pH 7.5 buffer solution (50 mM) containing *Pfu* was replaced with 50 mM of borate pH 8.5 buffer solution through a semi-permeable membrane for an hour to prevent the reaction between the NHS functional group and Tris–buffer that contains primary amines. The labelling reaction was initiated by adding 0.5 μl of fluorescein solution conjugated by dimethylformamide (DMF). After being mixed for an hour, the enzyme solutions combined with the fluorescein dye were dialyzed through a semi-permeable membrane for an hour to remove the non-reacted NHS-fluorescein. The ATTE was immersed for 12 h in 50 mM of borate pH 8.5 buffer solution containing fluorescein-dyed *Pfu*. The surface morphology of the bare and enzyme-deposited TiO_2 tubes were also investigated by atomic force microscopy (AFM) in conjunction with a scanning probe microscope (D3100, Veeco Instruments) in tapping mode using a Si tip. Linear sweep voltammetry was applied to evaluate the electrochemical behavior of the bare ATTE, *Pfu*-adsorbed ATTE and *Pfu*-crosslinked ATTE using a potentiostat (G300 w/PHE200 software, GAMRY Instruments Electrochemistry, PA, USA) with a platinum mesh electrode as the counter electrode and Ag/AgCl (saturated in 3.0 M KCl) as the reference electrode. The light source used for the hydrogen evolution reactions was a 1000 W xenon lamp (Oriel, USA), which was filtered through a 10-cm IR water filter. The irradiated light intensity was ca. 74 ± 3.4 mW cm^{-2} (at 350–450 nm with a portable radiometer with UM-10 and UM-400, from Minolta Co., Japan). The hydrogen and oxygen produced were analyzed by a gas chromatograph with a thermal conductivity detector (TCD at 260 °C, oven at 40 °C). The column used in the system was a molecular sieve 5 Å (Supelco, USA).

3. Results and discussion

In our previous studies, the morphology of the ATTE varied noticeably according to the electrolyte, applied voltage and bath temperature. This demonstrated showing the feasibility of using the ATTE made from Ti foil with a thickness of 0.25 mm anodized at an applied voltage of 20 V in 0.5 vol.% hydrofluoric acid and annealed at a temperature of 650 °C [17–20]. The resulting ATTE had TiO_2 tubes with a wall thickness of 14–20 nm and a length of ca. 620 nm forming a periodic ring structure. In the diffraction patterns, all of the as-anodized samples were found to be amorphous, while heat-treatment in dry O_2 ambient supported the formation of anatase phase at no higher than 650 °C and rutile phase at no lower than 475 °C [17].

The distribution of the enzymes immobilized on the ATTE is qualitatively shown in Fig. 3, compared with the bare ATTE. The image on the right-hand side showing the enzymes adsorbed on the ATTE reveals the presence of fluorescent material (green colour) arrayed linearly, which represents the well-organized TiO_2 tubes

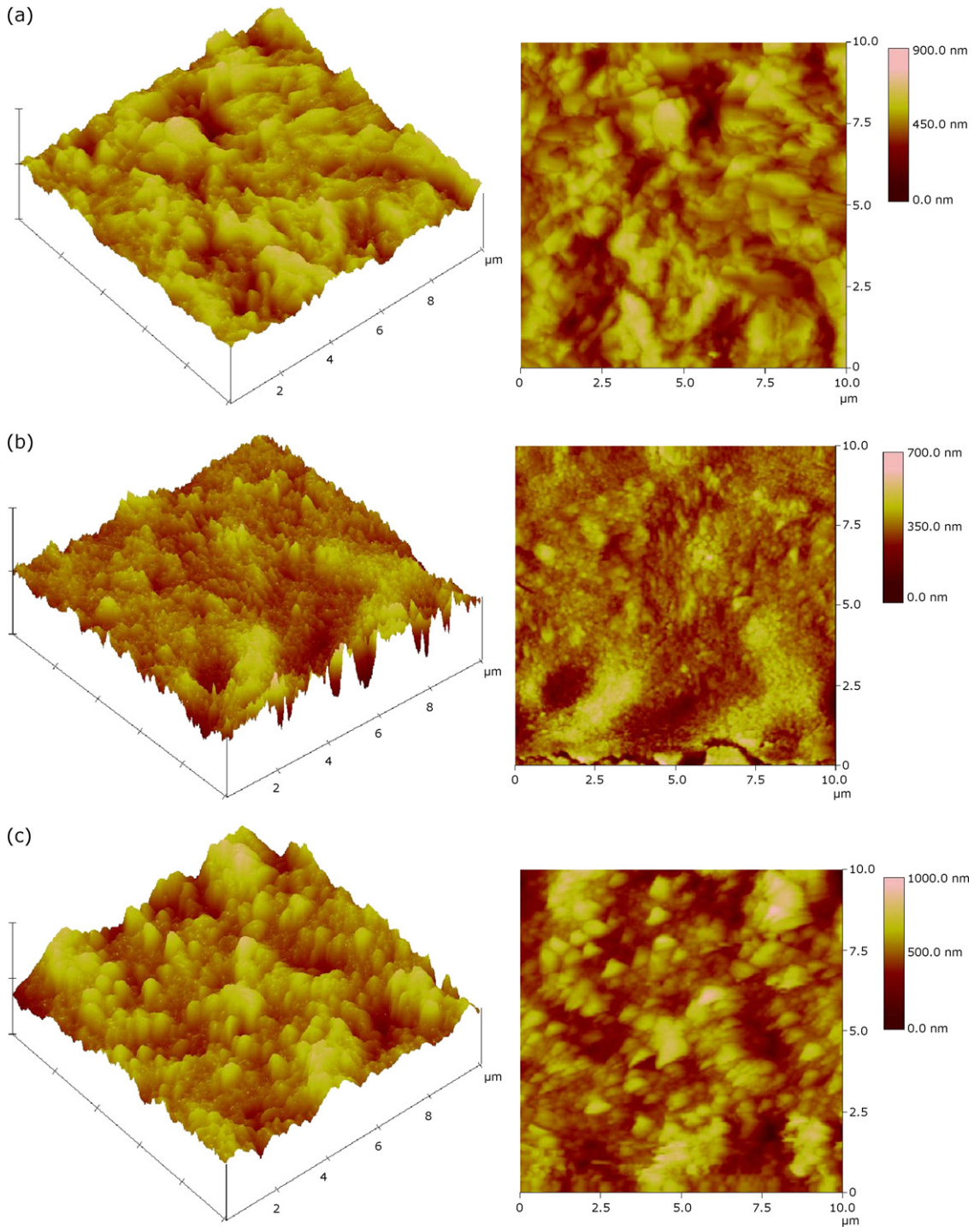

Fig. 4. AFM images of electropolymerized bare and enzyme-immobilized surfaces of (a) bare TiO_2, (b) *P. furiosus*-adsorbed TiO_2 (method 1), (c) *P. furiosus*-crosslinked TiO_2 (method 2) (ATTE at 20 V for 45 min in 0.5% HF at 5 °C and annealed at 650 °C for 5 h).

standing vertically with respect to the Ti foil. The entrapment of enzymes within polymer networks can preserve the catalytic activity of biomolecules, sustaining their activity when they are used in an electrolyte [26,27]. For the enzymes crosslinked on the ATTE, confocal laser spectroscopy was not able to be conducted, owing to the conflict between the labelling material and immobilizing crosslinker in the reaction with the ATTE. Therefore, AFM is employed to compare further the two methods, as shown in Fig. 4. Three-dimensional (3D) and two-dimensional (2D) AFM micrographs were recorded from three regions with areas of 500 nm × 500 nm, 2 μm × 2 μm and 10 μm × 10 μm, respectively. It can be seen that the surface morphology is strongly dependent on the method of immobilization employed. For the bare ATTE, the presence of electropolymerized pyrrole polymer without any agglomerates is observed, as shown in Fig. 4(a), while the enzyme-immobilized samples show many peaks caused by enzymes agglomerates, as shown in Fig. 4(b) for the *Pfu*-adsorbed ATTE and Fig. 4(c) for the *Pfu*-crosslinked ATTE. The *Pfu*-adsorbed ATTE exhibits a smoother surface than the *Pfu*-crosslinked ATTE. This might be due to the crosslinker which enabled the enzymes

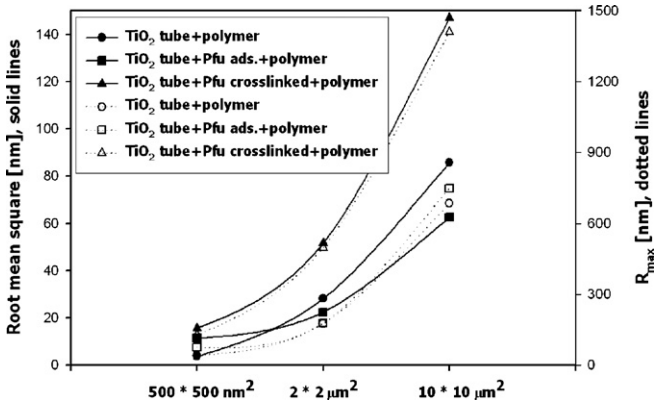

Fig. 5. Mean and maximum root-mean-square roughness (RMS) values of electropolymerized bare and enzyme-immobilized surfaces of TiO_2.

and ATTE to link more efficiently. This result is in agreement with the surface roughness. The surface roughness of the bare ATTE is lower than those of the other samples, having an average root mean square (RMS) value of about 3.7 nm (500 nm × 500 nm)–85.7 nm (10 μm × 10 μm), as shown in Fig. 5. The RMS roughness values are 11.4 nm (500 nm × 500 nm)–62.6 nm (10 μm × 10 μm) for the *Pfu*-adsorbed ATTE and 15.6 nm (500 nm × 500 nm)–147.0 nm (10 μm × 10 μm) for *the Pfu*-crosslinked ATTE. The maximum and mean RMS roughness values follow the same trend, as shown in Fig. 5.

Linear sweep voltammetry is applied to evaluate the electrochemical behavior of the bare ATTE, *Pfu*-adsorbed ATTE and *Pfu*-crosslinked ATTE in both 1.0 M KOH only at 25 °C and Tris–HCl buffer electrolyte at 25 and 70 °C (Fig. 6). In the case of the 1.0 M KOH electrolyte, measurements were unable to be conducted due to the instability of the reference electrode. A strong reduction peak begins to appear at a less negative potential for the *Pfu*-crosslinked ATTE than for the other samples, which is due mostly to the generation of hydrogen by the water decomposition reaction ($2H_2O + 2e^- \rightarrow H_2 + 2OH^-$) [28]. From this reason, the strong current at a lower potential means that the enzymes, which are chemically linked to the ATTE, catalyze the water-decomposition reaction efficiently by lowering the potential and accelerating the relay of electrons. In the electrolytes at 25 °C, the potential is −0.63 V in 1.0 M KOH and −0.25 V in Tris–HCl buffer as shown in Fig. 6(a) and (b), respectively. By contrast, the *Pfu*-adsorbed ATTE does not show a strong reduction peak at such a negative potential and behaves similarly to the bare ATTE or the polymerized ATTE. This might be due to the physically, not chemically, adsorbed enzyme which has a linkage that is unable to transfer electrons efficiently. This phenomenon also occurs in the Tris–HCl electrolyte at 70 °C, with the only difference being the amount of current measured. Since the activity of the *Pfu* enzyme is strongly dependent on the temperature of the electrolyte, the use of a higher temperature can enhance the catalytic activity, so that more electrons can be transferred and, consequently, the current is generated more efficiently. The effect of temperature on the activity of the enzyme is also supported by the following results. The *Pfu* immobilized by 'method 1' in 1 M KOH electrolyte shows an H_2 evolution rate of 81.1 μmol h^{-1} (≈1.82 ml h^{-1}) at room temperature, which is approximately 54.6% of that observed at 70 °C (148.6 μmol h^{-1} ≈3.33 ml h^{-1}) and is close to the result obtained without the enzyme (76.27 μmol h^{-1} ≈1.71 ml h^{-1}). This means that *Pfu* at room temperature scarcely catalyzes the reduction of protons to H_2 and that a higher temperature, but less than 110 °C, is a necessary condition for *Pfu* to catalyze the reduction of protons.

The AFM and linear sweep voltammetry results are also consistent with the observed hydrogen evolution rates (Fig. 7). In the

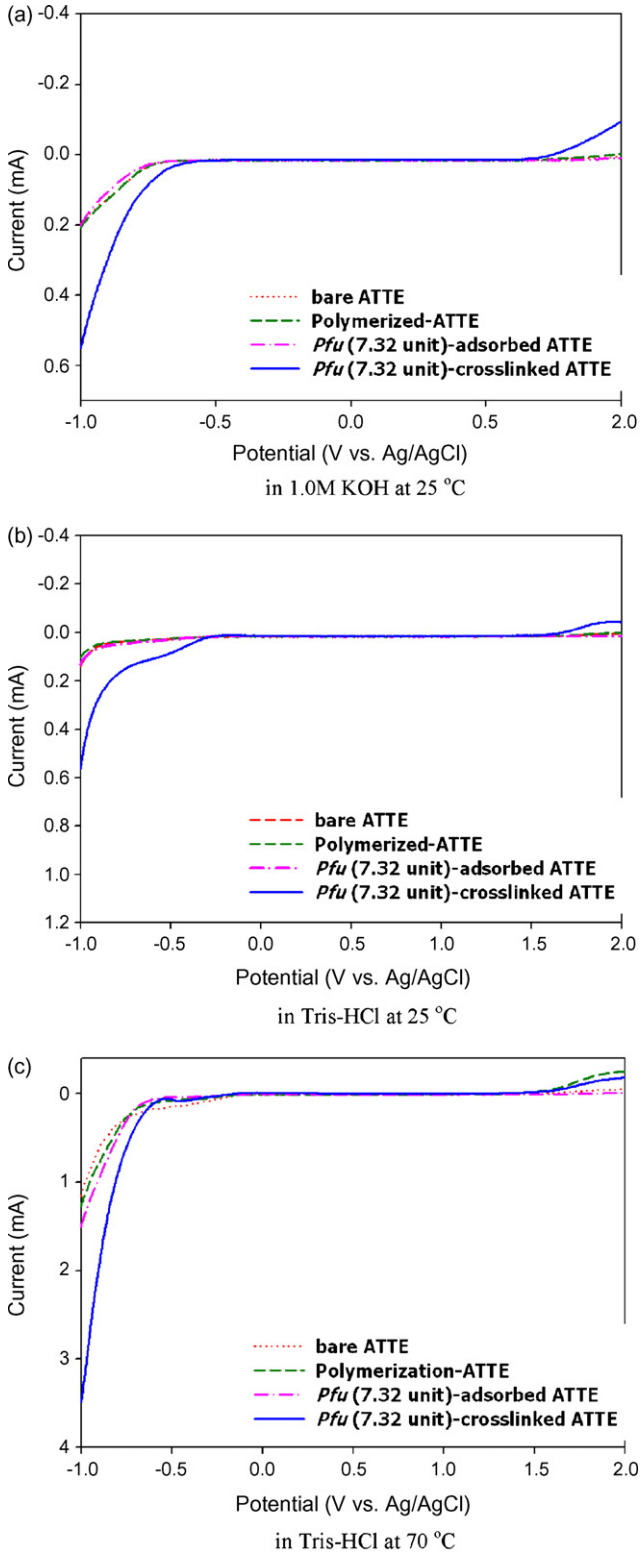

Fig. 6. Linear sweep voltammetry curves of prepared cathodes with or without enzyme in (a) 1 M KOH at 25 °C, (b) Tris–HCl at 25 °C and (c) Tris–HCl at 70 °C (ATTE at 20 V for 45 min in 0.5% HF at 5 °C and annealed at 650 °C for 5 h).

slurried enzyme system, approximately 33.1 μmol h^{-1} of hydrogen is evolved per cm^2 of the bare ATTE cathode (optimum *Pfu* amount of 10.98 units) with 1 cm^2 of the ATTE photoanode illuminated. On the other hand, the H_2 evolution rate drastically increases to 148.6 and 234.9 μmol h^{-1} for ATTEs with enzymes immobi-

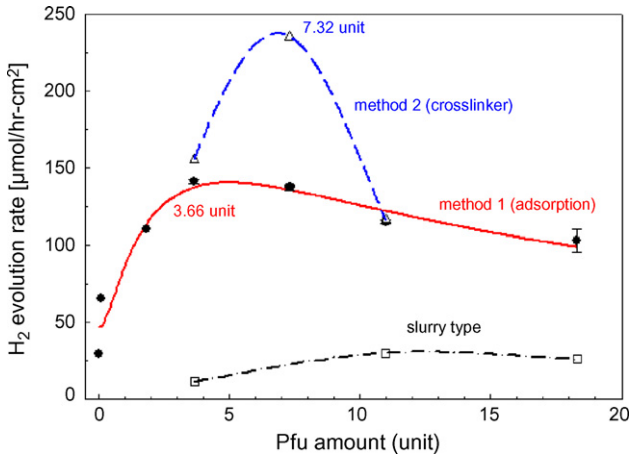

Fig. 7. Effect of immobilization method of enzyme on hydrogen evolution rate (external bias of 1.5 V from solar cell, ca. $74 \pm 3.4\,mW\,cm^{-2}$, ATTE at 20 V for 45 min in 0.5% HF at 5 °C and annealed at 650 °C for 5 h).

lized by methods 1 and 2, respectively. Most interestingly, the optimized amount of enzyme varies from 10.98 units for the slurried system, to 3.66 units for method 1 and 7.32 units for method 2. Once the enzyme is immobilized and has better contact with the ATTE, a greater amount of enzyme is necessary to produce the same amount of hydrogen because the resultant lower electrical resistance leads to a greater amount of electrons being made available to reduce protons to hydrogen. An excessive amount of enzymes, however, plays a detrimental role in producing hydrogen by accelerating the reverse reaction corresponding to hydrogen oxidation. Enlargement of the ATTE and increase in the amount of enzyme immobilized by 10 times (10 cm^2 and 36.6 units, respectively) produces about 13 ml h^{-1} of hydrogen at room temperature that resultis in a scale-up factor of 13/18.2 = 0.71.

4. Conclusions

Two different methods of enzyme immobilization are compared with each other and with a slurried enzyme method. When using anodized tubular TiO_2 as the substrate, the enzyme efficiently immobilized on the cathode enables electrons to be transferred with a promoted amperometric value. The increased current induced by the effective interaction between the interfaces allows more hydrogen to be produced with the optimum amount of the enzyme. The rate of hydrogen evolution is noticeably increased from 33.04 μmol h^{-1} for the slurried enzyme to about 148.58 μmol h^{-1} for method 1 and 234.88 μmol h^{-1} for method 2. Although enlargement of the photoanode and the cathode result in a loss of efficiency, 10 times larger electrodes have been fabricated and outdoor experiments with a flat-type cell are being conducted. The results are to be published elsewhere in the near future.

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