SUPPORTING INFORMATION FOR

IMMOBILIZED ENZYMES ON GRAPHENE AS NANO-BIO-CATALYST

Hathaichanok Seelajaroen,*[a] Aristides Bakandritsos,[b] Michal Otyepka,[b] Radek Zbořil,[b] and Niyazi Serdar Sariciftci[a]

[a] Linz Institute for Organic Solar Cells (LIOS), Institute of Physical Chemistry, Johannes Kepler University Linz, Altenberger Straße 69, 4040 Linz, Austria
[b] Regional Centre for Advanced Technologies and Materials, Department of Physical Chemistry, Faculty of Science, Palacký University Olomouc, listopadu 1192/12, 771 46, Olomouc, Czech Republic

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1. Experimental procedures

1.1. Chemicals, materials and methods

Alcohol dehydrogenase (ADH, EC 1.1.1.1 from *Saccharomyces cerevisiae*, 321 units·mg^{-1}), formate dehydrogenase (F_{ate}DH, EC 1.2.1.2 from *Candida boidinii*, 1.04 units·mg^{-1}), formaldehyde dehydrogenase (F_{ald}DH, EC 1.2.1.46 from *Pseudomonas* sp., 4 units·mg^{-1}) *N*-Ethyl-*N*´-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC) (~98%), *N*-hydroxysulfosuccinimide (sulfo-NHS), *β*-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH), *β*-nicotinamide adenine dinucleotide hydrate (NAD$^+$), ethanol, NaCN (p.a. ≥97%), graphite fluoride (>61 wt.% F, $C_1F_{1.1}$) and alginic acid sodium salt were purchased from Sigma-Aldrich. Acetone (pure) and ethanol (absolute) were purchased from Penta. Amine-free dimethylformamide (DMF) and nitric acid (Analupure®, 65%) were obtained from Lach-Ner. All other chemicals were of analytical grade and used without further purification. Carbon felt electrodes were purchased from SGL Carbon GmbH. A phosphate buffer solution was prepared from appropriate amounts of K_2HPO_4 and KH_2PO_4 to reach desired pH value. A TRIS-HCl buffer solution was prepared from certain amount of tris(hydroxymethyl)aminomethane and 0.1 M HCl solution to reach a certain pH value.

ATR-FTIR studies were performed using a Bruker IFS-66/S machine. The thin films of the samples were prepared by drop-casting 25 μL of those of aqueous solutions at the concentration of 1 mg·mL^{-1} on ZnSe crystal substrates and left for the water to dry at ambient condition. IR spectra were then acquired from 4000 to 500 cm^{-1} by averaging over 32 scans, using the N_2 flow through the ATR accessory. Later, blank subtraction of the bare ZnSe substrates was applied to the collected spectra.

High resolution X-ray photoelectron spectroscopy (HR-XPS) was carried out with a PHI VersaProbe II (Physical Electronics) spectrometer using an Al Kα source (15 kV, 50 W). The obtained data were evaluated with the MultiPak (Ulvac-PHI, Inc.) software package.

The absorbance measurement in enzymatic assay and protein determination was performed in 96-well microplates using a Thermo Scientific Multiskan FC microplate photometer at room temperature.

Thermal analyses were performed with an STA449 C Jupiter Netzsch instrument at a heating rate of 10 °C min^{-1}, under an N_2 flow in the sample compartment.

Transmission electron microscope (TEM) measurements were performed with a JEOL 2100 instrument by evaporating a drop of the G-ADH dispersion in water on the holey carbon coated copper grid.

Scanning electron microscope (SEM) images were taken using a JEOL JSM-6360 LV scanning electron microscope at the accelerating voltage of electron beam of 7.0 kV with a working distance of 15 mm.

1.2. Preparation of G-COOH

The carboxyl-graphene derivative was synthesized according to previously reported procedures.[1] First, fluorographene (FG) was reacted with NaCN to produce cyano-graphene (G-CN), from which the carboxyl derivative (graphene acid, G-COOH) was produced by acidic hydrolysis of G-CN using HNO_3.

1.3. ADH enzymatic activity assay

With a slight modification,[2] the enzymatic activity of ADH was determined spectrophotometrically by measuring the absorption increase at 340 nm corresponding to the formation of NADH concomitant with ethanol oxidation using a Multiskan microplate reader. The working solution contains ethanol aqueous solution (20 μL, 2.25 M), TRIS-HCl buffer solution pH 8.8 (200 μL, 0.1 M) and free ADH or G-ADH aqueous solution (20 μL). To initiate the reaction, NAD$^+$ aqueous solution (20 μL, 5 mM) was added into the mixture. While one unit of ADH enzyme activity is defined as the amount of ADH that converts 1 μmol of NAD$^+$ to NADH per minute at pH 8.8 at 25°C. The enzyme activity is determined by averaging the produced NADH per minute for the first 5 min.

1.4. F_{ate}DH enzymatic activity assay

The enzymatic activity of F_{ate}DH was determined spectrophotometrically by measuring the absorption increase at 340 nm corresponding to the formation of NADH concomitant with formate oxidation using a Multiskan microplate reader. The working solution contains formate aqueous solution (75 μL, 0.2 M), phosphate buffer solution pH 7.0 (150 μL, 0.1 M), deionized water (20 μL) and enzyme solution (25 μL). To initiate the reaction, NAD$^+$ aqueous solution (30 μL, 10.5 mM) was added into the mixture. While one unit of F_{ate}DH enzyme activity is defined as the amount of F_{ate}DH that converts 1 μmol of NAD$^+$ to NADH per minute at pH 7.0 at 25°C. The enzyme activity is determined by averaging the produced NADH per minute for the first 5 min.

1.5. F_{ald}DH enzymatic activity assay

The enzymatic activity of F_{ald}DH was determined spectrophotometrically by measuring the absorption increase at 340 nm corresponding to the formation of NADH concomitant with formaldehyde oxidation using a Multiskan microplate reader. The working solution contains formaldehyde solution (10 μL, 0.08%), phosphate buffer solution pH 7.5 (200 μL, 0.05 M), deionized water (15 μL) and enzyme aqueous solution (25 μL). To initiate the reaction, NAD$^+$ aqueous solution (50 μL, 5.7 mM) was added into the mixture. While one unit of F_{ald}DH enzyme activity is defined as the amount of F_{ald}DH that converts 1 μmol of NAD$^+$ to NADH per minute at pH 7.5 at 25°C. The enzyme activity is determined by averaging the produced NADH per minute for the first 5 min.

1.6. Protein determination

The amount of dehydrogenases is determined as the protein content using the reported bicinchoninic acid (BCA) assay.[3] The assay relies on two reactions. First, the peptide bonds in protein reduce Cu^{2+} to Cu^+ under basic condition. The resulting Cu^+ then chelates with two molecules of BCA, forming a purple complex (BCA-Cu^+ complex) with an absorbance maximum at 562 nm. The amount of protein can be quantified spectrophotometrically by measuring the absorbance at 562 nm. Two reagents (A and B) solutions were prepared separately. In 100 mL of reagent A aqueous solution consisted of sodium bicinchoninate (0.1 g), $Na_2CO_3·H_2O$ (2.0 g), sodium tartrate dihydrate (0.16 g), NaOH (0.42 g) and $NaHCO_3$ (0.95 g). Reagent B consisted of $CuSO_4·5H_2O$ (0.4 g) in 10 mL of deionized water. The working reagent is prepared by mixing 100 mL of reagent A and 2 mL of reagent B. The protein determination of sample was performed by mixing 100 μL of

sample with 2 mL of working reagent and incubation at 60°C for 30 min. The amount of protein was then determined by comparison with known protein standard, bovine serum albumin (BSA).

1.7 Chemical conversion of acetaldehyde to ethanol.

To prove the activity of ADH covalently attached on graphene, a set of samples was prepared including G-COOH, G-COOH mixed with ADH, G-ADH, free ADH and G-ADH immobilized in alginate matrix (G-ADH alginate beads). G-COOH mixed with ADH sample was prepared by incubation of G-COOH and ADH in 0.1 M phosphate buffer solution pH 7.4 at room temperature for 14 h and then centrifugal to remove unbound ADH. Following the reported procedure,[4] G-ADH alginate beads were prepared by dropping G-ADH alginate solution of 2 mL 0.1 M TRIS-HCl buffer solution pH 7.4 containing ~2 mg G-ADH and 0.05 g alginic acid sodium salt into 0.2 M $CaCl_2$ solution and the beads were left in the solution for 20 min. A mixture of acetaldehyde aqueous solution (0.5 mL, 0.3 M), 0.1 M TRIS-HCl buffer solution pH 7.4 (1.8 mL, 0.1 M) and the aqueous sample solution (0.2 mL) or the prepared gel beads was treated with NADH aqueous solution (0.5 mL, 2 mM) at room temperature for 2 h. For comparison, a blank experiment using deionized water as sample was done.

The product analysis for ethanol was done by using a Thermo Scientific Trace 1310 Gas Chromatograph (TR-V1 column) equipped with a flame ionized detector (FID). The liquid samples were filtered through a polyethersulfone (PES) filter (0.45 μm) before injecting using an autosampler. The measurements were performed in triplicate using split mode with the split flow of 10 mL·min^{-1} and He was used as a carrier gas with the constant flow of 1.5 mL min^{-1}. Since for each molecule of NADH, one molecule of acetaldehyde is reduced to one molecule of ethanol, the yields of the reactions were expressed by the chemical conversion efficiency (CE) of acetaldehyde to ethanol, calculated as:

$$\%CE_{ethanol} = \frac{moles\ of\ ethanol}{moles\ of\ NADH} \times 100$$

where moles of ethanol are calculated from the amount of ethanol produced in the system and moles of NADH are calculated from the amount of NADH added into the reaction.

1.8 Chemical conversion of CO_2 to methanol

To prepare the alginate beads containing three enzyme-functionalized graphene, G-F_{ate}DH, G-F_{ald}DH and G-ADH were dispersed in 0.1 M TRIS-HCl buffer solution pH 7 (0.5 mL, resulting ~4 mg·mL^{-1} solution). The graphene alginate solution was prepared by adding 0.5 mL of G-F_{ate}DH, 0.25 mL of G-F_{ald}DH and 0.25 mL of G-ADH into alginate solution (0.05 g alginic acid sodium salt dissolved in 1.75 mL buffer solution). Then, the alginate mixture was dropped into 0.2 M $CaCl_2$ aqueous solution and left for 20 min yielding alginate beads containing three enzyme-modified graphenes, G-DHs immobilized in alginate beads. The beads were transferred in 0.1 M TRIS-HCl buffer solution pH 7.0 (10 mL) containing NADH (9 mg, 0.13 mmol). The reaction was performed for 14 h under CO_2-saturated condition. The liquid sample was collected and analyzed using gas chromatograph as mentioned previously for ethanol analysis. As three NADH molecules are consumed to produce one methanol molecule, the chemical conversion efficiency toward the conversion of CO_2 to methanol could be calculated using the following equation:

$$\%CE_{methanol} = \frac{moles\ of\ methanol}{\frac{1}{3} \times moles\ of\ NADH} \times 100$$

where moles of methanol are calculated from the amount of methanol produced in the system and moles of NADH are calculated from the amount of NADH added into the reaction.

1.9 Headspace analysis

To analyze gas product(s) in headspace of an electrochemical setup, 2 mL of headspace gas was taken using a gas-tight syringe and injected to gas chromatograph (GC, Thermo Scientific Trace GC Ultra). The GC machine was equipped with two channels using two different carrier gases namely N_2 and He. Both channels were connected with thermal conductivity detector (TCD). The N_2 channel was used for quantification analysis of H_2, CH_4 and CO_2 while CO can be quantified in the He channel.

2. Additional results

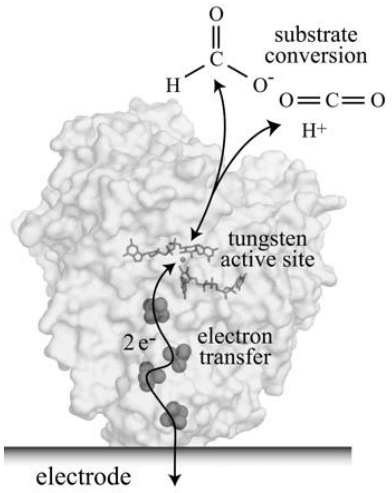

Figure S1. Schematic electrocatalytic interconversion of CO_2 and formate by a formate dehydrogenase adsorbed on the pyrolytic graphite edge electrode.[5]

Alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae* (E.C. 1.1.1.1) was reported that it contains 24 lysine units per molecule (PMID: 26743849).[6] B-factor values of nitrogen atom (referred as atom "NZ") in lysine units were extracted and plotted over amino acid residue index showing flexibility of each lysine unit (in chain A and B, Figure S2). Based on B-factor analysis, 18 lysine groups have higher B-factor, as compared to the reported average B-factor of 57 $Å^2$ for the whole structure. The analysis revealed that the highest flexible unit located at Lys17 (chain A) and Lys334 (chain B). This covalent conjugation resulted to the drop in the acetaldehyde conversion efficiency of 92% in free ADH's case to 64% in G-ADH's case as shown in Table 1, entry 1.

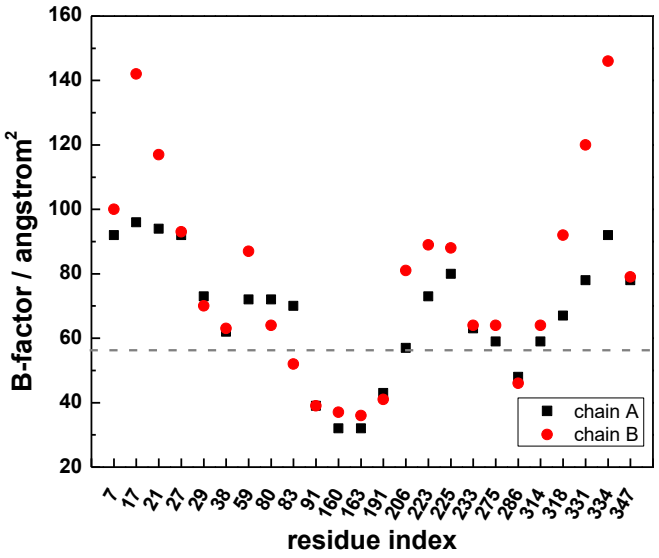

Figure S2. Plot of B-factor values of lysine units in ADH. The values were extracted from the published study.[6]

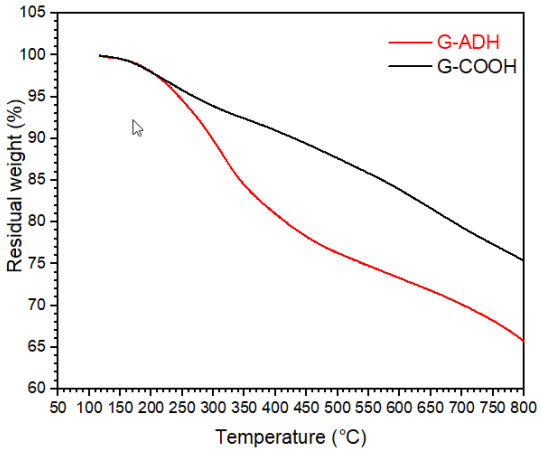

Figure S3. Thermogravimetric analysis results of the G-COOH before (black solid line) and after immobilization of the enzyme (red solid line).

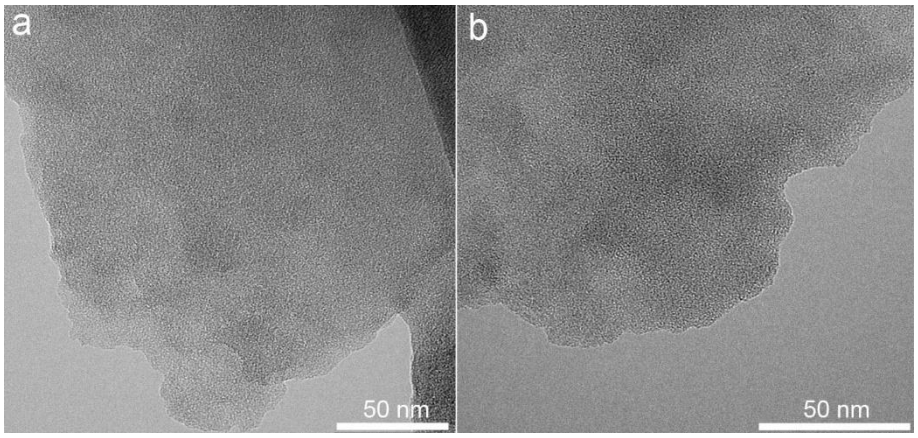

Figure S4. TEM images of G-COOH a) before and b) after covalent modification with ADH.

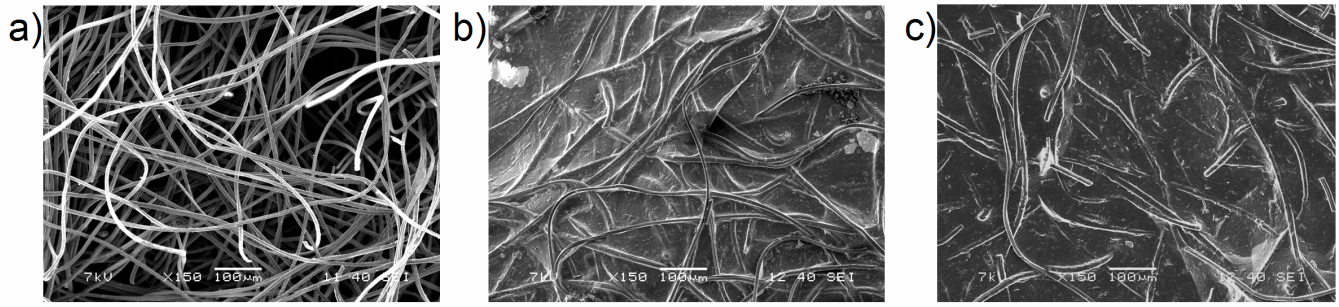

Figure S5. SEM images of a) bare carbon felt, G-DHs immobilized in alginate-Ca^{2+} matrix coated on carbon felt b) before electrolysis and c) after 20-h electrolysis.

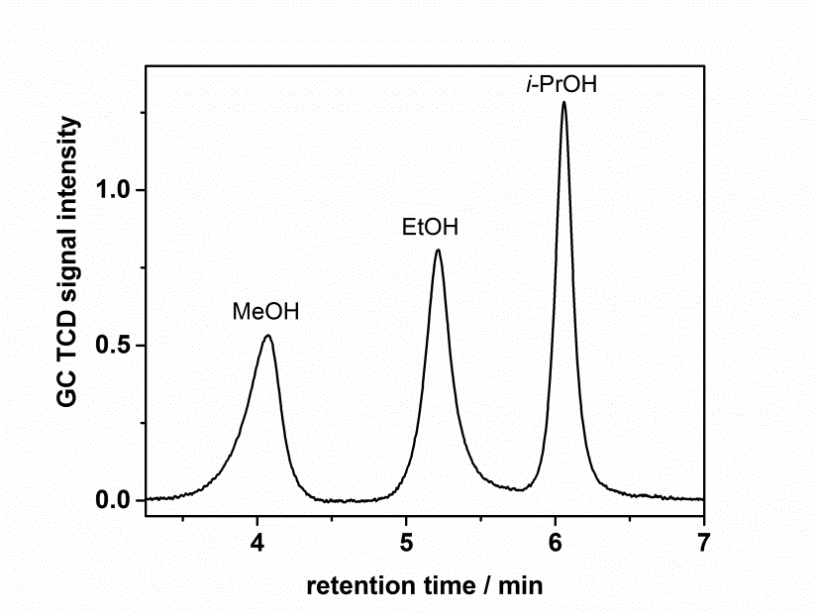

Figure S6. Liquid-injection gas chromatogram of standard alcohol mixture containing 49 ppm of methanol, 42 ppm of ethanol and 41 ppm of *i*-propanol.

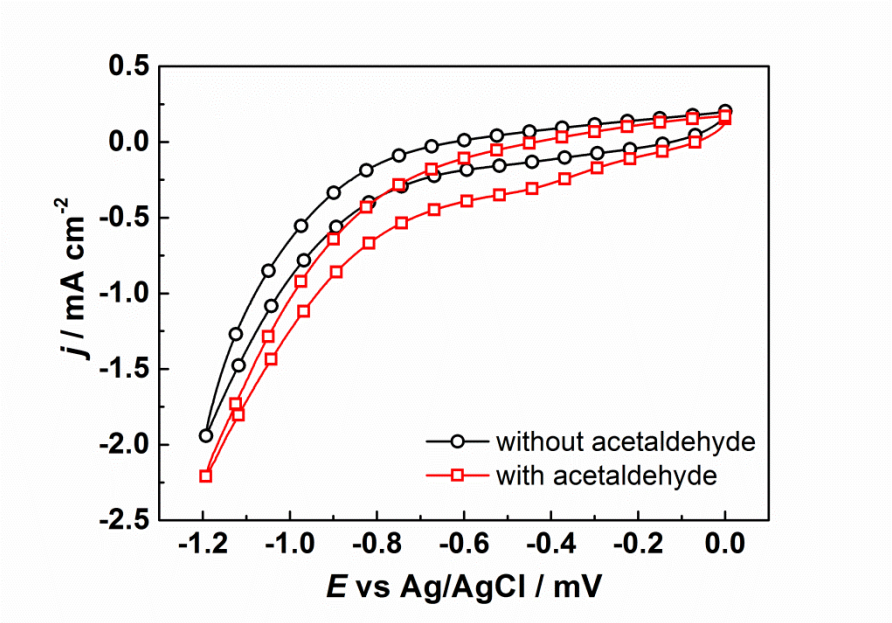

Figure S7. Cyclic voltammograms of a G-ADH immobilized in alginate coated on carbon felt were recorded at the potentials between 0 to -1.20 V vs Ag/AgCl (3 M KCl) with a scan rate of 10 mV·s^{-1} in 0.1 M TRIS-HCl solution pH 7.4 in the absence of acetaldehyde (black line) and in the presence of acetaldehyde (1 M, red line) under N_2-saturated condition.

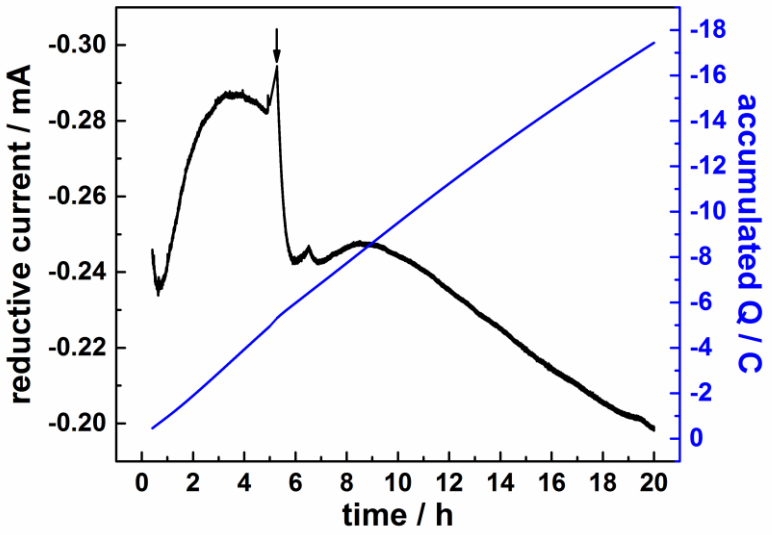

Figure S8. Transient curve of a constant-potential electrolysis of G-ADH immobilized in alginate coated on carbon felt at -1.00 V vs Ag/AgCl (3 M KCl) in 0.1 M TRIS-HCl buffer solution pH 7.4 containing 1 M acetaldehyde under N_2-saturated condition. Arrow indicates liquid solution sampling.

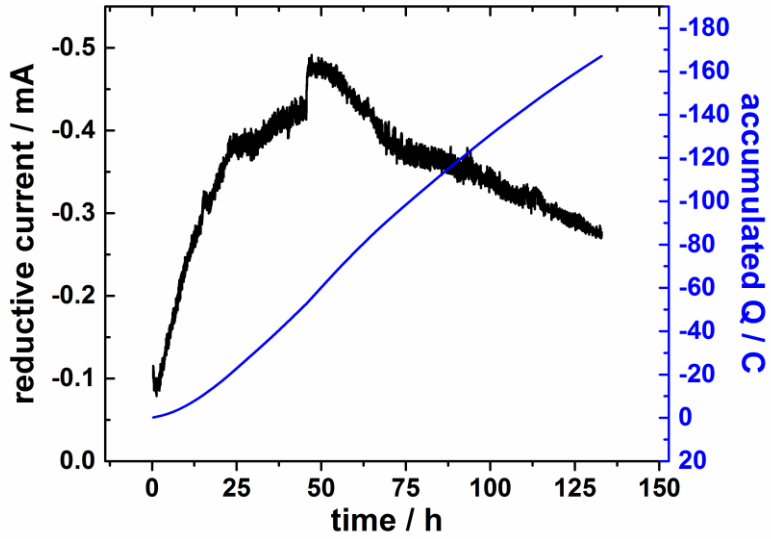

Figure S9. Transient curve and plot of accumulated charge of a constant-potential electrolysis of G-ADH immobilized in alginate coated on carbon felt at -1.00 V vs Ag/AgCl (3 M KCl) in 0.1 M TRIS-HCl buffer solution pH 7.4 containing 1 M acetaldehyde under N_2-saturated condition for 132 h.

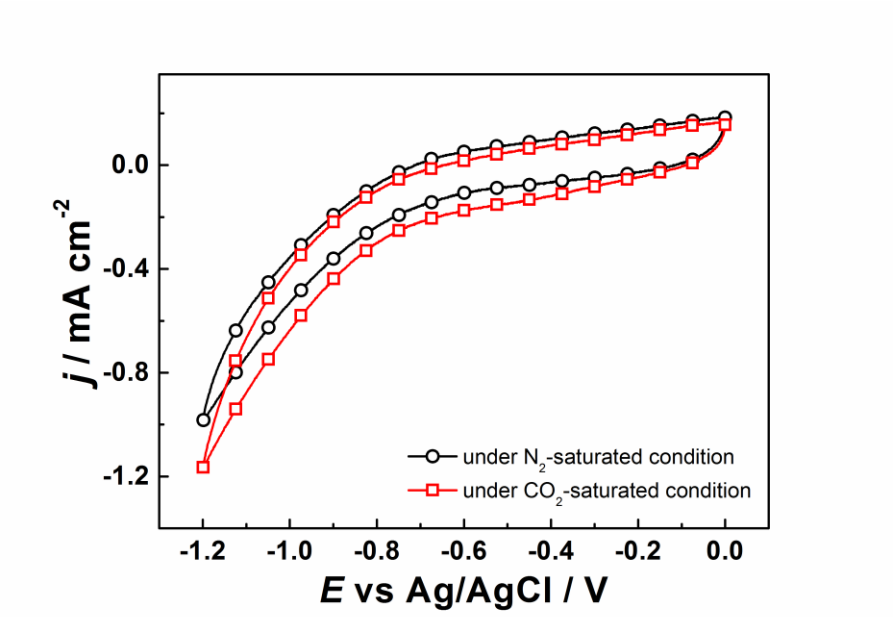

Figure S10. Cyclic voltammograms of a G-F_{ate}DH immobilized in alginate coated on carbon felt were recorded at the potentials between 0 to -1.20 V vs Ag/AgCl (3 M KCl) with a scan rate of 10 mV·s^{-1} in 0.1 M TRIS-HCl solution pH 7.0 under N_2- (black line) and CO_2-saturated conditions (red line).

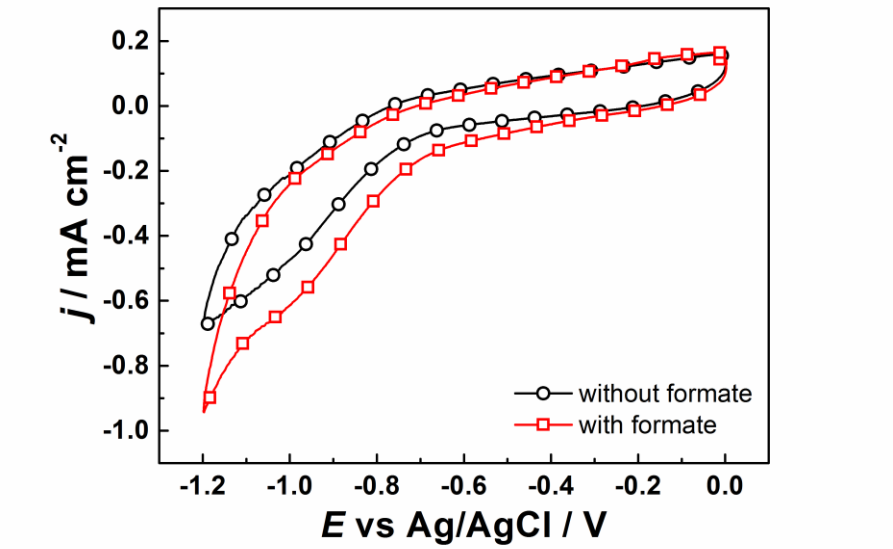

Figure S11. Cyclic voltammograms of a G-F_{ald}DH immobilized in alginate coated on carbon felt were recorded at the potentials between 0 to -1.20 V vs Ag/AgCl (3 M KCl) with a scan rate of 10 mV·s^{-1} in 0.1 M TRIS-HCl solution pH 7.0 in the absence of formate (black line) and in the presence of formate (0.1 M, red line) under N_2-saturated condition.

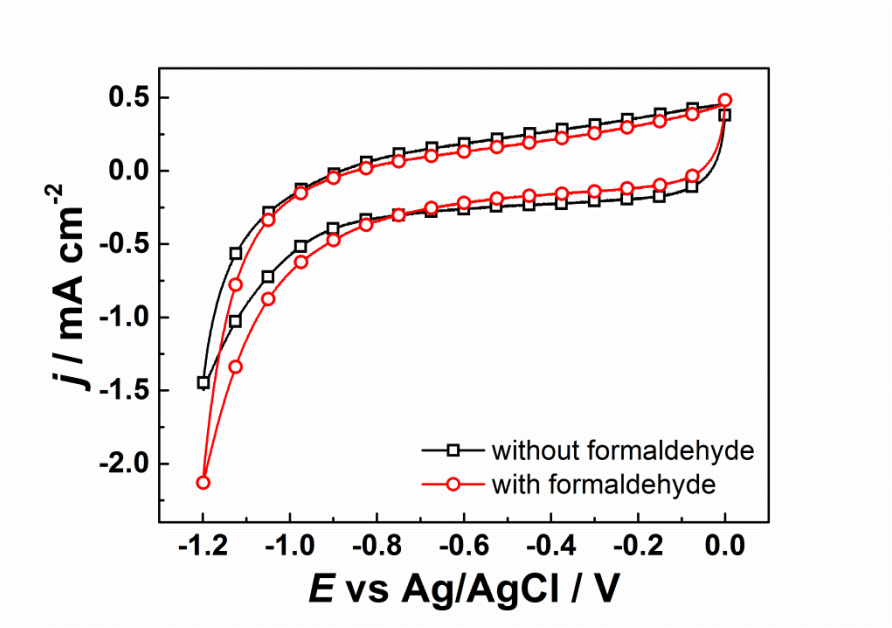

Figure S12. Cyclic voltammograms of a G-ADH immobilized in alginate coated on carbon felt were recorded at the potentials between 0 to -1.20 V vs Ag/AgCl (3 M KCl) with a scan rate of 10 mV·s^{-1} in 0.1 M TRIS-HCl solution pH 7.0 in the absence of formaldehyde (black line) and in the presence of formaldehyde (0.1 M, red line) under N_2-saturated condition.

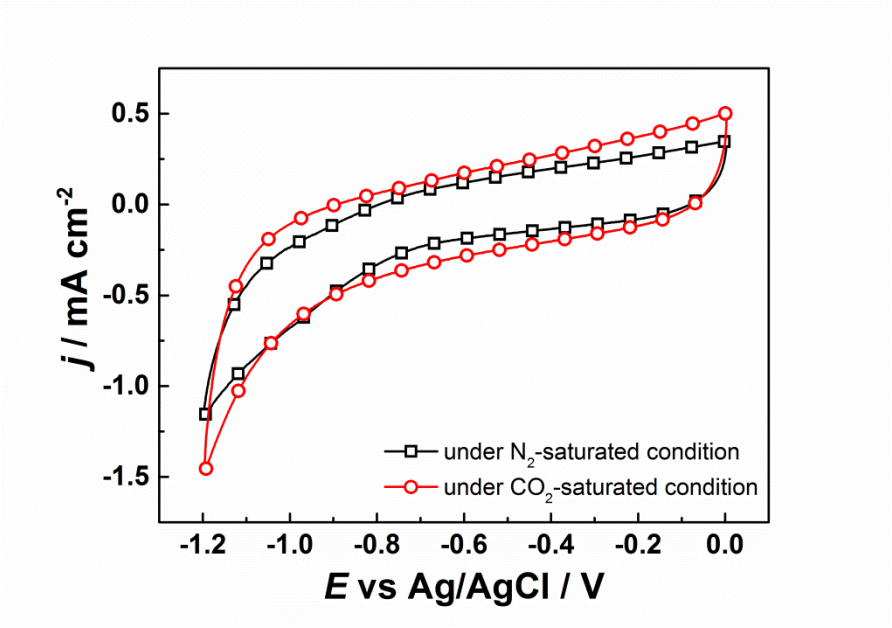

Figure S13. Cyclic voltammograms of a G-DHs immobilized in alginate coated on carbon felt were recorded at the potentials between 0 to -1.20 V vs Ag/AgCl (3 M KCl) with a scan rate of 10 mV·s^{-1} in 0.1 M TRIS-HCl solution pH 7.0 under N_2- (black) and CO_2-saturated conditions (red line).

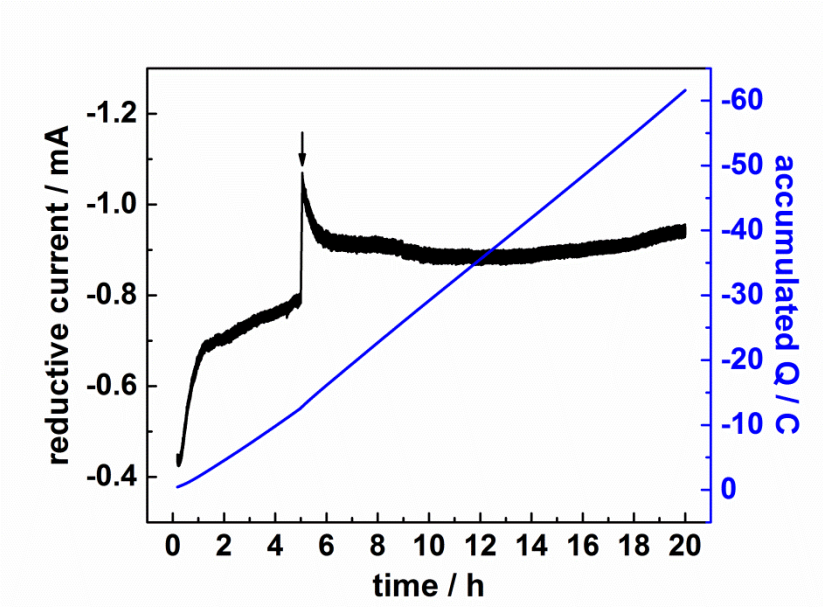

Figure S14. Transient curve and plot of accumulated charge of a constant-potential electrolysis of G-DHs immobilized in alginate coated on carbon felt at -1.20 V vs Ag/AgCl (3 M KCl) in 0.1 M TRIS-HCl buffer solution pH 7.0 under CO_2-saturated condition for 20 h. Arrow indicates liquid solution sampling.

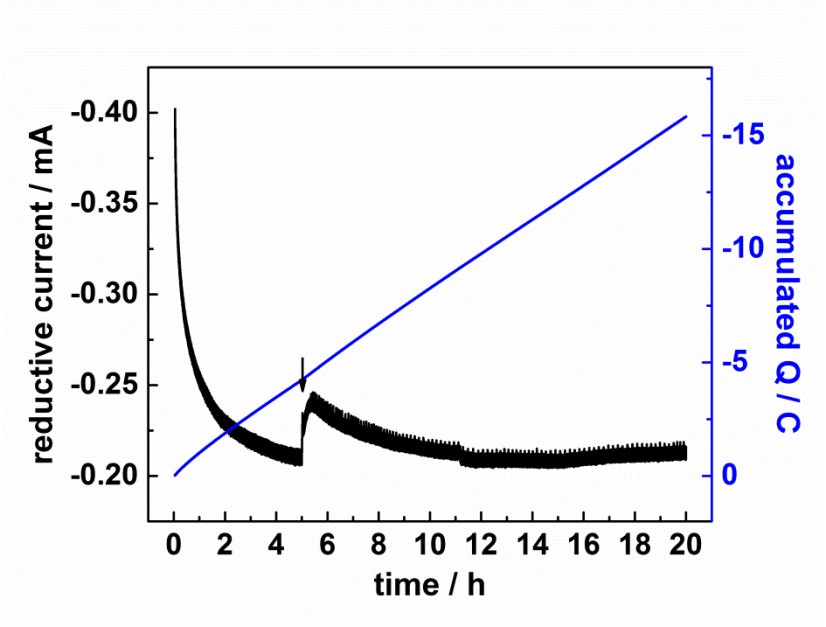

Figure S15. Transient curve and plot of accumulated charge of a constant-potential electrolysis of G-DHs immobilized in alginate coated on carbon felt at -1.20 V vs Ag/AgCl (3 M KCl) in 0.1 M TRIS-HCl buffer solution pH 7.0 under N_2-saturated condition for 20 h. Arrow indicates liquid solution sampling.

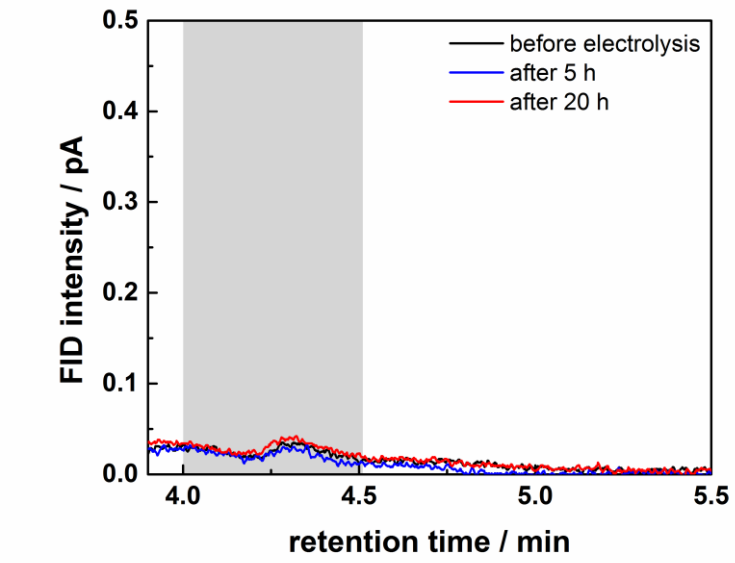

Figure S16. Methanol analysis of liquid samples collected before electrolysis (black line) and after 5-h (blue line) and 20-h (red line) electrolysis under N_2-saturated condition using G-DHs immobilized in alginate coated on carbon felt.

As reported in our previous work,[7] the G-COOH was subjected to inductively coupled plasma mass spectrometry (ICP-MS) analysis and the highest metal contents were found for Fe and Zn at around 70 ppm (Table S1). In the submitted manuscript (Table 1), we have included control experiments performed on this pristine G-COOH, which showed ~6% conversion efficiency, while the bio-catalyst shows 78%. Moreover, we added the control electrochemical experiment on bare G-COOH. The cyclic voltammograms (Figure S17) revealed no evidence of CO_2 reduction. The analysis of liquid samples from the electrolysis (Figure S18) shows only trace of methanol formed after 20-h electrolysis. (Figure S19) Based on this observation we could claim that metal impurities in G-COOH could hardly affect the electrocatalytic process.

Table S1. Inductively coupled plasma mass spectrometry (ICP-MS) trace metal analysis of the G-COOH sample (LTQL stands for: lower than the quantitation limit).[7]

Metal	Cr	Mn	Fe	Co	Ni	Cu	Zn	Pd	Pt
Content (μg/g)	4.4	2.7	76.3	LTQL	30.4	54.6	69.9	LTQL	LTQL

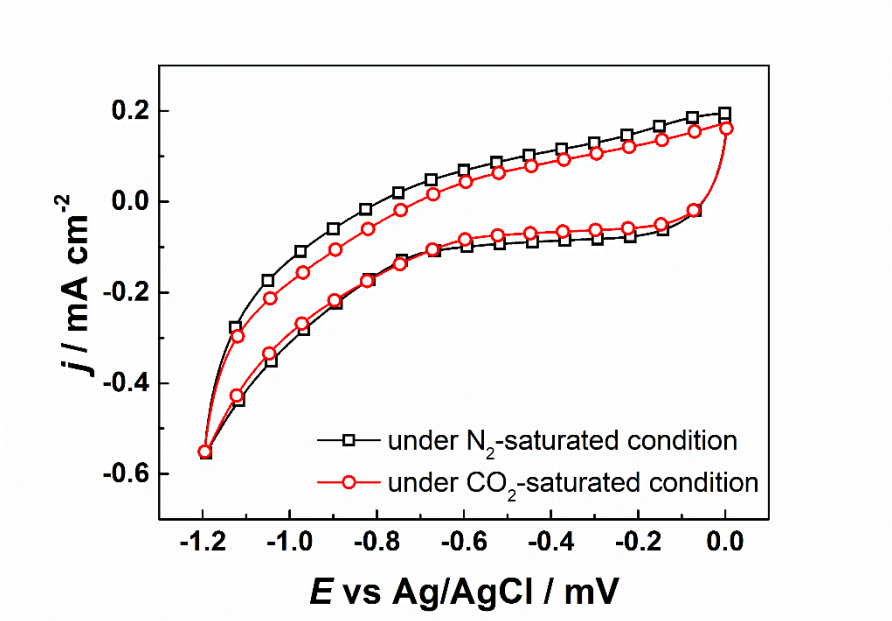

Figure S17. Cyclic voltammograms of a G-COOH immobilized in alginate coated on carbon felt were recorded at the potentials between 0 to -1.20 V vs Ag/AgCl (3 M KCl) with a scan rate of 10 mV·s^{-1} in 0.1 M TRIS-HCl solution pH 7.0 under N_2- (black) and CO_2-saturated conditions (red line).

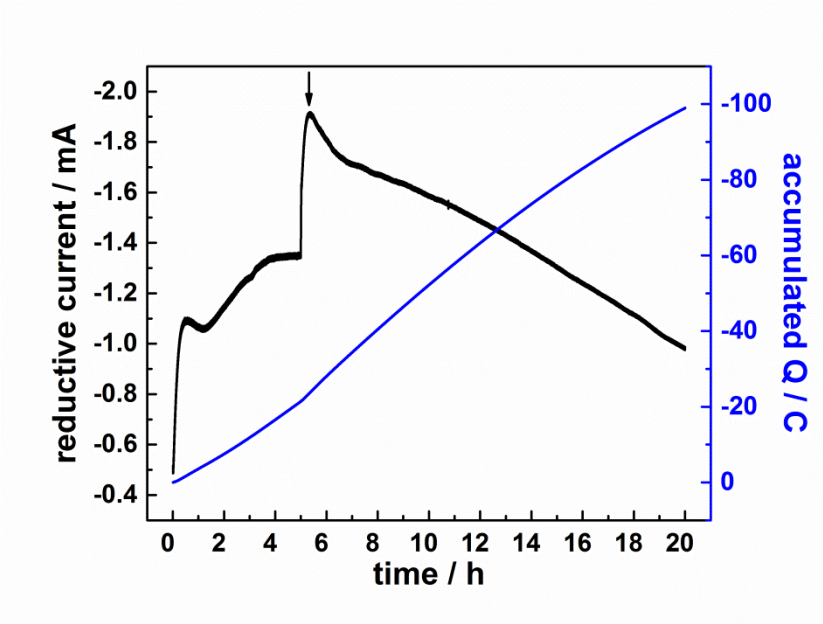

Figure S18. Transient curve and plot of accumulated charge of a constant-potential electrolysis of G-COOH immobilized in alginate coated on carbon felt at -1.20 V vs Ag/AgCl (3 M KCl) in 0.1 M TRIS-HCl buffer solution pH 7.0 under CO_2-saturated condition for 20 h. Arrow indicates liquid solution sampling.

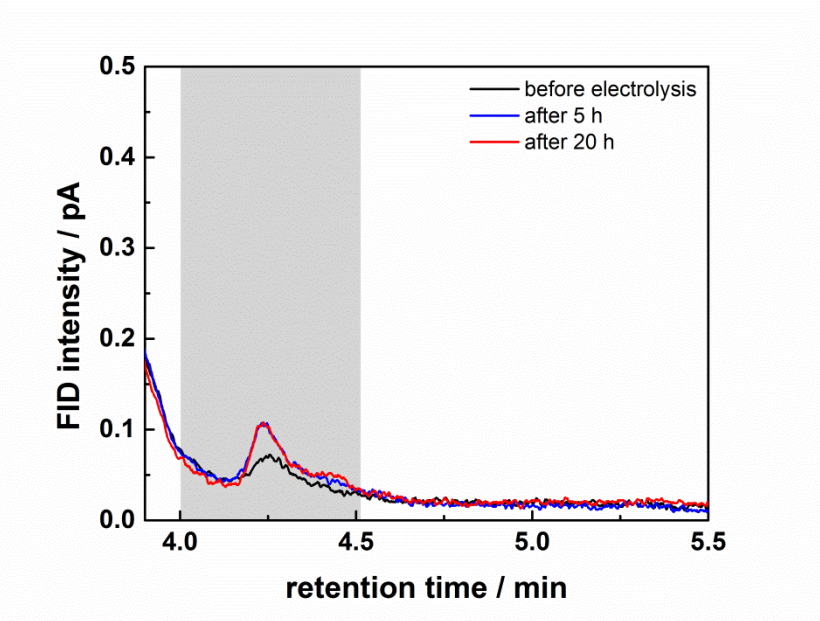

Figure S19. Methanol analysis of liquid samples collected before (black line) and after 5-h (blue line) and 20-h electrolysis (red line) under CO_2-saturated condition using G-COOH immobilized in alginate coated on carbon felt.

3. References

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