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Direct Electrochemistry of *Phanerochaete chrysosporium* Cellobiose Dehydrogenase Covalently Attached onto Gold Nanoparticle Modified Solid Gold Electrodes

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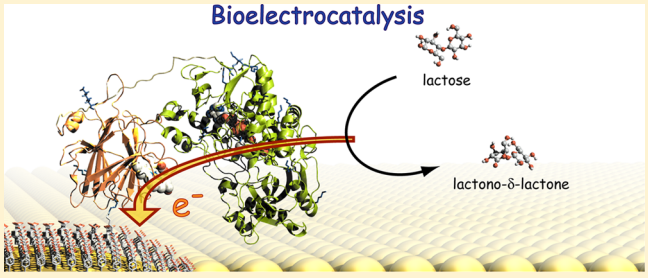
Supporting Information

ABSTRACT: Achieving efficient electrochemical communication between redox enzymes and various electrode materials is one of the main challenges in bioelectrochemistry and is of great importance for developing electronic applications. Cellobiose dehydrogenase (CDH) is an extracellular flavocytochrome composed of a catalytic FAD containing dehydrogenase domain (DH_{CDH}), a heme *b* containing cytochrome domain (CYT_{CDH}), and a flexible linker region connecting the two domains. Efficient direct electron transfer (DET) of CDH from the basidiomycete *Phanerochaete chrysosporium* (*Pc*CDH) covalently attached to mixed self-assembled monolayer (SAM) modified gold nanoparticle (AuNP) electrode is presented. The thiols used were as follows: 4-aminothiophenol (4-ATP), 4-mercaptobenzoic acid (4-MBA), 4-mercaptophenol (4-MP), 11-mercapto-1-undecanamine ($MUNH_2$), 11-mercapto-1-undecanoic acid (MUCOOH), and 11-mercapto-1-undecanol (MUOH). A covalent linkage between *Pc*CDH and 4-ATP or $MUNH_2$ in the mixed SAMs was formed using glutaraldehyde as cross-linker. The covalent immobilization and the surface coverage of *Pc*CDH were confirmed with surface plasmon resonance (SPR). To improve current density, AuNPs were cast on the top of polycrystalline gold electrodes. For all the immobilized *Pc*CDH modified AuNPs electrodes, cyclic voltammetry exhibited clear electrochemical responses of the CYT_{CDH} with fast electron transfer (ET) rates in the absence of substrate (lactose), and the formal potential was evaluated to be +162 mV vs NHE at pH 4.50. The standard ET rate constant (k_s) was estimated for the first time for CDH and was found to be 52.1, 59.8, 112, and 154 s^{-1} for 4-ATP/4-MBA, 4-ATP/4-MP, $MUNH_2$/MUCOOH, and $MUNH_2$/MUOH modified electrodes, respectively. At all the mixed SAM modified AuNP electrodes, *Pc*CDH showed DET only via the CYT_{CDH}. No DET communication between the DH_{CDH} domain and the electrode was found. The current density for lactose oxidation was remarkably increased by introduction of the AuNPs. The 4-ATP/4-MBA modified AuNPs exhibited a current density up to 30 $\mu A\ cm^{-2}$, which is ~70 times higher than that obtained for a 4-ATP/4-MBA modified polycrystalline gold electrode. The results provide insight into fundamental electrochemical properties of CDH covalently immobilized on gold electrodes and promote further applications of CDHs for biosensors, biofuel cells, and bioelectrocatalysis.



INTRODUCTION

Studies of DET reactions between redox enzymes and electrodes constitute an area of extensive research with important implications for understanding the fundamental features of proteins and for developing bioelectronics such as biosensors, biofuel cells, and bioelectrocatalysts.[1] However, DET has only been proven for a restricted number of redox enzymes due to the fact that for the majority of them the active site is deeply buried within the protein structure in combination with the lack of a built-in electron transfer pathway connecting the active site with the surface of the protein.[1−4] Recently, multidomain enzymes such as flavocytochromes, quinohemoproteins, and multicopper oxidases have attracted great interest in this field.[4−6] DET-type biosensors and biofuel cells have been constructed, because these enzymes generally have both a catalytic domain and a separate redox domain/electron transfer pathway acting as a built-in mediator connecting the active site of the catalytic domain with the surface of the enzyme and thus also with electrodes. CDH (EC 1.1.99.18) is an extracellular flavocytochrome secreted by a variety of fungi in the course of

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cellulose degradation. It is a monomeric enzyme, which carries a flavin adenine dinucleotide (FAD) containing dehydrogenase domain (DH_{CDH}) and a *b*-type heme containing cytochrome domain (CYT_{CDH}) connected by a Ser-Thr rich linker region. In the catalytic cycle, the DH_{CDH} catalyzes the dehydrogenation of cellobiose, cello-oligosaccharides, and lactose to their corresponding δ-lactones followed by intermolecular electron transfer (IET) from DH_{CDH} to CYT_{CDH}. Only recently has the natural electron acceptor of CDH been suggested, a copper containing monoxygenase;[7−9] however, a variety of electron acceptors, e.g., ferric and osmium complexes, as well as cytochrome *c*, are able to accept electrons from the reduced CYT_{CDH}. Alternatively, the electrons can be sequentially transferred from reduced CDH to electrodes such as graphite and thiol-modified gold electrodes via CYT_{CDH} in DET communication.[10]

During investigations on the possibility for DET between redox enzymes and electrodes, the combination of nanomaterials and redox enzymes has become an interesting field because of the unique properties of nanomaterials.[11−13] Nanomaterial-modified electrodes promote efficient DET reactions of redox enzymes due to the advantage of having a large surface area for protein loading resulting from large surface area-to-volume ratios and their intrinsic biocompatibility.[14−16] We reported recently that conventional electrodes, such as glassy carbon, graphite, screen-printed electrodes, modified with carbon nanomaterials such as single- and multiwalled carbon nanotubes were used for obtaining efficient DET reactions of CDHs because of the efficient CDH adsorption on carbon-based nanomaterials.[17−20] We have also previously shown that on thiol-modified Au electrodes we can register clear electrochemical response through DET communication with the CYT_{CDH}.[21−25] The current density at CDH-modified conventional solid Au electrodes is, however, rather restricted, and for applications in, e.g., biofuel cells, the current density is much too low. In an attempt to drastically increase the current density, we therefore explore the possibility to increase the surface area and construct a three-dimensional architecture on a solid Au electrode through deposition of AuNPs onto the surface of the solid Au electrode.

In contrast to carbon-based electrodes, no DET of CDHs on unmodified AuNP-modified electrodes have been reported because CDHs cannot be electrostatically bound to an unmodified gold surface. However, AuNPs take advantage of the facility of tailoring the size and controlling surface properties such as hydrophilicity and electrical charge through modification with SAMs.

In this study, we report on the DET reaction of *Phanerochaete chrysosporium* CDH (*Pc*CDH) covalently immobilized on AuNP electrodes. The CDH from the basidiomycete *Phanerochaete chrysosporium* is the most well studied CDH enzyme, and the crystal structures of the individual domains are so far the only available ones.[26,27] The formation of the assembly and surface coverage of *Pc*CDH on the gold surface were determined using surface plasmon resonance (SPR). Three-dimensional surface structured electrodes were prepared by casting concentrated citrate-reduced AuNPs onto the surface of polycrystalline gold electrodes. These electrodes were further modified with thiols forming a SAM onto which *Pc*CDH was covalently bound. The *Pc*CDH immobilized AuNPs electrodes showed well-defined reduction−oxidation peaks of heme *b* of the CYT_{CDH}, and the standard heterogeneous ET rate between the immobilized *Pc*CDH and gold electrodes was determined. The catalytic current density of the *Pc*CDH/AuNPs modified electrodes using lactose as enzyme substrate was remarkably increased compared with those electrodes based only on the basic polycrystalline gold electrodes.

EXPERIMENTAL SECTION

Chemicals and Materials. Recombinant wild-type *P. chrysosporium* CDH was heterologously expressed in the methylotropic yeast *Pichia pastoris* and purified as described previously.[28] The purified enzyme (volumetric activity = 74 U/mL) was stored in 50 mM sodium citrate buffer (pH 5.50) at −20 °C. The cytochrome *c* (cyt *c*) assay was performed to determine the activity of *Pc*CDH. The *Pc*CDH activity was specifically determined by following the reduction of 20 μM cyt *c* (ε_{550} = 19.6 mM^{-1} cm^{-1}) at 30 °C in sodium acetate buffer, pH 4.0, containing 30 mM lactose. One unit of enzymatic activity was defined as the amount of enzyme that oxidizes 1 μmol of lactose per minute under the assay conditions. The protein concentration was determined by the method of Bradford[29] using a prefabricated assay from Bio-Rad Laboratories (Hercules, CA, USA) and bovine serum albumin as the standard. The cyt *c* specific activity for *Pc*CDH was 17.1 U mg^{-1}. Cyt *c* from bovine heart, 4-aminothiophenol (4-ATP), 4-mercaptobenzoic acid (4-MBA), 4-mercaptophenol (4-MP), 11-mercapto-1-undecanoic acid (MUCOOH), and 11-mercapto-1-undecanol (MUOH) were purchased from Aldrich. 11-Mercapto-1-undecanamine ($MUNH_2$) was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Gold-coated SPR chips were obtained from Xan Tec Bioanalytics GmbH (Düsseldorf, Germany). Absolute ethanol (>99%) was purchased from Solveco Chemicals AB (Stockholm, Sweden). β-Lactose was obtained from Fluka (Buchs, Switzerland). All chemicals were of analytical grade. All solutions were prepared with water (18.2 MΩ cm) purified with a PURELAB UHQ II system from ELGA Labwater (High Wycombe, UK). Argon (Ar) and nitrogen (N_2), additionally purified using a Gas Clean Filters from Varian BV (Middelburg, The Netherlands), were obtained from AGA Gas AB (Sundbyberg, Sweden).

Electrode Preparation and Equipment. Polycrystalline gold disk electrodes (BAS, West Lafayette, IN, USA, 0.031 cm^2) were cleaned by incubation in freshly made Piranha solution (3:1 v/v H_2SO_4/H_2O_2; CAUTION: *Piranha solution is especially dangerous, corrosive and may explode if contained in a closed vessel, it should be handled with special care*) for 5 min and then washed with Milli-Q water. The surface of the gold electrodes was mechanically polished on Microcloth (Buehler, Lake Bluff, IL, USA) in aqueous alumina FF slurry (0.1 μm, Struers, Copenhagen, Denmark) and rinsed with water, followed by ultrasonication for 5 min in Milli-Q water. The electrodes were cleaned by cyclic voltammetry in 0.5 M H_2SO_4 between 0 and +1950 mV vs NHE for 20 cycles at a scan rate of 0.1 V s^{-1}. Cyclic voltammetry was performed using an AUTOLAB PGSTAT 30 (Eco Chemie, Utrecht, The Netherlands) equipped with *GPES 4.9* software. A three-electrode cell was used with the *Pc*CDH modified electrode as a working electrode, an Ag|AgCl (sat'd KCl) as reference electrode, and a platinum foil as a counter electrode. All measurements were performed in 0.1 M acetate buffer (pH 4.50) saturated with N_2 at room temperature. During the measurements, a N_2 atmosphere was kept in order to maintain O_2-free conditions.

Preparation of AuNPs Modified Electrodes. AuNPs were prepared according to a method described by Frens.[30] Briefly, 12.5 mL of a 38.8 mM sodium citrate solution was added to a boiling 1.0 mM solution of 125 mL aqueous $HAuCl_4$ under vigorous stirring. After addition, the reaction mixture turned into a deep red color. Boiling and stirring were continued for 15 min. The solution was then allowed to cool to room temperature. The particle diameter of the AuNPs was determined by UV−vis spectroscopy to be 19 nm with error values of at least 10%[31] and confirmed using scanning electron microscopy (SEM). To increase the number of particles per volume, the AuNP solution was centrifuged (10 000 × *g*, 30 min), and then, 98% volume of the supernatant was removed. The precipitated AuNPs were resuspended by ultrasonication and were stored at 4 °C.

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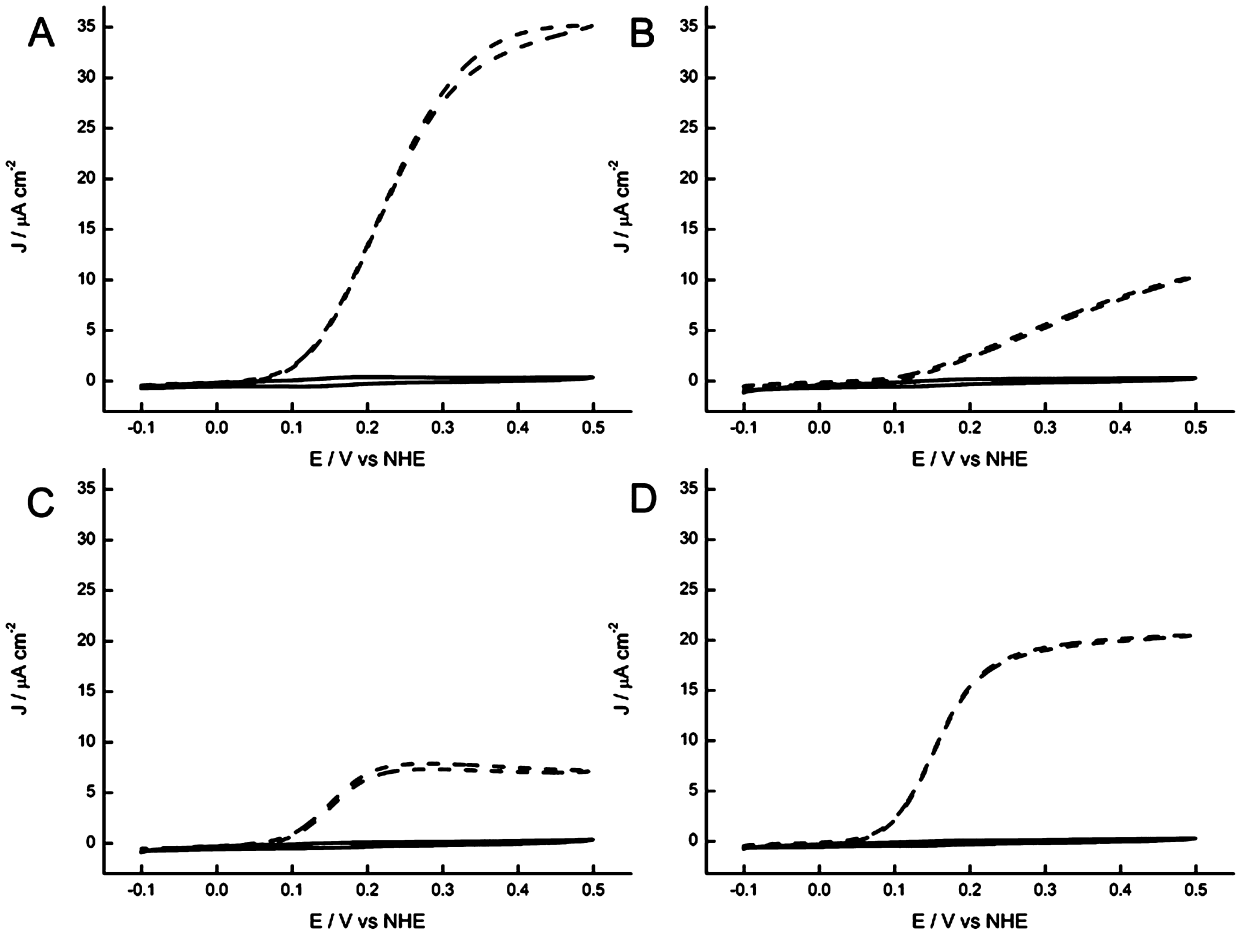

Figure 1. CVs of *Pc*CDH-modified gold electrodes at pH 4.50 in the presence (dashed line) and absence (solid line) of 10 mM lactose. *Pc*CDH was trapped under a permselective membrane and the gold electrode modified with a SAMs composed of (A) MUOH, (B) MUCOOH, (C) 4-MP, and (D) 4-MBA, respectively. Scan rate: 2 mV s^{-1}.

The polycrystalline gold electrodes were polished as described above. Then, to obtain AuNPs modified electrodes, an aliquot of 2 μL of the concentrated AuNPs was cast onto the surface of the polycrystalline gold electrode and dried at room temperature. The casting and evaporation steps were repeated three times to increase the number of particles at the electrode. The AuNPs modified electrodes were electrochemically cleaned by cycling in 0.5 M H_2SO_4 according to the procedure for polycrystalline gold electrodes described above, thoroughly rinsed with Milli-Q water, and finally allowed to dry under a stream of N_2.

Electrode Modification with *Pc*CDH and Mixed SAMs. To form mixed SAMs at the electrode surface two different ethanolic solutions of thiols were mixed in a proportion of 1/50 v/v. The mixtures used were 1 mM 4-ATP and 1 mM 4-MBA, 1 mM 4-ATP and 1 mM 4-MP, 1 mM $MUNH_2$ and 1 mM MUCOOH, and finally 1 mM $MUNH_2$ and 1 mM MUOH. The electrodes were immersed in the mixture solutions for 1 h at room temperature. Then, the modified electrode surfaces were thoroughly rinsed with ethanol to remove physically adsorbed thiol molecules. To form covalent bonds between *Pc*CDH and the electrodes, 2 μL of enzyme solution (74 U/mL) were placed on the entire surface of the electrode and then allowed to react with 1 μL of an aqueous 0.25% GA solution for 2 h at room temperature. Before each measurement, the electrodes were thoroughly rinsed with acetate buffer at pH 4.50 to remove physically adsorbed enzyme molecules.

Preparation of Permselective Membrane Gold Electrodes. Gold disk electrodes were prepared as described above. In order to confirm the cleanness of the electrodes, the last scan of the CVs run in 0.5 M H_2SO_4 was compared to that previously reported as a clean gold surface.[32] The electrode was rinsed abundantly with water between every step. A SAM of thiols was prepared by immersion into 1 mM thiol in ethanolic solution for 1 h at room temperature, followed by rinsing with ethanol, and dried with Ar. Modification of the electrode with CDH was made by trapping 11 μL of CDH solution in a Teflon holder between the electrode and a soaked permselective dialysis membrane (MWCO < 12 000−14 000 Da, Spectrum laboratories, USA).[33]

SPR Analysis of Immobilized *Pc*CDH. SPR measurements were performed using a double-channel Autolab ESPRIT instrument (Eco Chemie, Delft, The Netherlands). The solutions were delivered using a syringe pump into SPR cuvettes. The polarized laser light (λ = 670 nm) was directed to the bottom side of the gold disk via a hemispheric lens and the reflected light was detected using a photodiode. SPR curves were scanned on the forward and backward movements of the mirror and the minima in reflectance were determined. The gold chips were modified with SAMs and *Pc*CDH according to the protocol used for gold electrodes as above. The immobilization of *Pc*CDH on the mixed SAM-modified gold was followed continuously and measured as the SPR angle shifted among a dynamic range of 4000 mdeg and analyzed with the AUTOLAB SPR software. The SPR response can be converted to a mass loading according to the relationship provided by the manufacturer: 120 mdeg = 1 ng/mm^2.

Scanning Electron Microscopy. Scanning electron microscopy (SEM) was performed with an ultrahigh-resolution scanning electron microscope (Zeiss Leo 1560, Oberkochen, Germany) using the Inlens detector at an acceleration voltage of 15 kV and a probe current of 300 pA.

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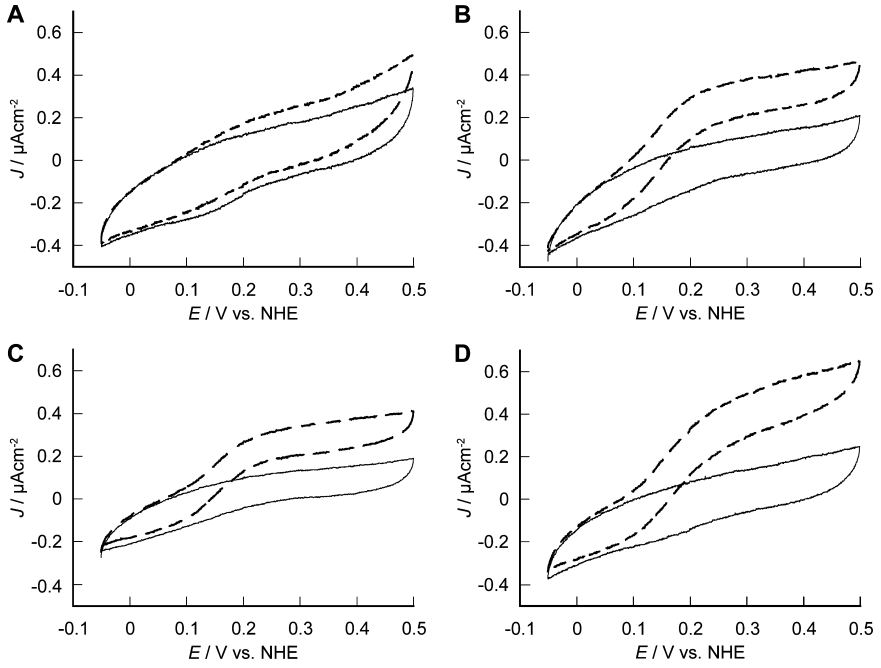

Figure 2. CVs of *Pc*CDH modified gold electrodes at pH 4.50 in the presence (dashed line) and absence (solid line) of 10 mM lactose. *Pc*CDH was covalently bound with 0.25% GA at mixed SAMs monolayer of (A) 4-MP/4-ATP, (B) 4-MBA/4-ATP, (C) MUOH/$MUNH_2$, and (D) MUCOOH/$MUNH_2$, respectively. Scan rate: 2 mV s^{-1}.

RESULTS AND DISCUSSION

Electrochemistry of *Pc*CDH at Mixed SAMs on Gold Electrodes. To construct the *Pc*CDH covalently bound gold electrodes with SAMs, we initially examined cyclic voltammograms obtained for *Pc*CDH at various SAM-modified solid gold electrodes covered with a permselective membrane (molecular weight cutoff 12 000−14 000 Da) according to our previously reported methods.[22,23,33] The thiols with a long chain or a phenyl group, with a basic or an acidic terminal group, and with a hydrophilic or a hydrophobic character were used to build SAMs on the surface of the gold electrode. In this study, lactose was chosen as substrate because lactose does not exhibit substrate inhibition for *Pc*CDH in contrast to the natural substrate, cellobiose.[34] The K_m value for lactose is relatively high (1.1 mM),[35] and the 10 mM lactose concentration used is enough to work at 90% V_{max}. The electrode was modified with 4-MBA, 4-MP, MUCOOH, and MUOH and used with a permselective membrane to trap the enzyme between the membrane and the SAM-modified electrode. Using any of the thiols resulted in electrodes exhibiting direct electron transfer communication between *Pc*CDH and the electrode revealed through cyclic voltammetry (CV) both in the absence and in the presence of substrate (Figure 1). The onset of the catalytic currents started at around 100 mV vs NHE in agreement with previous reports,[21] and the highest current density was observed for MUOH-modified gold electrodes. When the permselective membrane was removed from the electrode, an unstable catalytic electrochemical response was observed that slowly decreased with time. CVs registered for an electrode modified with MUOH are shown in the Supporting Information Figure S1, and similar behaviors were observed for MUCOOH, 4-MP, and 4-MBA (not shown). The catalytic response was followed for 20 cycles at 10 mV/s, and a response still not stabilized was observed at the last cycle, indicating that the interaction between the enzyme and the SAM suffered from excessively weak adsorption. This behavior is in agreement with our previously reported electrochemical properties for CDH on SAM-modified Au electrodes.[21−23]

In order to obtain a stable electrochemical response in the absence of any membrane, *Pc*CDH was covalently attached to the SAM modified gold electrode using GA as cross-linker. GA is composed of two reactive aldehydes that react spontaneously in mild aqueous conditions with primary amino groups. Au electrodes were modified with 4 different mixtures of thiols. In all mixed SAM combinations, a $-NH_2$ terminated thiol acted as a binder between GA and an amino group in the enzyme. The other thiol helped both orient the enzyme and facilitate the ET to the electrode. The combinations used were the following in the proportion 1/50 (v/v): 4-ATP/4-MBA, 4-ATP/4-MP, $MUNH_2$/MUCOOH, and $MUNH_2$/MUOH. In Figure 2, the CVs of *Pc*CDH modified gold electrodes with the four combinations of thiols in the presence and the absence of lactose are shown. Upon addition of the substrate, well-defined catalytic anodic currents were observed starting at about 100 mV vs NHE at all modified electrodes, which is in accordance with the data obtained using the permselective membrane (Figure 1). Control electrodes with no *Pc*CDH showed no anodic wave in this potential range, indicating that the increases in the currents were caused by the DET-type electrocatalytic communication of *Pc*CDH in the presence of lactose. When control electrodes prepared with *Pc*CDH and the mixed SAMs gold electrodes without GA were studied, no electrocatalytic current was observed. This suggests that only covalently bound *Pc*CDH molecules on the gold electrode are working efficiently. In comparison to mixed SAMs with a terminal alcohol group (4-MP/4-ATP and MUOH/$MUNH_2$), higher catalytic current densities can be seen with mixed SAMs with a terminal carboxylic acid group (4-MBA/4-ATP, MUCOOH/$MUNH_2$). This could be due to a more ordered or improved enzyme orientation that minimizes any repulsion between enzymes arising from an electrostatic interaction between the negatively

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charged enzyme surface and the terminal carboxylic acid group of the thiols.

The enzyme modification procedures with respect to the ratios of mixed thiols and GA concentration were optimized. As shown in Figure 3A, optimum catalytic currents of all the

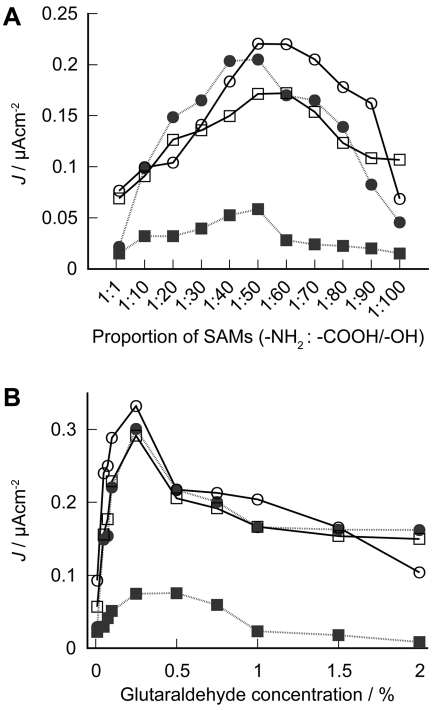

Figure 3. Dependence of the catalytic current on the molar ratio of mixed SAMs and GA concentration at (■) 4-MP/4-ATP, (●) 4-MBA/4-ATP, (□) MUOH/MUNH$_2$, and (○) MUCOOH/MUNH$_2$. (A) *Pc*CDH was covalently bound using 1% GA at various ratios of mixed SAMs and (B) with various GA concentrations at a fixed ratio 1:50 v/v of SAMs; thiol−NH$_2$/thiol−COOH or thiol−OH.

*Pc*CDH modified electrodes prepared with 1% GA were obtained for the mixed SAMs with a molar ratio of about 1/50 (−NH$_2$/−OH or −NH$_2$/−COOH). Note that the composition of the SAM on the surface will not be the same as the alkanethiol mixture on solution used for immobilization.[36−39] The dependence of the catalytic currents of *Pc*CDH modified electrodes with a mixed SAM with a molar ratio of 1/50 on the concentration of GA is shown in Figure 3B. The results demonstrate that the optimum concentration of GA for cross-linking is 0.25%.

Analysis of Attachment of *Pc*CDH on gold surface with SPR. To observe whether *Pc*CDH is covalently attached on the gold surface, the attachment of the enzyme onto the following mixed SAMs, viz., 4-ATP/4-MBA, 4-ATP/4-MP, MUNH$_2$/MUCOOH, and MUNH$_2$/MUOH, was studied with time using SPR. Figure 4 (solid line) shows the typical change in the SPR signal for *Pc*CDH modification of a gold disk with a 1/50 solution of 4-ATP/4-MBA. During SPR experiments, the first 600 s (Figure 4, section a) was used for baseline stabilization with the SAM-modified gold in 0.1 M acetate buffer at pH 4.5. The addition of *Pc*CDH and GA solution to the mixed SAMs (Figure 4, section b) resulted in an increased SPR signal. After 2 h, no further binding of GA or CDH was observed, and then, the *Pc*CDH modified 4-ATP/4-MBA gold disk was washed with acetate buffer (0.1 M, pH 4.5) in order to remove any weakly adsorbed enzyme molecules. Finally, buffer

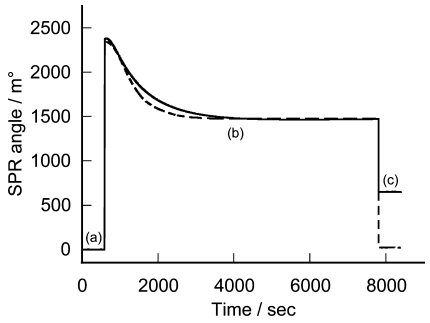

Figure 4. Variation in SPR signal vs time showing covalent immobilization of *Pc*CDH on the 4-ATP/4-MBA (1/50 v/v) modified gold with (solid line) 0.25% GA and (dashed line) without GA: (a) baseline correction with mixed SAMs in acetate buffer (0.1 M, pH 4.50); (b) formation of covalent bond between *Pc*CDH and mixed SAMs with GA; (c) after washing with 0.1 M acetate buffer (pH 4.50).

was introduced into the SPR cuvettes (Figure 4, section c) and compared with the original signal before 600 s. After washing with acetate buffer, the decrease in SPR signal is attributed to the change in the optical properties of the solution and the removal of nonspecifically bound enzyme molecules. While an increase in the SPR signal for *Pc*CDH with 0.25% GA was observed after *Pc*CDH modification, the SPR signal remained unchanged for the modification without GA. The results prove that GA reacted as a cross-linker between *Pc*CDH and the amine group of the modified SAMs and the enzyme could covalently attach onto the surface. The SPR response can be converted into an equivalent mass loading according to the relationship provided by the manufacturer: 120 mdeg = 1 ng/mm^2. According to this relation, the surface coverage, Γ, of immobilized *Pc*CDH was 5.6 ng/mm^2 (5.7 pmol/mm^2) from the difference between SPR signals before and after the loading with *Pc*CDH and GA. The different Γ-values of *Pc*CDH on the various gold surfaces with the mixed SAMs are summarized in Table 1. No significant difference can be observed in the Γ-

Table 1. Electrochemical Properties of *Pc*CDH at the Mixed SAM Modified Gold Electrodes[a]

	polycrystalline gold	AuNPs				SPR
mixed SAMs	J/μA cm^{-2}	J/μA cm^{-2}	$E^{\circ\prime}$/ mV	ΔE/ mV	k_s/s^{-1}	Γ/ pmol mm^{-2}
4-ATP/4-MP	0.26	4.0	161.7	14.7	59.8	5.79
4-ATP/4-MBA	0.40	29.3	161.5	14.6	52.1	5.71
MUNH$_2$/MUOH	0.34	11.6	161.8	14.7	154.0	5.67
MUNH$_2$/MUCOOH	0.49	15.2	161.3	14.6	112.0	5.65

[a]Current densities, J, for polycrystalline gold and AuNPs were obtained at 300 mV vs. NHE (see Figures 2 and 8, respectively). Formal potentials, $E^{\circ\prime}$, and peak separations, ΔE, at 0.2 V s^{-1} (see Figure 5).

values for all the mixed SAMs. The analysis shows that the covalent bonding of *Pc*CDH by GA was not influenced by the electric charge of SAMs at the pH used.

Electrochemical Properties of *Pc*CDH at Mixed SAMs on AuNP Modified Electrodes. To improve the DET coupling of *Pc*CDH to the electrode and to increase the catalytic current for lactose, AuNPs were employed for

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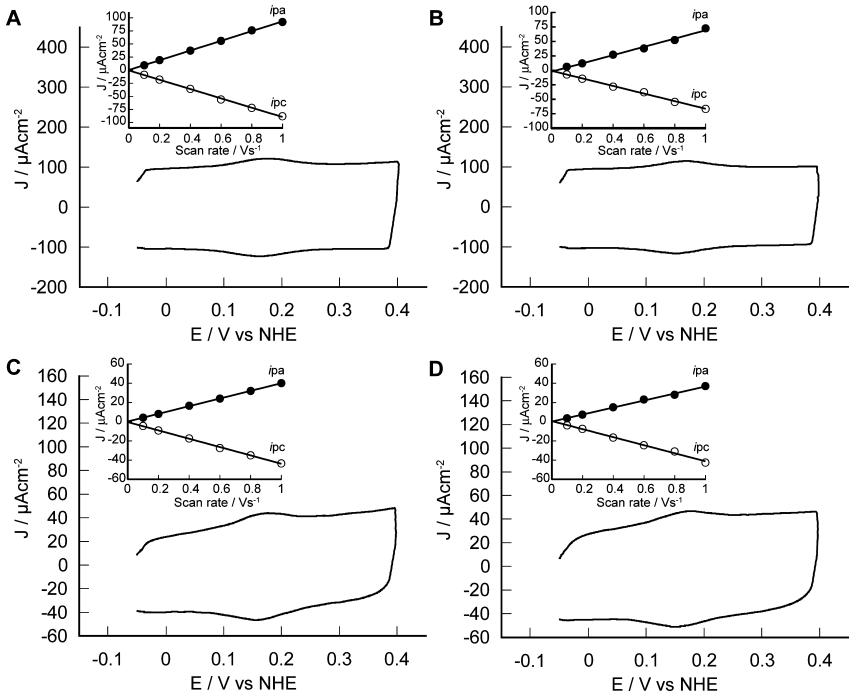

Figure 5. CVs of *Pc*CDH covalently immobilized on mixed SAM modified AuNPs electrode. *Pc*CDH was attached using 0.25% GA at mixed SAM monolayer of (A) 4-MP/4-ATP, (B) 4-MBA/4-ATP, (C) $MUOH/MUNH_2$, and (D) $MUCOOH/MUNH_2$. The CVs were measured in buffer solution (0.1 M acetate buffer, pH 4.50) at a scan rate of 0.2 V s^{-1}. The insets show the cathodic (i_{pc}) and anodic peak (i_{pa}) currents versus scan rate.

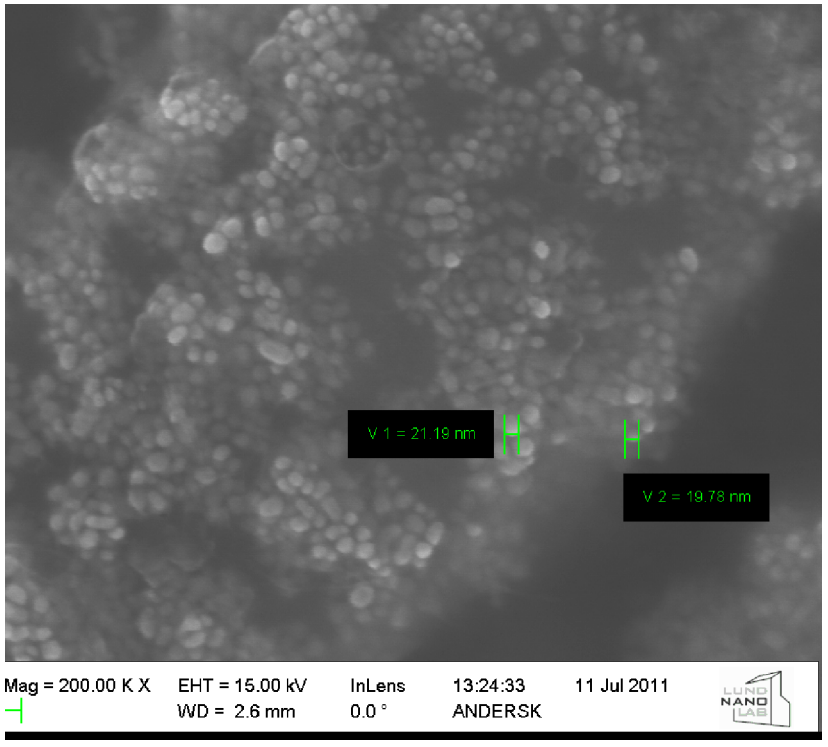

Figure 6. Typical SEM image (200 000×) of the top view of the AuNP-modified gold electrode.

preparing the enzyme modified electrodes. The CV of an AuNP modified electrode in 0.5 M H_2SO_4 clearly exhibits two oxidation waves (formation of surface oxides) with peaks at about 1.44 and 1.6 V and one reduction wave (reduction of surface oxides) with a peak at about 1.06 V corresponding to the so-called surface redox reaction of gold,[32] while these peaks were not as clearly recognized at the polycrystalline gold electrode (Supporting Information Figure S2 and Figure S3).

As shown in Figure 5, CVs of *Pc*CDH modified AuNP electrodes show well-defined anodic and cathodic waves in acetate buffer (pH 4.50) under anaerobic conditions. While no significant peaks of the heme *b* of the CYT_{CDH} of *Pc*CDH were observed at the mixed SAM modified polycrystalline gold

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electrodes, all the *Pc*CDH modified mixed SAM AuNP electrodes exhibit clear redox waves with similar redox potentials. Because the electrochemical effective surface area of the electrodes modified three times with AuNPs estimated by cyclic voltammetry by CV was about 70 times greater than the geometric surface area of the substrate electrode,[40] the clear redox waves were mainly attributed to an increase in the number of *Pc*CDH molecules attached to the electrode surface. However, there could possibly also be an effect that the enzyme molecule immobilized onto the nanoparticle modified surface has a higher probability to be oriented for efficient DET than when immobilized onto the original solid Au electrode. The drop-cast method creates a really porous structure formed by adsorbed AuNPs having a diameter of approximately 19 nm. Figure 6 shows a SEM picture of the structure created. At all the AuNP modified electrodes, the formal potential, $E^{\circ\prime}$, determined by evaluating the midpoint of the oxidation and reduction peak potentials, was about +162 mV vs NHE, which is assigned to the Fe^{3+}/Fe^{2+} redox couple of the heme *b* in *Pc*CDH. No shift in the $E^{\circ\prime}$ was detected compared with those of *Pc*CDH entrapped under a permselective membrane and the values are consistent with previously reported values for the CYT_{CDH} of *Pc*CDH in solution.[23] The results reveal that using GA for the immobilization onto the mixed SAMs did not induce any protein denaturation, and the DET between *Pc*CDH and the AuNP modified electrodes takes place via the CYT_{CDH} of the enzyme. In addition, the peak-to-peak separation between the anodic and the cathodic peak potentials, ΔE_p, of ca. 15 mV and the linear dependence of the peak currents on scan rate show a surface-controlled electrochemical behavior (see Figure 5). The values of the standard ET rate constants (k_s) were calculated according to Laviron.[41] The variation of the reduction and oxidation peak currents analyzed as a function of the logarithmic scan rate is shown in Figure 7.

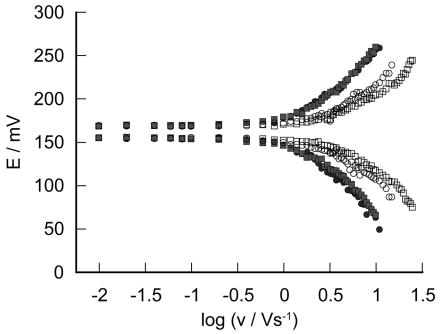

Figure 7. Variations of the peak potentials versus the logarithmic scan rate (log *v*) for *Pc*CDH at the (■) 4-MP/4-ATP, (●) 4-MBA/4-ATP, (□) $MUOH/MUNH_2$, and (○) $MUCOOH/MUNH_2$ modified AuNPs electrode.

For all electrodes, the plots exhibit a typical trumpet shape, in which the peak currents separate symmetrically from the $E^{\circ\prime}$-value with a slope of about 120 mV/log unit as the scan rate is increased. This indicates a kinetically limited heterogeneous ET reaction with a transfer coefficient, α, of 0.5. The analysis gave the following k_s values; 52.1, 59.8, 112, and 154 s^{-1} when using SAMs based on 4-ATP/4-MBA, 4-ATP/4-MP, $MUNH_2$/MUCOOH, and $MUNH_2$/MUOH, respectively. Obviously, the k_s value is dependent on the length of the carbon chain and on the terminal groups of the SAMs. When *Pc*CDH was covalently immobilized on the mixed SAMs with aliphatic 11-hydrocarbon chain thiol electrodes, the ET rates were almost 2 times higher than the mixed SAMs composed of aromatic rings. According to the Marcus theory, the aliphatic 11-hydrocarbon chain thiol modified electrodes would be expected to show the slowest k_s values, because they are farther away from the electrode than the aromatic ring thiols. The explanation for the opposite behavior could be the SAMs formed by thiolic aromatic rings repulsing the electronic cloud of the enzyme. It has been shown that longer aliphatic chains due to van der Waals interactions between the long carbon chains create a more stable SAM.[42] SAMs with negatively charged surfaces showed lower k_s values than those based on −OH (uncharged). This effect could be due to electrostatic repulsions between the negatively charged SAM and the *Pc*CDH molecules immobilized close to them. Moreover, the k_s at all mixed SAM AuNPs are much higher compared with the intramolecular ET rate between the DH_{CDH} and the CYT_{CDH} domains (30 s^{-1}), which has been proposed to be the rate-limiting step in the overall *Pc*CDH reaction.[43] Thus, the maximum electrocatalytic density would be controlled by the intrinsic enzymatic dehydrogenase activity on the electrodes. To the best or our knowledge, the heterogeneous ET rate constant of CDH on a thiol-modified gold electrode has been estimated for the first time.

In Figure 8, the catalytic current responses of *Pc*CDH on the mixed SAM AuNP electrode to lactose are presented. As

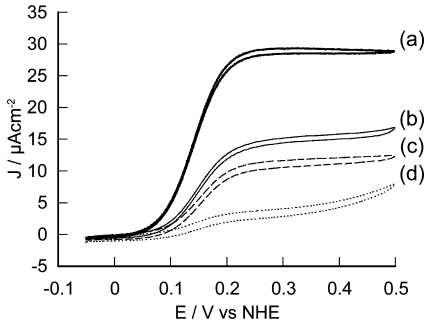

Figure 8. Electrocatalytic currents of *Pc*CDH covalently immobilized on mixed SAMs (1/50 v/v) on modified AuNP electrode: (a) 4-MBA/4-ATP, (b) $MUCOOH/MUNH_2$, (c) $MUOH/MUNH_2$, and (d) 4-MP/4-ATP. The measurements were performed at a scan rate of 2 mV s^{-1} in 0.1 M acetate buffer at pH 4.50 in the presence of 10 mM lactose.

already shown in Figure 5, in the absence of lactose, the redox waves of the heme *b* of the CYT_{CDH} can be seen at around 160 mV. On the addition of substrate, the electrocatalytic currents start at the beginning of the anodic wave of the CYT_{CDH}. Therefore, the electro-oxidation of lactose can be observed via the CYT_{CDH} domain of *Pc*CDH as shown above on the polycrystalline gold electrode (Figure 2). The catalytic current densities obtained for the 4-MBA/4-ATP or $MUCOOH/MUNH_2$ based SAMs were higher than those for 4-MP/4-ATP and $MUOH/MUNH_2$, which is in accordance with the observations at the mixed SAMs on polycrystalline gold electrode. Additionally, in the presence of the AuNPs, significant differences in current densities between the mixed SAMs can be found. Notably, the 4-MBA/4-ATP electrode exhibited a current about 6 times higher compared with that obtained for the 4-MP/4-ATP based one. Obviously, the current density was dependent on the terminal groups of the SAMs. When *Pc*CDH was covalently immobilized on the mixed SAMs with carboxyl terminated thiol electrodes, the electro-

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catalytic currents of lactose were higher than when the mixed SAMs with alcohol electrodes were used. We have previously reported on the electrocatalytic reaction of *Pc*CDH for lactose at single-walled carbon nanotubes (SWCT) modified electrode on pyrolytic graphite using the flow injection analysis mode.[18] In comparison with the SWCT modified electrode (~13 μA cm^{-2}), the electrocatalytic current density with the 4-MBA/4-ATP modified electrode was twice as high. Even higher currents are expected if the AuNP electrodes are studied in the flow injection mode.

CONCLUSIONS

*Pc*CDH was covalently attached onto polycrystalline gold electrodes and AuNP-modified polycrystalline gold electrodes with GA as cross-linker. The polycrystalline gold electrodes as well as the AuNP modified polycrystalline gold electrodes were further modified with mixed SAMs, viz., 4-ATP/4-MBA, 4-ATP/4-MP, $MUNH_2$/MUCOOH, and $MUNH_2$/MUOH in a ratio of 1/50. The terminal amino group of the SAM was used to react with GA, while the terminal alcohol or acid group can facilitate DET reaction between *Pc*CDH and the gold electrodes. *Pc*CDH on the mixed SAM modified polycrystalline electrodes obviously show the DET-type electrocatalytic currents for lactose. The SPR signal revealed covalent immobilization of *Pc*CDH on the gold surface. Additionally, by using the three-dimensionally structured surface of the AuNP-modified electrodes, a clear redox wave in the CVs of heme *b* of the CYT_{CDH} in *Pc*CDH can be observed, whereas the corresponding *Pc*CDH modified polycrystalline gold electrodes did not show any redox waves in the absence of substrate. The AuNPs electrodes also exhibit remarkably high heterogeneous ET rates (~50−150 s^{-1}). The current density for lactose oxidation with 4-ATP/4-MBA modified AuNPs reached 29.3 μA cm^{-2} at 300 mV vs NHE. A further increase in the current density can be expected by improvement of the architecture of the AuNPs, and the signal-to-background ratio was sufficiently low compared with previously used carbon electrodes. Thus, *Pc*CDH attached to AuNP modified electrodes is of high interest for the development of third-generation biosensors and biofuel cells.

ASSOCIATED CONTENT

Supporting Information

CV stability for adsorbed *Pc*CDH on MUOH gold electrodes and comparison of AuNP modified and nonmodified Au electrodes in H_2SO_4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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