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Gold single-crystal electrode surface modified with self-assembled monolayers for electron tunneling with bilirubin oxidase

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Abstract

Using Au(111) and Au(100) single-crystal electrodes modified with self-assembled monolayers (SAMs), the direct electron transfer reaction of bilirubin oxidase (BOD) adsorbed onto their surfaces was investigated. The BOD adsorbed onto the Au(111), Au(100) and gold/mica electrodes, and the BOD adsorbed onto Au(111) electrodes modified with C₃-SO₃H and Cₙ-COOH (n = 2, 5 and 7), showed the electrocatalytic currents of dioxygen reduction based on the direct electron transfer reaction. The BOD adsorbed onto Au(111) electrodes modified with C₆-NH₂, C₆-OH and C₅-CH₃ did not show any electrocatalytic current. Negatively charged electrode surfaces can give a suitable molecular orientation for the direct electron transfer of BOD. The $k^*$ values evaluated by an analysis of the steady-state voltammogram with a simulated fitting method did not depend on the crystal structure of the gold electrode surface. Using a Cₙ-COOH (n = 2, 5, 7) modified Au(111) electrode, the $k^*$ values decreased with an increasing alkyl chain length of Cₙ-COOH. Based on the $k^*$ values obtained from the Cₙ-COOH (n = 2, 5, 7) modified Au(111) electrodes, the electron tunneling distance was evaluated. The average distance between the type 1 Cu site of BOD and the outside of the BOD protein structure was evaluated to be 17 (± 2) Å.

Keywords

Bilirubin oxidase; electron transfer; catalytic current, self-assembled monolayer; single crystal electrode
1. Introduction

Bilirubin oxidase (BOD) contains multi active center copper sites: type 1, type 2 and type 3 Cu (in a ratio of 1:1:2).\textsuperscript{1-3} Type 1 Cu site accepts electron from the substrate, which is then transferred through an intramolecular electron transfer to the type 2-3 cluster. Dioxygen is reduced to water at type 2-3 Cu cluster sites. BOD is one of the few enzymes that directly reduces dioxygen to water in a four-electron transfer reaction in neutral solution without generating any dioxygen derived intermediates.\textsuperscript{1-3} Thus, this enzyme is an interesting and promising candidate for applications in biofuel cells and biosensors. The biofuel cell was reported for the first time by Katz et al., and was fabricated from cytochrome c and a cytochrome c oxidase system.\textsuperscript{4} Kano and Ikeda group reported a dihydrogen-dioxygen biofuel cell using BOD as the cathode, which could be operated under physiological conditions and at room temperature.\textsuperscript{5,6} Heller and coworkers investigated the glucose-dioxygen biofuel cell using BOD.\textsuperscript{7} In these biofuel systems, an electron transfer mediator such as 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) and a redox polymer containing osmium complexes was required for electron transfer between BOD and the electrode. Such a mediated electron transfer system has several disadvantages in terms of thermodynamic loss between the mediator and the enzyme, decreased efficiency due to the mediator leaking and difficulty with a simplified system.

Recently, the direct electron transfer reaction of BOD at carbon material electrodes under anaerobic and aerobic conditions has been reported.\textsuperscript{8-14} Fuel cells without a mediator for BOD have also been reported.\textsuperscript{9,13,15} Furthermore, some researchers are investigating to extend the lifetime of the BOD modified electrode. BOD immobilized into the nano-sized pores of carbon aerogels showed a very stable catalytic current for more than 10 days,\textsuperscript{16} which was significantly better in comparison with those previously reported. Furthermore, it has been reported that a
cubic phase lyotropic liquid crystal reduces the urate electrooxidation-associated chemical damage for BOD in the presence of dioxygen and serum urate.\textsuperscript{17}

The direct electron transfer reaction of BOD has been performed only at carbon material electrodes. Except for highly ordered structural surfaces such as the basal plane of HOPG, the surface of carbon materials typically has many functional groups such as carboxy, carbonyl and quinone groups. Thus, it is very difficult to investigate the interactions between a BOD molecule and the electrode surface for direct electron transfer reactions. In particular, the molecular orientation of the enzyme on the electrode surface is one of the most important requirements for fast direct electron transfer reactions, because the redox center of the enzyme is buried deeply within the protein shell. The electron transfer rate is exponentially dependent on the distance between the redox active centers, as predicted by the Marcus theory.\textsuperscript{18,19}

Self-assembled monolayer (SAM)-modified electrodes have been widely used for protein electrochemistry.\textsuperscript{20,21} A SAM-modified electrode has advantages for the investigation of the molecular orientation of an enzyme on the electrode surface, because when a single crystal gold surface is used, it has a well-ordered and atomically flat surface.\textsuperscript{20-43} The investigation for the molecular orientation of BOD on a single-crystal gold electrode modified with SAMs has not been reported, although the slightly investigation using polycrystal gold\textsuperscript{44} and SAM-modified gold electrodes\textsuperscript{45} have been reported. In the present study, using Au(111) and Au(100) single-crystal electrodes modified with SAMs, the electrocatalytic current of dioxygen reduction based on the direct electron transfer reaction of BOD adsorbed onto their surfaces was investigated. The obtained results clearly indicated that the negatively charged electrode surfaces give a suitable molecular orientation for the direct electron transfer of BOD with the electrode. The average distance between the type 1 Cu site of BOD and the outside of the BOD protein structure was evaluated by an analysis of the electron tunneling rate versus its distance. The obtained distance was within the reported physiological range for electron tunneling.
2. Experimental Section

2.1. Materials

Bilirubin oxidase (BOD, EC 1.3.3.5, from *Myrothecium verrucaria*) was purchased from MP Biomedicals Inc., France, and used without further purification. Water was purified with a Millipore Milli-Q water system. All other chemicals were of analytical grade and were used as received.

The SAMs used were 3-mercaptopropionic acid (C$_2$-COOH, 99% <, Aldrich), 5-carboxy-1-pentanethiol (C$_5$-COOH, 97 %, Dojin Chem. Co., Japan), 7-carboxy-1-heptanethiol (C$_7$-COOH, 97 %, Dojin Chem. Co., Japan), 1-carboxy-1-decanethiol (C$_{10}$-COOH, 97 %, Dojin Chem. Co., Japan), 3-mercapto-1-propane sulfonic acid (C$_3$-SO$_3$H, 80 %, Tokyo Kasei), 6-hydroxy-1-hexanethiol (C$_6$-OH, 98 %, Dojin Chem.), 6-amino-1-hexanethiol (C$_6$-NH$_2$, 90%, Dojin Chem. Co., Japan), and 1-hexanethiol (C$_5$-CH$_3$, Aldrich).

2.2. Instrumentation

Quartz crystal microbalance (QCM) measurements were carried out using a QCM2000 (Initium Co., Japan). Gold film was deposited onto an AT-cut quartz crystal with a 27 MHz fundamental resonance frequency, and its surface was polished to a mirror-like finish. The QCM gold electrode surface (0.038 cm$^2$) was cleaned by a piranha solution (1:3 H$_2$O$_2$ (30 %) + H$_2$SO$_4$ (conc.)) for 3 min, and rinsed with Milli-Q water.

A multimode NanoScope III (Digital Instruments) was utilized for tapping-mode atomic force microscopy (AFM) imaging. Standard phosphorus doped silicon cantilevers were used. The calibration was performed using an imaging standard grating. The tapping-mode AFM measurements were performed under an air atmosphere.
The electrochemical measurements were performed with an Electrochemical Analyzer, Model 700B, CHI instrument (BAS, Tokyo) and using the hanging meniscus method in a three-compartment electrochemical cell under high purity argon. The instrument was combined with a rotating electrode system when a steady-state catalytic current was obtained. An Ag/AgCl (saturated KCl) electrode and a Pt plate were used as the reference and auxiliary electrodes, respectively. All potentials are reported with respect to the Ag/AgCl (saturated KCl) electrode. Prior to the cyclic voltammetric measurements, the buffer solution was de-aerated with high purity argon, and a positive pressure of argon gas was kept over the solution during all electrochemical experiments.

2.3. Preparation of electrode

Au(111) and Au(100) single-crystal electrodes were used as the working electrodes for cyclic voltammetry, which were prepared by the Clavilier method. The Au(111) and Au(100) surfaces were verified by the observation of the cyclic voltammograms in 0.01 mol dm$^{-3}$ HClO$_4$ solution. The voltammogram shapes observed were in good agreement with that reported in the literature.

The substrates of gold/mica for the AFM were prepared by the vapor deposition of 150 nm of gold (99.999% purity) onto freshly cleaved mica sheets (Nilaco Co., Japan) at a reduced pressure, $< 0.1 \times 10^{-6}$ Torr. The temperature of the mica sheet was maintained at 300 °C during the vapor deposition. To verify that the gold thin film surface was enriched with Au(111) facets, cyclic voltammetry was performed on the Au/mica in 0.01 mol dm$^{-3}$ HClO$_4$ solution.

2.4. Preparation of SAM-modified electrodes

The SAM-modified gold electrodes were prepared according to the following procedures. First, the Au(111) electrode and gold evaporated substrate were pretreated by flame-annealing and quenching in ultra-pure water saturated with hydrogen. The pretreated
gold electrode, Au/mica substrate surface and QCM gold electrode were then modified with one of the SAMs. To prepare the SAM-modified electrodes, the single crystal gold, gold/mica substrate and QCM gold electrodes were immersed into a 1 mmol dm\(^{-3}\) alkaline aqueous solution (C\(_2\)-COOH, C\(_5\)-COOH, C\(_7\)-COOH, C\(_{10}\)-COOH, C\(_3\)-SO\(_3\)H and C\(_6\)-NH\(_2\)) or ethanol solution (C\(_6\)-OH and C\(_5\)-CH\(_3\)) for 20 min. The surface coverage of each SAMs onto the Au(111) electrode was estimated by voltammograms in a 0.5 mol dm\(^{-3}\) KOH solution.\(^{49}\) From the charge integration of the reductive desorption curve of voltammograms for the SAMs, the surface coverage of C\(_2\)-COOH, C\(_5\)-COOH, C\(_7\)-COOH, C\(_{10}\)-COOH, C\(_3\)-SO\(_3\)H, C\(_6\)-OH, C\(_6\)-NH\(_2\) and C\(_5\)-CH\(_3\) on the electrode was estimated to be 9.1 (±0.4), 6.9 (±0.3), 6.5 (±0.3), 7.6 (±0.2), 7.0 (±0.4), 7.0 (±0.5), 7.7 (±0.5), 8.0 (±0.2) \(\times\) 10\(^{10}\) mol cm\(^{-2}\), respectively, indicating that full coverage monolayers had been formed on the gold surface.\(^{49}\)

To immobilize the BOD on the SAMs, the SAM-modified gold electrodes were immersed into a phosphate buffer solution (pH 6.8, ionic strength: \(\mu = 0.05\)) containing 40 units ml\(^{-1}\) BOD for 10 min. Prior to the measurements, this electrode was gently rinsed with the buffer solution.

3. Results and discussion

3.1. Electrocatalytic current based on BOD at single crystal gold electrodes

Fig. 1a shows cyclic voltammograms for BOD immobilized onto a bare Au(111) electrode in phosphate buffer (pH 6.8) in the presence of saturated dioxygen. A well-defined dioxygen reduction catalytic current was observed from around 0.55 V (vs. Ag/AgCl/saturated KCl). Such a catalytic current was not observed in the absence of dioxygen or a BOD unmodified electrode. These facts indicate that the observed catalytic current was due to the direct electron transfer reaction of BOD at the electrode. A similar result was also obtained when BOD was immobilized on a bare Au(100) electrode. To evaluate the heterogeneous electron transfer kinetics between BOD and the gold single-crystal
electrodes, the steady-state voltammetry was carried out. Fig. 1b shows the background current corrected steady-state linear sweep voltammogram for a BOD modified Au(111) electrode in a buffer solution. The voltammogram was analyzed by Equations 1-3 as follows:

\[
I = \frac{nFk_c \Gamma}{1 + k_c/k_i + k_b/k_i}
\]  

(1)

\[
k_i = k^\circ \exp[- \alpha (nF/RT)(E-E')] \]

(2)

\[
k_b = k^\circ \exp[(1- \alpha) (nF/RT)(E-E'')] \]

(3)

Where, \(n\), \(F\) and \(\Gamma\) are the number of electrons (\(n = 1\) for type 1 Cu of BOD), the Faraday constant and the total surface concentration of the electrically active BOD, respectively. \(k_c\) is a function of the intermolecular electron transfer rate constant from type 1 Cu to type 2-3 Cu active centers and the intermolecular electron transfer rate constant for dioxygen reduction at the type 2-3 active centers. In this study, the \(k_c\) value was used as a maximum catalytic constant for BOD of 250 s\(^{-1}\) in solution, according to a previous study.\(^8\) \(E''\), \(k^\circ\) and \(\alpha\) are the formal potential (0.46 V) for the BOD type 1 Cu site, the heterogeneous electron transfer rate constant at \(E''\) and the transfer coefficient, respectively. In this study, \(k^\circ\), \(\Gamma\) and \(\alpha\) were adjustable parameters.\(^50,51\)

Table 1 shows the summarized results including the results for Au(100), Au(100) and gold/mica electrodes. Overall, the \(k^\circ\) value did not show any dependence on the crystal structure of the gold electrode surface, although the \(k^\circ\) value at the Au(100) electrode was somewhat larger in comparison with those at the Au(111) and gold/mica electrodes.

3.2. BOD adsorption onto SAM-modified gold electrodes
Fig. 2 shows typical tapping-mode AFM images for BOD adsorbed onto SAM-modified gold/mica surfaces. These results clearly indicated that BOD was present on their surfaces. Aggregated BOD was not confirmed. To estimate the quantity of BOD on the modified surface, QCM measurements were carried out because this method is highly sensitive to mass changes.\(^{52}\) The surface coverage of BOD on the electrode was investigated using gold films deposited onto polished quartz crystals with a 27 MHz fundamental resonance frequency. A frequency change of 1 Hz corresponds to a mass change of 0.6 ng cm\(^{-2}\).\(^{53,54}\) For example, the C\(_2\)-COOH-modified QCM electrode was immersed into a phosphate buffer solution. After stabilization of the frequency, 100 \(\mu\)L containing 40 units ml\(^{-1}\) BOD was added into a buffer solution of 8 mL in a cell at 25 °C. The frequency quickly decreased in a few minutes, and then slowly decreased. The surface was almost saturated \(ca.\) 60 min later. The frequency did not change, even if the BOD solution was added again. The frequency decrease was evaluated to be \(-650 (\pm 150)\) Hz, corresponding to a mass change of 0.39 (\(\pm\) 0.09) \(\mu\)g cm\(^{-2}\). Since the molecular weight of BOD is 52,000,\(^{55,56}\) the obtained value \((\Gamma_{\text{QCM}})\) corresponded to 7.5 (\(\pm\) 1.7) \(\times\) 10\(^{-12}\) mol cm\(^{-2}\), which was in good agreement with the reported value (5~7 \(\times\) 10\(^{-12}\) mol cm\(^{-2}\)).\(^9\) The same procedure was performed for other SAM-modified electrodes, and the adsorption behaviors were similar to the case of C\(_2\)-COOH-modified QCM. The results are summarized in Table 2.

The crystallographic structure of BOD has not been reported. Here, we evaluated the size of BOD by FE-SEM, and determined it to be ca. 6 nm in diameter. This evaluated size was close to other enzymes of members of the multi-copper oxidase family.\(^{57,58}\) Assuming BOD is spherical in shape with a 6 nm diameter, the theoretical number \((\Gamma_{\text{cal}})\) of BOD molecules based on a simple model for monolayer packing on a planar substrate is calculated to be 5.3 \(\times\) 10\(^{-12}\) mol cm\(^{-2}\). The ratio \((\Gamma_{\text{QCM}}/\Gamma_{\text{cal}})\) of \(\Gamma_{\text{QCM}}\) to \(\Gamma_{\text{cal}}\) is also shown in Table 2. For bare, C\(_2\)-COOH, C\(_6\)-NH\(_2\) and C\(_5\)-CH\(_3\) modified QCM electrodes, the \(\Gamma_{\text{QCM}}/\Gamma_{\text{cal}}\) values were around 1 and/or larger than 1, indicating that the BOD was present in a monolayer and/or multilayers. In contrast, for C\(_2\)-SO\(_3\)H and C\(_6\)-OH modified QCM electrodes, the \(\Gamma_{\text{QCM}}/\Gamma_{\text{cal}}\) values were much lower than 1, indicating that BOD was present at less than a full monolayer.
3.3. Electrocatalytic current based on BOD at SAM-functionalized Au(111) electrodes

The electrochemical activity of BOD adsorbed onto SAM-modified Au(111) electrodes was investigated by cyclic voltammetry. The results are shown in Fig. 3. The BOD adsorbed onto C₂-COOH and C₃-SO₃H modified Au(111) electrodes showed well-defined electrocatalytic currents due to dioxygen reduction. In contrast, the BOD adsorbed onto C₆-NH₂, C₆-OH and C₅-CH₃ modified Au(111) electrodes did not show any electrocatalytic current. These results give several important findings. First, the molecular orientation of BOD adsorbed onto SAM-modified electrodes is one of the most important requirements for heterogeneously direct electron transfer reactions with an electrode. Second, negatively charged electrode surfaces give a suitable molecular orientation for the direct electron transfer of BOD with the electrode. Third, a suitable charge (positive or negative) of the electrode surface for the molecular orientation does not depend on the whole molecular charge. The isoelectric point of BOD has been reported to be 4.1, indicating that the overall charge of BOD molecule is negative in neutral solution. Thus, from the viewpoint of the overall charge, it has been postulated that a positively charged surface is suitable for the electrode reaction of BOD. However, in the case of BOD, a negatively charged surface was required for the electrode reaction. This means the charge of the amino acid residues near the active site of the enzyme is important for the correct molecular orientation to achieve direct electron transfer with the electrode.

To evaluate the heterogeneous electron transfer kinetics \( k' \) for BOD adsorbed onto C₂-COOH and C₃-SO₃H modified Au(111) electrodes, steady-state voltammetry was carried out. Table 3 shows the results obtained from an analysis of the background current corrected steady-state linear sweep voltammogram in a buffer solution using Equations 1-3. The \( k' \) values at the C₂-COOH and C₃-SO₃H modified Au(111) electrodes were similar to those obtained at bare Au(111), Au(100) and gold/mica electrodes, although the value at C₃-SO₃H
modified electrode was somewhat scattered. One should note here the electroactive enzyme ratio against the total enzyme number adsorbed onto the electrode. The electroactive enzyme ratio given by $\Gamma/\Gamma_{QCM}$ is shown in Table 3, where the electroactive enzyme ($I$) number was obtained by an analysis of the steady-state linear sweep voltammogram, and the $\Gamma_{QCM}$ was evaluated by the QCM measurements. The $\Gamma/\Gamma_{QCM}$ values were less than 10%. This fact suggests that the BOD adsorbed onto the C$_2$-COOH and C$_3$-SO$_3$H modified Au(111) electrodes showed an electrocatalytic current: however, almost all BOD molecules (ca. 90%) were still present under the electroinactive conditions.

3.4. C$_n$-COOH chain length dependence on electron tunneling of BOD

Fig. 4 shows the steady-state linear sweep voltammogram for BOD adsorbed onto C$_n$-COOH (n = 2, 5, 7 and 10) modified Au(111) electrodes in a phosphate buffer (pH 6.8) in the presence of dioxygen. The electrocatalytic currents of dioxygen reduction decreased with increasing chain length of C$_n$-COOH, and eventually for the C$_{10}$-COOH modified Au(111) electrode, the electrocatalytic current was no longer detectable. The kinetic analysis results for the steady-state linear sweep voltammogram are summarized in Table 4. The $k^*$ and the $\Gamma$ values decreased with increasing chain length of C$_n$-COOH. This behavior can be understood as follows: the electron tunneling rate of BOD on an electrode surface through the insulating barrier becomes slower upon increasing with the tunneling distance between the type 1 copper site of BOD and the modified electrode, since the electron transfer rate is proportional to $\exp(-\beta R)$, where $R$ is the edge-to-edge distance and $\beta$ is proportional to the square root of the barrier height.$^{18,59}$ Furthermore, upon increasing the distance between the type 1 Cu site of BOD and the modified electrode surface, a more ordered molecular orientation is required to shorten the electron transfer. Thus, the $\Gamma$ values also decreased with increasing chain length of C$_n$-COOH.
From the obtained \( k^* \) values, we evaluated the electron tunneling distance between the type 1 Cu site of BOD and the modified electrode surface using the Equation (4) reported by Dutton and coworkers as follows:\(^{60}\)

\[
\log_{10} k_{et} = 13.0 - (1.2 - 0.8\rho)(R - 3.6) - 3.1(\Delta G + \lambda)^2/\lambda
\]  

(4)

where, \( k_{et} \) is the electron tunneling rate, \( \rho \) is the packing density of protein atoms in the volume between the redox centers to account for the \( \beta \) variations in an exergonic electron tunneling rate, \( R \) is the edge-to-edge distance, \( \lambda \) is the energy required to reorganize the nuclear coordinates upon electron transfer, and \( \Delta G \) is the driving force for the electron transfer. \( \beta \) value is included as a function of \( \rho \).\(^{61}\) Here, assuming that \( \rho = 0.78, k_{et} = k^* \) under \( \Delta G = -\lambda \), and \( R \) is the distance between the type 1 Cu site of BOD and the electrode surface, \( R \) can be calculated to be 23 (± 1), 23 (± 1) and 24 (± 1) Å for the C\(_2\)-COOH, C\(_5\)-COOH and C\(_7\)-COOH modified Au(111) electrodes, respectively, where \( R \) is the included thickness \( (L_{SAM}) \) of the SAMs on the electrode, as shown in Fig. 5. Thus, the distance \( (L) \) between the type 1 Cu site of BOD and the outside of the BOD protein structure can be evaluated as follows:

\[
L = R - L_{SAM}
\]  

(5)

The \( L_{SAM} \) term can be calculated from the CPK model of C\(_n\)-COOH adsorbed onto a Au(111) surface, where the alkyl chain structure is present on the surface with an average tilt of 30° from the surface normal. The \( L_{SAM} \) values are 4, 7 and 9 Å for C\(_2\)-COOH, C\(_5\)-COOH and C\(_7\)-COOH, respectively (Table 5). Therefore, the \( L \) values are 19, 16 and 15 Å for C\(_2\)-COOH, C\(_5\)-COOH and C\(_7\)-COOH. The obtained average \( L \) value, 17 (± 2) Å, is within the reported physiological range for electron tunneling.\(^{60}\) This result also indicates that the molecular
orientation of BOD on C₇-COOH modified Au(111) electrodes may be similar to physiological molecular orientation for electron tunneling between the redox coupled enzymes. The $k^+$ values for BOD adsorbed onto bare Au(111), Au(100) and gold/mica electrodes should be much higher than the obtained values (~80 cm s⁻¹). The much lower $k^+$ values for BOD adsorbed onto bare gold electrodes may be due to the fact that the BOD molecular orientation is not similar to the physiological molecular orientation for electron tunneling.

4. Conclusions

The electrocatalytic current of dioxygen reduction based on the direct electron transfer reaction of BOD adsorbed onto Au(111), Au(100) and gold/mica, and Au(111) electrodes modified with C₃-SO₃H and C₇-COOH (n = 2, 5 and 7), was demonstrated. In contrast, the BOD adsorbed onto Au(111) electrodes modified with C₆-NH₂, C₆-OH and C₃-CH₃ did not show any electrocatalytic current. These facts indicate that the negatively charged electrode surfaces give a suitable molecular orientation for the direct electron transfer of BOD with the electrode. The $k^+$ values were evaluated by an analysis of the steady-state voltammogram with a simulated fitting method. From the results using bare Au(111), Au(100) and gold/mica electrodes, the $k^+$ value did not show any dependence on the crystal structure of the gold electrode surface. On the other hand, the $k^+$ values decreased upon increasing the chain length of Cₙ-COOH (n = 2, 5 and 7). From the obtained $k^+$ values at C₇-COOH (n = 2, 5 and 7) modified Au(111) electrodes, the electron tunneling distance was evaluated. The average distance between the type 1 Cu site of BOD and outside of the BOD protein structure was evaluated to be 17 (±2) Å, which is within the reported physiological range for electron tunneling. Taking into account the obtained $k^+$ values and the electron tunneling distance, it was recognized that for BOD adsorbed onto bare gold electrodes, the $k^+$ values were much
lower than expected. This behavior may reflect the difference in BOD molecular orientation between the bare gold electrodes and the C₆-COOH modified Au(111) electrodes.

Acknowledgements

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References


 3559.


30 A. Ulman (Ed.), Characterization of Organic Thin Films, Butterworth-Heinemann, 

31 A. Ulman (Ed.), An Introduction to Ultrathin Organic Films from Langmuir-Blodgett to 

32 H. O. Finklea (Ed.), Electrochemistry of organized monolayers of thiols and related 
 molecules on electrodes, Electroanalytical Chemistry: A Series of Advances, 
 Electroanalytical Chemistry 19, Marcel Dekker, NY, 1996.


 93, 1367.


The $\rho$ can range from values of 1 to 0, corresponding to a fully packed medium ($\beta = 0.9 \AA^{-1}$) and the interstitial space in the protein structure outside the united van der Waals atomic radii ($\beta = 2.8 \AA^{-1}$), respectively. The $\rho$ weighting of $\beta$ and $\beta = (\rho)0.9 \AA^{-1} + (1 - \rho)2.8 \AA^{-1}$ generates the $(1.2 - 0.8\rho)$ coefficient in equation (4). The log of the optimal rate at 3.6 Å van der Waals contact is 13.
Figure captions

Fig. 1. (a) Cyclic voltammograms for BOD immobilized onto Au(111) electrodes in a phosphate buffer (pH 6.8) solution in the presence (solid line) and absence (broken line) of dioxygen at a potential sweep rate of 5 mV s$^{-1}$; (b) background-current corrected steady-state liner voltammograms for BOD onto immobilized Au(111) electrodes in a dioxygen-saturated phosphate buffer (pH 6.8) solution at a potential sweep rate of 5 mV s$^{-1}$. The open circles represent the regression curves based on Eqs. (1)-(3) with the parameters from Table 1.

Fig. 2. Tapping-mode AFM images for BOD immobilized onto a bare gold/mica surface (a), and a gold/mica electrode surface modified with C$_2$-COOH (b), C$_3$-SO$_3$H (c), C$_6$-OH (d), C$_6$-NH$_2$ (e), and C$_5$-CH$_3$ (f).

Fig. 3. Cyclic voltammograms of dioxygen reduction at BOD immobilized Au(111) electrodes modified with C$_2$-COOH (a), C$_3$-SO$_3$H (b), C$_6$-OH (c), C$_6$-NH$_2$ (d) and C$_5$-CH$_3$ (e) in a dioxygen-saturated phosphate buffer (pH 6.8) solution. The potential sweep rate was 5 mV s$^{-1}$.

Fig. 4. Background-current corrected steady-state liner voltammograms (a-c) and cyclic voltammograms (d) for BOD immobilized onto Au(111) electrodes modified with C$_2$-COOH (a), C$_5$-COOH (b), C$_7$-COOH (c) and C$_{10}$-COOH (d) in a dioxygen-saturated phosphate buffer (pH 6.8) solution at a potential sweep rate of 5 mV s$^{-1}$. The open circles represent the regression curves (a-c) based on Eqs. (1)-(3) with the parameters from Table 4.

Fig. 5. Schematic illustration of the proposed BOD molecule orientation on the C$_n$-COOH (n=2,5,7)-modified Au(111) electrode surface.
Table 1. Kinetic analysis of BOD at Au(100), Au(111) and gold/mica electrodes.

Table 2. QCM measurement for BOD adsorption onto bare and SAM-modified gold electrodes.

Table 3. Kinetic analysis of BOD adsorbed onto Au(111) electrodes modified with C$_2$COOH and C$_3$-SO$_3$H.

Table 4. Kinetic analysis of BOD adsorbed onto Au(111) electrodes modified with C$_n$COOH (n = 2, 5, 7, 10).

Table 5. Electron transfer distance evaluated by Eqs. (4) and (5).
Direct electron transfer reaction of bilirubin oxidase adsorbed onto Au(111) and Au(100) single-crystal electrodes modified with self-assembled monolayers was investigated.

Graphical content
**Fig. 1**

- a) [Graph showing electrochemical data with E/V vs. Ag/AgCl axis ranging from 0 to 0.6 and current density from 0.4 μA/cm² to 10 μA/cm².]

- b) [Graph showing electrochemical data with E/V vs. Ag/AgCl axis ranging from 0 to 0.6.]

**Fig. 2**

- a) Bare
- b) C₂-COOH
- c) C₂-SO₃H
- d) C₆-OH
- e) C₆-NH₂
- f) C₆-CH₃

[Images of surface analysis with corresponding graphs showing depth profiles.]
Fig. 3

Fig. 4
Fig. 5

Table 1

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<th>Electrode</th>
<th>( k^\circ /\text{cm s}^{-1} )</th>
<th>( \Gamma \times 10^{-12} / \text{mol cm}^2 )</th>
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<td>Au(100)</td>
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</tr>
<tr>
<td>Au(111)</td>
<td>50 (± 10 )</td>
<td>1.10 (± 0.2 )</td>
<td>0.5</td>
</tr>
<tr>
<td>Au/mica</td>
<td>40 (± 10 )</td>
<td>1.70 (± 0.5 )</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Table 2

<table>
<thead>
<tr>
<th>Electrode</th>
<th>$\Delta f$/ Hz</th>
<th>$\Gamma_{QCM} \times 10^{-12}$/ mol cm$^2$</th>
<th>$\Gamma_{QCM}/\Gamma_{cal}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare</td>
<td>450 (± 50)</td>
<td>5.2 (± 0.6)</td>
<td>1.0 (± 0.10)</td>
</tr>
<tr>
<td>C$_2$-COOH</td>
<td>650 (± 150)</td>
<td>7.5 (± 1.7)</td>
<td>1.5 (± 0.40)</td>
</tr>
<tr>
<td>C$_3$-SO$_3$H</td>
<td>150 (± 50)</td>
<td>1.7 (± 0.6)</td>
<td>0.3 (± 0.1)</td>
</tr>
<tr>
<td>C$_6$-OH</td>
<td>130 (± 70)</td>
<td>1.5 (± 0.8)</td>
<td>0.25 (± 0.15)</td>
</tr>
<tr>
<td>C$_6$-NH$_2$</td>
<td>630 (± 70)</td>
<td>7.9 (± 1.4)</td>
<td>1.5 (± 0.3)</td>
</tr>
<tr>
<td>C$_5$-CH$_3$</td>
<td>580 (± 120)</td>
<td>6.7 (± 1.4)</td>
<td>1.25 (± 0.25)</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Electrode</th>
<th>$k^*$ / cm s$^{-1}$</th>
<th>$\Gamma \times 10^{-12}$/ mol cm$^2$</th>
<th>$\alpha$</th>
<th>$\Gamma_{QCM} \times 10^{-12}$/ mol cm$^2$</th>
<th>$\Gamma/\Gamma_{QCM}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_2$-COOH</td>
<td>60 (± 10)</td>
<td>0.48 (± 0.10)</td>
<td>0.5</td>
<td>7.6 (± 1.7)</td>
<td>0.04-0.1</td>
</tr>
<tr>
<td>C$_3$-SO$_3$H</td>
<td>70-200</td>
<td>0.24 (± 0.10)</td>
<td>0.3</td>
<td>1.7 (± 0.6)</td>
<td>0.06-0.3</td>
</tr>
</tbody>
</table>
Table 4

<table>
<thead>
<tr>
<th>Electrode</th>
<th>$k^0$/cm s$^{-1}$</th>
<th>$\Gamma \times 10^{-12}$/mol cm$^{-2}$</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_2$-COOH</td>
<td>60 ($\pm$ 10)</td>
<td>0.48 ($\pm$ 0.1)</td>
<td>0.5</td>
</tr>
<tr>
<td>C$_5$-COOH</td>
<td>50 ($\pm$ 10)</td>
<td>0.20 ($\pm$ 0.1)</td>
<td>0.3</td>
</tr>
<tr>
<td>C$_7$-COOH</td>
<td>30 ($\pm$ 10)</td>
<td>0.17 ($\pm$ 0.1)</td>
<td>0.2-0.3</td>
</tr>
<tr>
<td>C$_{10}$-COOH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5

<table>
<thead>
<tr>
<th>Electrode</th>
<th>$k^0$/cm s$^{-1}$</th>
<th>$R$/Å</th>
<th>$L_{SAM}$/Å</th>
<th>$L$/Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_2$-COOH</td>
<td>60 ($\pm$ 10)</td>
<td>23</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>C$_5$-COOH</td>
<td>50 ($\pm$ 10)</td>
<td>23</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>C$_7$-COOH</td>
<td>30 ($\pm$ 10)</td>
<td>24</td>
<td>9</td>
<td>15</td>
</tr>
</tbody>
</table>