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Electrochemical Control of Bioluminescence by Blocking the Adsorption of the Bacterial Luciferase Using a Mercaptobipyridine Self-assembled Monolayer

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An *N*-butyl-*N*′-(4-mercaptobutyl)-4,4′-bipyridinium (4BMBP) was modified on a gold electrode to improve the electrochemical control of the bacterial luciferase (BL) luminescence system. The 4BMBP-modified gold electrode (4BMBP/Au) was able to prevent the adsorption of BL on the electrode surface, and enhanced the electrochemical regeneration rate of the reduced flavin mononucleotide ($FMNH_2$), which is one of the substrates of the BL luminescence reaction. By using the 4BMBP/Au, the luminescence intensity increased by about 27% compared to that of a bare gold electrode (bare Au). Moreover, the modified electrode improved the time required for analysis because the modified layer prevented BL adsorption. Even without a refreshing procedure for each measurement, a constant luminescence intensity could be observed, and the analysis time was reduced to half (about 10 min) for one sample. The 4BMBP/Au is not only useful to control of the BL luminescence system, but also for electrochemical measurements in the presence of proteins.

Keywords Electrochemically-controlled luminescence, flavin mononucleotide, cyclic voltammetry, protein adsorption

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Introduction

Luminescence enzymes extracted from the firefly and bacteria have been used as a model of a membrane protein to elucidate the inhibitory mechanism of general anesthesia, which is in the category of hydrophobic molecules.[1-6] The effect of hydrophobic molecules, such as terminally-substituted normal alkyl compounds, on the bacterial luciferase (BL) bioluminescence has been reported in our previous studies.[5,6] The BL reaction requires a reduced flavin mononucleotide ($FMNH_2$) as one of the substrates.[7] However, the $FMNH_2$ is rapidly oxidized to oxidized flavin (FMN) by dissolved oxygen in an aqueous solution. To overcome this problem, we have established the electrochemical regeneration system of FMN to generate the BL luminescence shown by the following equations:[8,9]

$$FMN + 2H^+ + 2e^- \longrightarrow FMNH_2 \text{ (electrode reaction)} \quad (1)$$

$$FMNH_2 + O_2 + C_{11}H_{23}CHO \longrightarrow FMN + H_2O + C_{11}H_{23}COOH + h\nu \quad (2)$$

When a metal electrode, such as bare Au, was used as the working electrode, the BL reaction was inhibited by the adsorption of BL molecules on the electrode surface. The adsorbed BL blocked the FMN reduction based on Eq. (1). It produces a poor accuracy and time consuming analysis of the inhibitory potency of the hydrophobic molecules on the BL luminescence reaction. Therefore, cleaning the electrode surface has been required for each measurement. To improve the BL system, we focused on a modification of the electrode surface. The self-assembled monolayer (SAM) modified electrode is a well-examined technique for various electrochemical systems. By introducing the functional thiol compound on the electrode surface, various improvements can be achieved.[10]

In the present study, we used the *N*-butyl-*N*′-(4-mercaptobutyl)-4,4′-bipyridinium ion (4BMBP), shown in Fig. 1, as a component of SAM, and applied it for electrochemical control of the BL luminescence system. It can be expected that the bipyridinium

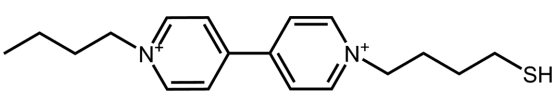

Fig. 1 Structure of *N*-butyl-*N*′-(4-mercaptobutyl)-4,4′-bipyridinium ion (4BMBP). The counter anions were dihexafluorophosphate in the present study.

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unit functions as a mediator for the reduction process of the FMN. The standard redox potential of the bipyridinium compound is about –0.31 V[11] (*vs.* Ag/AgCl), and that of FMN is about –0.43 V.[12] Although the redox potential of 4BMBP is higher than that of FMN, electron transfer from 4BMBP to FMN is expected to be possible at a potential lower than the FMN reduction:

$$\text{bpy(Ox)} + 2e^- \rightleftharpoons \text{bpy(red)} \quad (3)$$

$$\text{bpy(red)} + \text{FMN} + 2H^+ \rightleftharpoons \text{bpy(Ox)} + \text{FMNH}_2 \quad (4)$$

where bpy(ox) and bpy(red) indicate the redox state of 4BMBP modified on the electrode surface. The SAM modified on the electrode is also expected to prevent the adsorption of BL on the electrode surface. By using the 4BMBP SAM modified gold electrode (4BMBP/Au), we examined (1) the redox behavior of the bipyridinium unit in 4BMBP/Au, (2) the effect of SAM on the redox behavior of FMN, (3) the blocking of the BL adsorption on the electrode surface by SAM, and (4) the usefulness of 4BMBP/Au for the BL luminescence system.

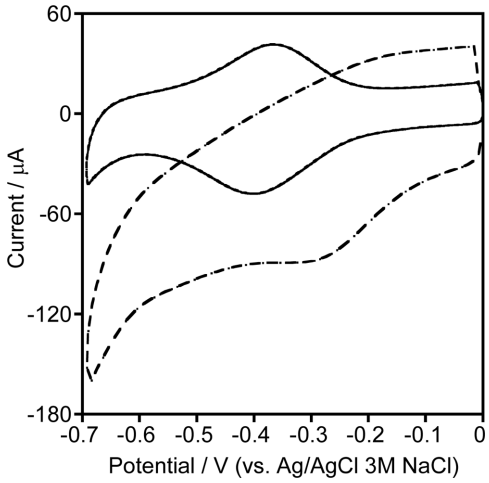

Fig. 2 Cyclic voltammogram of 4BMBP/Au-mesh in 50 mmol dm^{-3} phosphate buffer and 100 mmol dm^{-3} KCl solution. The solid line and dashed line indicate the result of the 4BMBP/Au and the bare Au, respectively. The pH value was adjusted to 7.0. The scan rate was 100 mV s^{-1}. The Au mesh electrode was used in this experiment.

Experimental

SAM preparation

For the voltammetric measurement, the SAM was modified on a gold disk (ϕ = 1.6 mm) electrode (SAM/Au) by immersing it in 1 mmol dm^{-3} thiol in 10 cm^3 of acetonitrile for 3 days at room temperature (25 ± 1°C). For the bioluminescence measurement, the SAM was modified on a gold mesh (100 mesh) electrode (SAM/Au). The effective electrode area was determined to be 4.72 cm^3 by using $K_4Fe(CN)_6$. After washing the gold mesh electrode in a piranha solution (30% H_2O_2:H_2SO_4 = 1:3 (v/v), 60°C) for 1 h, the electrode was immersed in the 4BMBP solution for 3 days at room temperature. To examine the effect of 4BMBP on the BL luminescence intensity, hexane thiol (HXT) was mixed in the 4BMBP solution for a control experiment. Three kinds of thiol solutions were prepared, as follows: 4BMBP:HXT = 1:0 (4BMBP/Au), 1:1 (4BMBP-HXT/Au) and 0:1 in molar ratio (HXT/Au).

Cyclic voltammetry of SAM modified electrode

The electrode reaction of 4BMBP/Au was evaluated by cyclic voltammetry. The SAM/Au was used as the working electrode. The scan rate was 10 mV s^{-1}. Pt wire and a Ag/AgCl (in 3 mol dm^{-3} NaCl solution) electrode were used as the counter and reference electrodes, respectively. A 50 mmol dm^{-3} phosphate buffer solution adjusted at pH 7.0 containing 100 mmol dm^{-3} KCl as the supporting electrolyte was used in this measurement. For all of the experiments in this study, the pH value was kept constant at 7.0, because the optimal pH of BL is between 8.5 and 6.5, and many physicochemical examinations using BL have been done at pH 7.0.[13]

Effect of SAM on FMN redox behavior and BL adsorption

Cyclic voltammetry was performed using a solution of 1.0 mmol dm^{-3} FMN containing 0.1 mg cm^{-3} BL, 100 mmol dm^{-3} KCl and 50 mmol dm^{-3} phosphate buffer (pH 7.0). Either the SAM/Au or the bare Au electrode was used as the working electrode. Pt wire and Ag/AgCl were used as the counter and reference electrodes, respectively. The measurement was started 5 min after immersing the working electrode into the solution in order to examine the effect of BL adsorption on the electrode surface. The scan rate was 10 mV s^{-1}.

Application to BL luminescence system

The BL luminescence intensity was measured by a previously reported system.[6] BL extracted from *Vibrio fischeri* [EC: 1.14.14.3] (Sigma-Aldrich Co. LLC., USA) was used in this study. The 4BMBP/Au was used as a working electrode. The luminescence measurement was performed using a homemade flow electrochemical luminescence cell. FMN was reduced by linear sweep voltammetry and the corresponding BL luminescence was observed by a photomultiplier. The potential was scanned from –0.30 to –0.65 V (*vs.* Ag/AgCl) at a scan rate of 10 mV s^{-1}.

Effect of a SAM modified electrode on repeated BL luminescence measurements

The 4BMBP/Au was applied to the BL luminescence system. In order to examine the effectiveness of the electrode in the repetition experiments, the variation in the maximum luminescence intensity was measured during repeated measurements. The 4BMBP/Au and bare Au were used as the working electrode, and the BL luminescence was recorded without refreshing the electrode. The measurement was repeated 4 times for each electrode. The BL luminescence reaction was controlled by constant potential electrolysis at –0.70 V (*vs.* Ag/AgCl).

Results and Discussion

Electrochemical behavior of SAM modified electrode

Figure 2 shows a cyclic voltammogram of the 4BMBP/Au in a 50 mmol dm^{-3} phosphate buffer solution. The dashed line indicates the result of the bare mesh electrode, and the solid line indicates that of the 4BMBP/Au. The cathodic peak around –0.3 V for the bare electrode resulted from the reduction of dissolved oxygen. On the other hand, the redox peaks corresponding to the redox reaction of the bipyridinium unit on the electrode surface were observed at around –0.34 V. This result clearly indicated that the redox peaks were caused by the reaction of the electroactive species adsorbed on the electrode surface. The redox behaviors of similar bipyridinium SAM

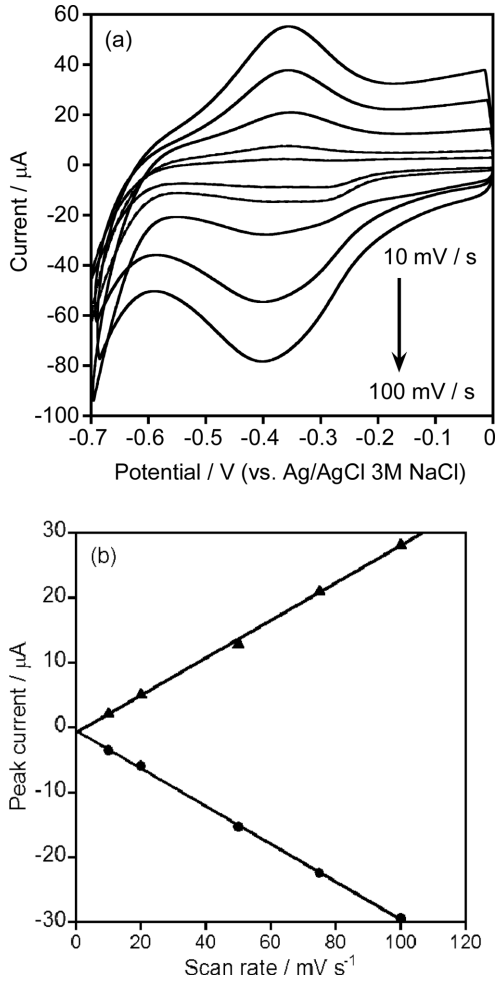

Fig. 3 (a) Cyclic voltammograms of the 4BMBP/Au mesh electrode in 50 mmol dm^{-3} phosphate buffer and 100 mmol dm^{-3} KCl solution at pH 7.0. The scan rates were 10, 20, 50, 75 and 100 mV s^{-1}. (b) Scan rate dependence on the redox currents. The closed circles indicate the cathodic peak currents and the closed triangles indicate the anodic peak currents. The redox currents were obtained from Fig. 3(a). The solid lines indicate a first-order regression line.

modified electrodes have been reported.[11,14] Although the bipyridinium thiol having a different alkyl chain length of the terminal group was used in the previous study, the redox peaks obtained in the present study were similar to those of the previous one[11] (–0.31 V, corrected for Ag/AgCl electrode). In the literature,[11] the redox peaks correspond to the one-electron reduction of a bipyridinium unit shown in Eq. (5), where bpy^{2+} and $bpy^{\bullet+}$ denote the divalent bipyridinium cation and the monovalent bipyridinium radical, respectively.

$$bpy^{2+} + e^- \rightleftharpoons bpy^{\bullet+} \quad (5)$$

The values of the anodic and cathodic currents were almost the same as shown in Fig. 2. Therefore, the $bpy^{\bullet+}$ radical formed by the cathodic reaction was not spontaneously reoxidized to the bpy^{2+}. Figure 3(a) shows cyclic voltammograms of 4BMBP/Au in 50 mmol dm^{-3} phosphate buffer at scan rates of 10, 20, 50, 75 and 100 mV s^{-1}, respectively. Both the cathodic peak currents and the anodic peak currents increased with the scan rate. The peak currents were plotted *versus* the scan rate, as shown in Fig. 3(b). Linear lines having an intercept of zero are shown in Fig. 3(b); the potential difference between the anodic peak and cathodic peak was less than 10 mV, indicating the redox reaction of the adsorbed SAM rather than the diffusion of the redox active species.

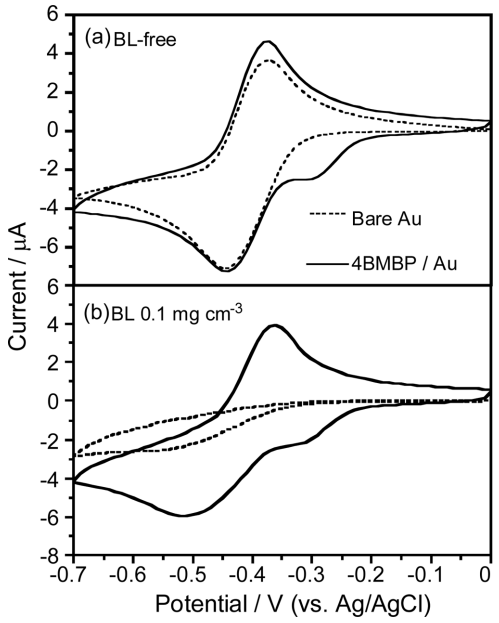

Fig. 4 Cyclic voltammograms of 4BMBP/Au (solid line) and bare Au (dotted line) in 1.0 mmol dm^{-3} FMN solution containing no BL (a), and 0.1 mg cm^{-3} BL (b). A 50 mmol dm^{-3} phosphate buffer and 100 mmol dm^{-3} KCl were added to the sample solution as a pH buffer and supporting electrolyte, respectively. The scan rate was 10 mV s^{-1}.

Effect of a SAM modified electrode on the FMN redox reaction and BL luminescence reaction

After the 4BMBP was successfully immobilized on the electrode surface, the redox behavior of FMN was then measured using 4BMBP/Au. Figure 4(a) shows cyclic voltammograms of the 1.0 mmol dm^{-3} FMN solution obtained using bare Au and 4BMBP/Au. In the case of the bare Au electrode (dotted line), only one cathodic peak corresponding to the reduction of FMN was observed. On the contrary, two cathodic peaks (–0.30 and –0.45 V) and one anodic peak (–0.39 V) were observed for the 4BMBP/Au. The cathodic peak at around –0.30 V can be ascribed to a reduction of the bipyridinium unit, as shown in Fig. 2. These results indicated that a small molecule, such as FMN, is able to penetrate into the 4BMBP SAM because of the loosely packed SAM layer. The redox behavior of the small compound has also been confirmed using the $Fe(CN)_6^{4-}$ ion, and clear redox currents could be observed in addition to those of the thiol SAM (data are shown in Fig. S1, Supporting Information).

Figure 4(b) shows cyclic voltammograms of the 1 mmol dm^{-3} FMN solution containing 0.1 mg cm^{-3} BL using a bare Au (dashed line) and 4BMBP/Au (solid line). In the presence of BL, the cathodic peak at –0.52 V was small for bare Au as compared to the 4BMBP/Au. These voltammograms were recorded after a 5-min immersion of the electrodes in the BL solution. Therefore, the BL was adsorbed onto the bare Au surface, which prevented the redox reaction of FMN. At the 4BMBP/Au, in contrast, a well-defined current response was observed. Although the modified SAM is considered to reduce the BL adsorption, the FMN redox reaction was not reversible

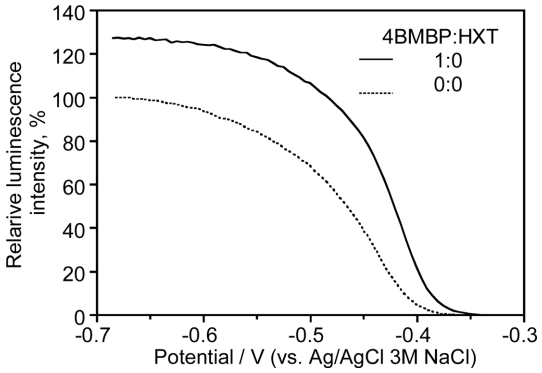

Fig. 5 Effect of the electrolysis potential on the relative luminescence intensity. Mixing ratios of 4BMBP and HXT were 1:0 (4BMBP/Au, solid line), 0:0 (bare Au, dotted line). The results of 1:1 (4BMBP-HXT/Au) and 0:1 (HXT/Au) are not shown (no luminescence).

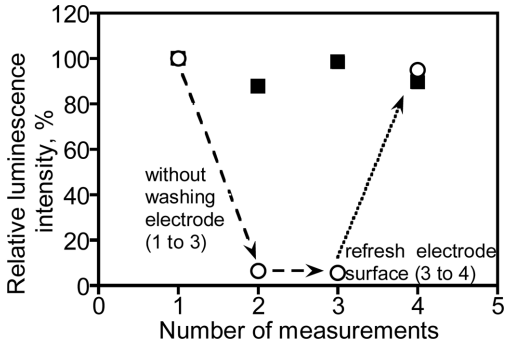

Fig. 6 Effect of electrode cleaning on the reproducibility of the luminesence intensity for 4BMBP/Au (closed square) and bare Au (open circle). For the 4BMBP/Au, the reproducible luminescence intensities were observed without washing the electrode surface. The variation in the luminescence intensity was about 5% for 4 measurements, which is in the acceptable range. In the case of the bare Au, the electrode surface was washed with ethanol before the first and fourth measurements. If the bare Au was not washed during each measurement, the luminescence intensity significantly decreased as shown in the second and third measurements. The washing of the bare Au surface regenerates the BL reaction, as shown in the fourth measurement.

(anodic peak: –0.38 V and cathodic peak: –0.52 V). This result indicated that although the SAM prevented the adsorption of BL, it also affected the FMN transport near the electrode surface. Although the reaction rate of FMN at 4BMBP/Au decreased by the presence of BL, it still proceeded. This will be a great advantage for the application of the 4BMBP/Au to the BL luminescence system to maintain the regeneration of $FMNH_2$ and, in addition, prevent contamination of the electrode surface.

Application of 4BMBP modified electrode for the BL luminescence reaction

The 4BMBP/Au was applied to the BL luminescence system. Figure 5 shows the effect of the electrode potential on the BL luminescence intensity. A potential sweep was performed by linear sweep voltammetry at a 10 mV s^{-1} scan rate. The luminescence intensity was normalized by the luminescence intensity at –0.65 V using the bare Au. In the case of the bare Au, although the reduction peak of FMN was not observed, as shown in Fig. 4(b), the BL luminescence was observed at a negative potential lower than –0.38 V (Fig. 5, dashed line). The luminescence intensity at the bare Au reached a constant value at around –0.65 V, in good agreement with the reduction potential of FMN. In the case of 4BMBP/Au, a similar potential dependence of the luminescence intensity was also observed. However, the maximum luminescence intensity was 27% higher at the 4BMBP/Au than at the bare Au. This result indicated the effective reduction of FMN at the modified electrode, as shown in Fig. 4(b). Although this enhancement of the luminescence intensity seems to disagree with the FMN reduction rate observed in the cyclic voltammograms shown in Fig. 4(b), the 4BMBP/Au is useful to effectively promote the BL luminescence reaction. To clarify the effect of 4BMBP/Au, the 4BMBP SAM was replaced with the hexane thiol (HXT) SAM and 4BMBP-HXT mixed SAM; however, the BL luminescence was not observed at any of the HTX/Au and 4BMBP-HXT/Au electrodes. This is probably caused by the formation of a densely packed HXT SAM in the gap of 4BMBP SAM, even in the case of 4BMBP:HXT = 1:1. In the other case, the HXT may preferentially adsorb on the electrode compared to 4BMBP under the conditions of the present thiol concentration.

Finally, to examine the practicality of the 4BMBP/Au, repeated measurements were performed. Figure 6 shows the effect of rinsing the electrode surface for each measurement. The open circle and the closed square indicate the results of a bare Au and the 4BMBP/Au, respectively. The luminescence intensity was normalized by the luminescence intensity of the first measurement. In the case of the bare Au, the luminescence intensity significantly decreased with repeated measurements without washing the electrode surface (from first to third measurements). After the third measurement, the bare Au was rinsed for 5 min with sonication in ethanol. This procedure resulted in regenerating the luminescence intensity during the fourth measurement. In the case of the 4BMBP/Au, almost the same luminescence intensity was observed during repeated measurements without washing the electrode surface. The relative standard deviation of 4 repeated measurements was 5.4%, which is not significantly different from those of the bare Au with electrode cleaning for each measurement. Therefore, the 4BMBP/Au is applicable to the BL luminescence system and is strongly expected to reduce the required time for analysis by half (about 10 min) compared to the previous method.[5,6]

Conclusions

The 4BMBP-modified Au electrode (4BMBP/Au) was prepared to prevent the adsorption of BL on the electrode surface and to promote FMN reduction. The 4BMBP/Au showed well-defined redox reactions of the bipyridinium unit and FMN, even in the presence of BL. The 4BMBP/Au increases the reduction rate of FMN to form $FMNH_2$. The BL luminescence intensity increased about 27% at the 4BMBP/Au as compared to that of the bare Au. These results can be acsribed to the effective reduction of FMN. In addition, the 4BMBP/Au showed an effective FMN reduction by protecting the BL adsorption on the electrode surface. It can be concluded that 4BMBP/Au is a useful electrode to control the BL luminescence reaction. Moreover, the redox-active bipyridinium unit of 4BMBP/Au will be applicable for other electrochemical analyses of samples containing proteins.

Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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