Electron-Link between Bacterial Luciferase and Electrochemically Sensitized $Ru(bpy)_3^{2+*}$-Methylviologen System within Nafion Membrane

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Bacterial luciferase was linked with the electrochemically induced electron transport system, involving the $d\pi^*$ triplet state tris(2,2′-bipyridine)ruthenium(II) and N,N'-dimethyl-4,4′-bipyridinium (MV^{2+}), within a Nafion membrane.

Bacterial luciferase that catalyzes the light emitting reaction (1), involving reduced riboflavin 5′-phosphate ($FMNH_2$), molecular oxygen, and long-chain aldehyde (RCHO), has been postulated to be electronically linked with the respiratory electron flow within the intermembrane space,[1]

$$FMNH_2 + O_2 + RCHO \rightarrow FMN + H_2O + RCOOH + h\nu\ (\lambda_{max} \approx 490\,nm). \quad (1)$$

In the link of interest, the luciferase is presumably fed with electrons from the respiratory chain, leading to bioluminescence. To study the electron-link, it would be useful and functionally interesting to construct the artificial electron-link systems in the specific microenvironments. On the basis of this concept, we have constructed a basic model within a Nafion (perfluorinated ion exchanger) membrane, involving the $d\pi^*$ triplet state $Ru(bpy)_3^{2+}$($Ru(bpy)_3^{2+*}$)-MV^{2+} electron transport system.[2] To produce $Ru(bpy)_3^{2+*}$, electrochemical excitation, as well as photoexcitation, has been worthy remark.[3] $Ru(bpy)_3^{2+*}$ can also be electrochemically formed in acidified Nafion matrices with either $C_2O_4^{2-}$ or $S_2O_8^{2-}$, freely accessible to the matrices.[4] In this study, we have modified the electrochemical sensitization method to be favorable for characterization of the electron-link.

The Nafion membrane was prepared on either an indium tin-oxide (ITO) sputtered glass plate or a Au sputtered quartz crystal (9-MHz, AT-cut) by syringing a Nafion (Aldrich; equiv wt, 1100) ethanol solution. A Nafion coated electrode was then exposed to aqueous 2.5 mmol dm^{-3} (=mM) $Ru(bpy)_3^{2+}$, prepared from $Ru(bpy)_3Cl_2{\cdot}6H_2O$, for 15 min. The resulting membrane thickness was determined to be about 0.4 μm by the quartz crystal microbalance (QCM).[5] The amount of $Ru(bpy)_3^{2+}$ incorporated was spectroscopically evaluated to be about 0.6 M ($\varepsilon_{452} = 14.6\,cm^{-1}mM^{-1}$). Before each measurement, a $Ru(bpy)_3^{2+}$-Nafion membrane was equilibrated with a sample solution for 30 min. Absorption and emission spectra were recorded while applying a potential to the Nafion coated ITO electrode, on which a 100-mesh Au gauze and a quartz glass plate were overlapped in this order. Insertion of the Au gauze was effective in penetration of a sample solution between the two plates. The assembled thin layer electrode was fixed perpendicularly in a sample cup (400-μdm^3). An auxiliary electrode was a Pt wire and potentials were monitored with respect to a Ag wire, unless otherwise noted. The luciferase was purified from the cells of *Photobacterium phosphoreum*.[6] All measurements were carried out at 23 °C.

Absorption spectroscopy showed that the amounts of $Ru(bpy)_3^{2+}$ incorporated do not vary before and after equilibration with different pH solutions (3.0, 5.7, and 8.2). On applying +1.3 V, sufficiently positive to oxidize $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$, absorbance at 452 nm (A_{452}) characteristic of $Ru(bpy)_3^{2+}$ decreased with time and by stepping back to the initial potential (0 V) the A_{452} value returned to the original level, irrespective of pHs (Figure 1). These results indicate that the $Ru(bpy)_3^{3+/2+}$ couple is held during the electrode process and that the hydrophobic interaction with the Nafion matrix, as well as the electrostatic force due to its SO^{3-} head groups, plays an important role in holding the $Ru(bpy)_3^{3+/2+}$ couple.[7]

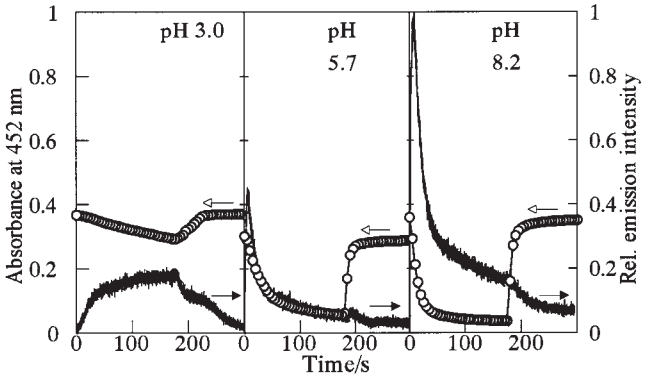

Figure 1. Changes in A_{452} of $Ru(bpy)_3^{2+}$-Nafion coated on ITO electrode during double potential step application and corresponding emission intensity (I). Potential was stepped from 0 to +1.3 V (vs Ag) then held for 180 s before being stepped back to 0 V. I, separately recorded by a luminometer, was normalized to the maximal intensity at pH 8.2 (= 1). Membranes were pre-equilibrated with 0.1 M acetic acid (pH 3.0) and 0.1 M Na/K phosphate buffer solutions (pHs, 5.7 and 8.2).

It is noting that applying +1.3 V makes the $Ru(bpy)_3^{2+}$-Nafion membrane emissive (Figure 1). Emission at any time t exhibited a single band spectrum peaking around 600 nm, identical as the phosphorescence arising from the lowest $d\pi^*$ triplet state $Ru(bpy)_3^{2+}$.[8] Since the water molecules adsorbed on the electrode are converted to the adsorbed OH groups around +1 V,[9] there is a possibility that the surface-active OH will reduce the remaining $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{+}$, followed by the $Ru(bpy)_3^{2+*}$ formation via the annihilation reaction between $Ru(bpy)_3^{+}$ and $Ru(bpy)_3^{3+}$.[3]

Subsequently, the QCM measurements, combined with simultaneous emission monitoring and cyclic voltammetry (CV), were performed. For the $Ru(bpy)_3^{2+}$-Nafion membrane equilibrated with 0.1 M Na/K phosphate buffer (pH 7.0), the cyclic scan gave a quasi-reversible CV curve centering around +1 V for the $Ru(bpy)_3^{3+/2+}$ couple and a corresponding emission peak (Figure 2, A and B). The cathodic wave around +0.5 V is due to the reduction of the surface-oxide formed during the forward scan.[9] The Δf ($= f_E - f_{E\text{initial }(=0\,V)}$) curve, obtained in the same cyclic scan (Figure 2 C), began to rise at about +1 V during the forward scan and reached maximum at about +1.1 V during the negative scan

back. To the contrary, in the presence of MV^{2+}, emission was no longer detected (Figure 2 B), due to the oxidative quenching by MV^{2+} to form its monocation radical ($MV^{+}\cdot$).[7] Moreover, neither rise in Δf nor cathodic peak current for the reduction of $Ru(bpy)_3^{3+}$ was definite (Figure 2, A and C).

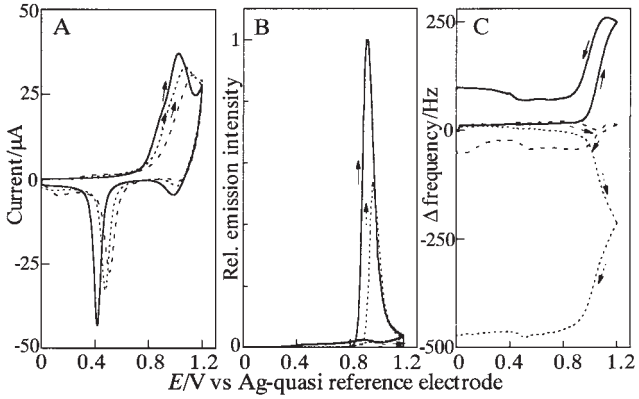

Figure 2. Cyclic voltammograms (A) for $Ru(bpy)_3^{2+}$-Nafion coated on Au-sputtered quartz crystal and corresponding responses for emission (B) and Δfrequency ($=f_E - f_{E\text{initial }(=0\,\text{V})}$) (C). Scan rate $= 20\,\text{mV s}^{-1}$. Samples: solid curve, 0.1 MNa/K phosphate buffer solution (ph 7.0); dotted curve, the same buffer containing 1.0 mM MV^{2+}; and dashed curve, the same buffer containing 0.50 mM FMN, 0.24 mM tetradecanal, 47 μM bacterial luciferase, 1.0 mM MV^{2+}, and 10 mM EDTA.

When the luciferase reaction mixture, containing MV^{2+} and ethylenediaminetetraacetic acid (EDTA) as a suicide electron donor, was present in the $Ru(bpy)_3^{2+}$-Nafion membrane, emission was produced during the forward scan (Figure 2 B). As reported previously,[10] $MV^{+}\cdot$ presumably possesses the ability to reduce FMN. The resulting $FMNH_2$ is then utilized by luciferase to produce emission. No emission was present in the luciferase reaction mixture containing MV^{2+} but with no EDTA. This is certainly because the back electron-transfer from $MV^{+}\cdot$ to remaining $Ru(bpy)_3^{3+}$ disrupts the electron transport from $Ru(bpy)_3^{2+*}$ to luciferase.

The Δf responses (Figure 2 C) can be discussed in terms of a contraction of a gel-like Nafion membrane. Assuming that at rather positive potentials the generation of $Ru(bpy)_3^{3+}$ is facilitated as compared with that of $Ru(bpy)_3^{+}$, both hydrated Na^+ and K^+ ions in the Nafion matrix, equilibrated with the Na/K phosphate buffer, may be driven out to maintain the charge balance, resulting in the membrane contraction. The slower mechanical contraction seems to be responsible for the delayed appearance of the Δf peak. The back electron-transfer reactions mentioned above will tend to eliminate the excess positive electric charge in the Nafion matrix, equilibrated with the same buffer solution containing MV^{2+}, being causative of holding Na^+ and K^+ ions, as reflected by little change in Δf.

It should be noted that the Δf curve exhibited relatively large negative number in the potential region where the luciferase reaction was triggered. At such potentials, the weakly anionic bacterial luciferase[1] is postulated to be attracted to the Nafion membrane and to penetrate into it. The electrostatic penetration of large bacterial luciferase molecules ($M_r \approx 78$ kDa) appears to have the Nafion membrane expanded. The maximal contraction is evaluated to be about 8 nm,[5] comparable to the size of a few luciferase molecules.[11] Since the Δf value remains almost constant during the negative scan back, the luciferase appears to be firmly retained in the Nafion matrix.

Emission spectra, recorded while applying +1.3 V to the $Ru(bpy)_3^{2+*}$-Nafion membrane, pre-equilibrated with the luciferase reaction mixture, exhibited two bands with maxima at about 520 and 590 nm (Figure 3). The former band is attributed to the luciferase associated light emission, being red-shifted. This shift is possibly due to the energy transfer from the excited luciferase intermediate to FMN present in excess. The decrease in the emission at 520 nm occurs with time mainly because the O_2 uptake into the Nafion membrane between the two glass plates is strictly restricted. Since the bacterial luciferase turnover is considerably slow,[1] $FMNH_2$ will be accumulated with time and oppositely the amount of FMN will decrease. This may delay the regeneration of MV^{2+}, due to the electron transfer from $MV^{+}\cdot$ to FMN, possibly leading to the rise in the $Ru(bpy)_3^{2+*}$ phosphorescence, appearing around 590 nm in this system. Moreover, the consumption of O_2 by luciferase seems to enhance the phosphorescence susceptible to the quenching by O_2.

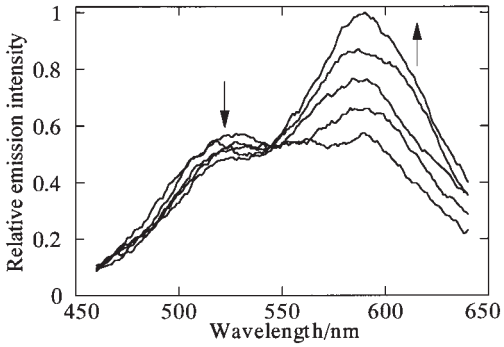

Figure 3. Emission spectra for $Ru(bpy)_3^{2+}$-Nafion membrane pre-equilibrated with 0.1 M Na/K phosphate buffer solution (pH 7.0) containing 0.25 mM FMN, 0.24 mM tetradecanal, 40 μM luciferase, 1.0 mM MV^{2+}, and 5.1 mM EDTA. $E_{\text{applied},}$ +1.3 V (vs Ag) for 3 min.

In conclusion, the bacterial luciferase reaction was triggered via the electron transport, starting from the electrochemically sensitized $Ru(bpy)_3^{2+*}$ within the Nafion membrane. The sensitization with no excitation light was also favorable to eliminate the photoreduction of FMN to $FMNH_2$.

References and Notes

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