Supporting information for

Electrochemiluminescence of $[Ru(bpy)_3]^{2+}$/tri-*n*-propylamine to visualize different lipid compositions in supported lipid membranes

Kaoru Hiramoto*[a, b], Ayumi Hirano-Iwata[b,c], Kosuke Ino[d], and Hitoshi Shiku[d]

[a]*Frontier Research Institute for Interdisciplinary Sciences, Tohoku University, Japan*
[b]*Research Institute of Electrical Communication, Tohoku University, Japan*
[c]*Advanced Institute for Materials Research, Tohoku University, Japan*
[d]*Graduate School of Engineering, Tohoku University, Japan*

Corresponding Author: Kaoru Hiramoto*[a, b]
E-mail: kaoru.hiramoto.b4@tohoku.ac.jp

Materials and Methods

Regents

Phospholipids used in this study included 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), and 18:1 NBD-PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)), purchased from Avanti Polar Lipids, Inc. (United States). Cholesterol, sourced from Wako Pure Chemical Industries (Osaka, Japan), was purified using methanol before use. $[Ru(bpy)_3]Cl_2$, tri-*n*-propylamine, isopropanol, and chloroform were purchased from Wako Pure Chemical Industries Ltd. For the buffer, 10× Dulbecco's phosphate-buffered saline (D-PBS; Nacalai Tesque, Japan) was diluted to 1× D-PBS with Milli-Q water and filtered through cellulose acetate membrane (ADVANTEC© pore size 0.20 μm, Toyo Roshi Kaisha, Ltd., Japan) before use.

Preparation of liposome solutions

The specified amount of lipids was transferred to a glass vial (Wako Pure Chemical) to obtain a total lipid concentration of 4 mg/mL in chloroform. Chloroform was evaporated by gently circulating nitrogen gas over the solution while rotating the vial, thus depositing a lipid film on its inner wall. The lipid film was then placed overnight in a vacuum desiccator to eliminate any residual chloroform. The following day, the film was rehydrated with 2 mL of D-PBS for an hour, resulting in a 2 mg/mL lipid solution. A stock solution of the lipid vesicles was obtained by vortexing. This could be stored at -80°C for future use.

Unilamellar vesicles were prepared through freeze-thaw cycles and subsequent extrusion. The lipid vesicle stock solution was brought to room temperature and vortexed for 1 min. The vial was then immersed in nitrogen liquid for 3 min, followed by immersion in warm water (40~60˚C) for 3 min. This cycle was repeated five times. The solution was then extruded using a mini-extruder (Avanti Polar Lipids) with a 100 nm pore size filter (Avanti Polar Lipids), adhering to the manufacturer's instructions. This extrusion process was repeated 11 times to yield the liposome solution. The prepared liposome solution was utilized within two weeks post-extrusion.

Supported lipid membrane formation on an ITO electrode

Indium tin oxide (ITO) coated glass slides (2.5 × 2.5 mm, 9-13 Ω, Sigma, United States) were initially ultrasonicated with mild detergent in Milli-Q, followed by rinsing Milli-Q and isopropanol for 20 min each and dried with nitrogen gas. For ECL imaging, a small pillar (2 mm diameter) made of polydimethylsiloxane (PDMS; Silpot 184, DuPont Toray Specialty Materials K. K., Japan) was positioned at the center of the electrode to create a region without lipid membranes. The electrodes were then treated with air plasma (Yamato, Japan) at a flow rate of 100 mL/min for 5 min. Conversely, for general electrochemical measurements (CV and EIS), the entire electrode area was subjected to plasma treatment. A PDMS well, sealed with insulation tape (Elegrip, Denka, Japan), was stacked on the ITO surface to form a central well. The insulating tape had an opening diameter of 6 mm which defines the electrode area as 0.28 cm^2. For electrical connectivity, a conducting wire was attached to the corner of the ITO electrode using copper tape.

The liposome solution was diluted to 0.2 mg/mL using D-PBS and 100 μL of this solution was applied onto the ITO electrode and incubated at 40˚C for 60 min. The liposomes spontaneously ruptured and fused, forming a bilayer on the ITO surface (vesicle fusion method). After incubation, the ITO electrode was rinsed with 1 mL D-PBS to remove excess liposomes. Throughout the washing and buffer replacement processes, care was taken to prevent the ITO surface from drying, as this could disrupt the bilayer structure. To create a DOPC-DOPS membrane, ITO electrodes without air plasma treatment were used. Additionally, during vesicle fusion, D-PBS with 1 mM $CaCl_2$ was used as a buffer to stabilize the DOPC-DOPS lipid membrane formation. Before replacing the buffer with the ECL reagent, the PDMS pillar was carefully removed using tweezers.

Electrochemical measurement

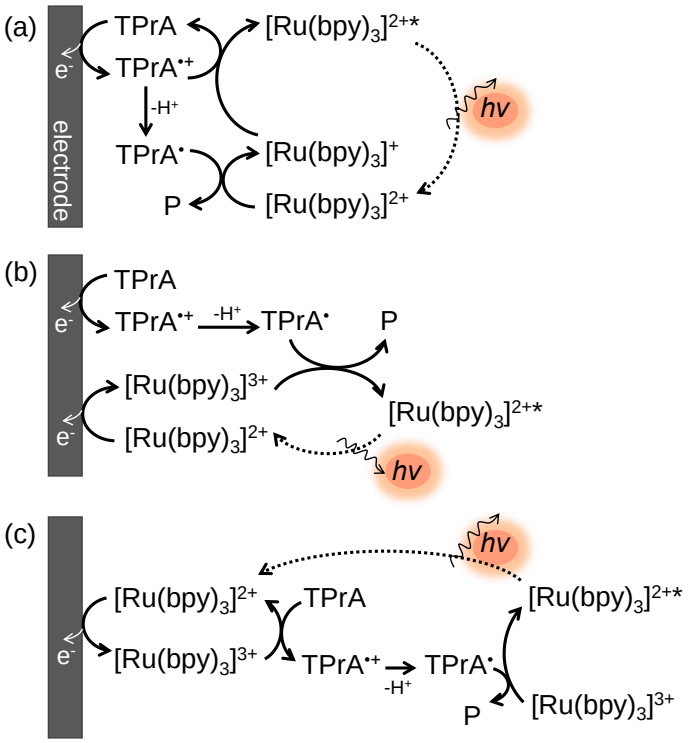
For electrochemical characterization of the lipid membrane-formed electrodes, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were performed using a potentiostat (Versastat, Ametek, Germany). An Ag/AgCl (saturated KCl) electrode (BAS, Japan) and a Pt foil served as the reference and counter electrodes, respectively. A PDMS pillar was not utilized for electrochemical evaluation. Following vesicle fusion and washing, the D-PBS buffer was substituted

with a 2 mM $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ solution at a 1:1 ratio in D-PBS. CV was performed at a scan rate of 100 mV/s. EIS was conducted at a holding potential of 0.26 V vs Ag/AgCl, applying alternating voltage ranging from 0.1 to 10^5 Hz with 10 mV amplitude. The EIS spectrum was analyzed using the PyZwx open-source software provided by the National Institute for Materials Science, Japan.

Electrochemiluminescence (ECL) imaging

For ECL imaging, the D-PBS buffer on the ITO electrode was replaced with an ECL buffer containing 400 μM $[Ru(bpy)_3]^{2+}$ and 25 mM TPrA in D-PBS. This assembly was then placed on a microscope stage (Olympus, Japan) (Fig. S2). The water immersion objective of the microscope (UMPLFLN20XW, Olympus, Japan) was carefully lowered toward the ITO surface and immersed in the ECL solution. Homemade Ag/AgCl and Pt wire electrodes were introduced into the solution. A potentiostat (EC-400, EC Frontier, Japan) was used to apply the required potential, and ECL images were captured using a Zyla 3.5 sCMOS camera (Andor, United Kingdom). During imaging, external light was excluded using a blackout curtain. To focus the objective on the lipid membrane surface, a fast CV scan (2 V/s, 0.7 to 1.2 V) was continuously performed while monitoring the live images. During these fast scan cycles, the SLB area appeared dark, whereas the lipid-free area appeared bright, facilitating focus adjustment at the point where the boundary between the SLB and lipid-free areas became distinctly visible. After setting the focus, CV and amperometric measurements were performed alongside ECL imaging. For CV, the potential was scanned from 0 to 1.4V at 100 mV/s and the camera captured 60 frames at a frame rate of 1.906 with an exposure time of 0.5 s. For the amperometric measurements, the same camera setting was maintained, while the potential was incrementally increased from 0 to a predetermined voltage (0.9–1.3 V).

The ECL images were analyzed using the ImageJ software. The 10 regions of interest (20 μm diameter each) were selected for the lipid membrane-formed and lipid-free areas in each image. The "Multi Measurements" function of ImageJ was utilized to acquire a series of ECL intensities during the scanning. To enhance image visibility, the minimum and maximum intensity values of the ECL images were adjusted from 0 to 300.



Scheme S1
General ECL reaction routes. (a) The low-oxidation potential route involves formation of reductive TPrA radical cations through the electrochemical oxidation of TPrA, generating the emitting species $[Ru(bpy)_3]^{2+*}$. (b) The oxidative-reduction route entails simultaneous oxidation of $[Ru(bpy)_3]^{2+}$ and TPrA on the electrode, resulting in ECL emission. (c) In the catalytic route, $[Ru(bpy)_3]^{2+}$ is electrochemically oxidized to generate TPrA radical cations, which eventually generate $[Ru(bpy)_3]^{2+*}$ which emits ECL.

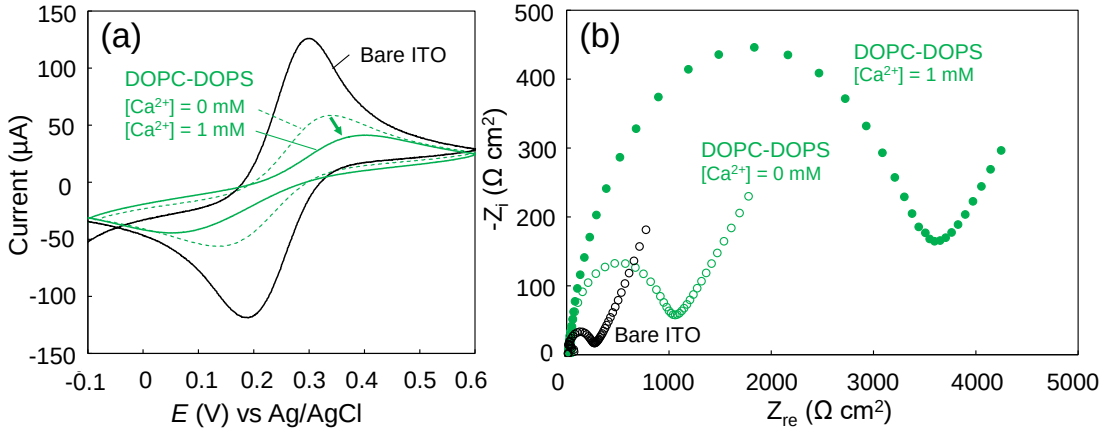
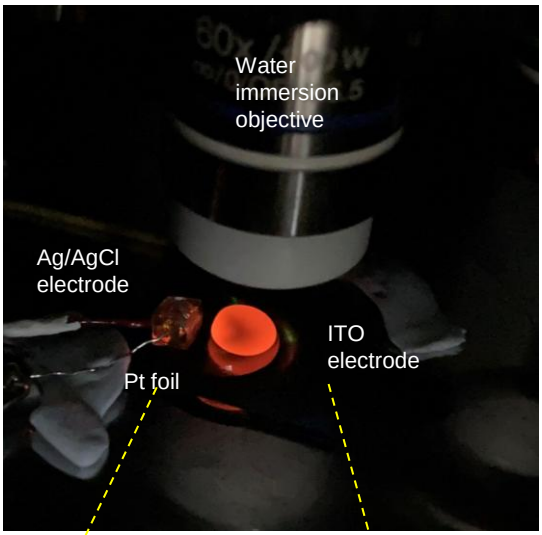


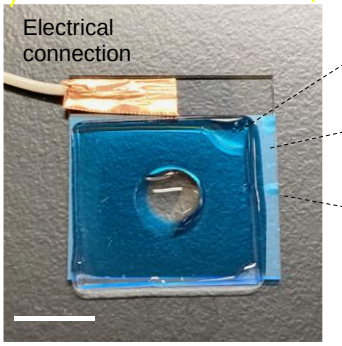
Figure S1

Electrochemical evaluation of DOPC-DOPS membrane on ITO electrodes formed with 0 or 1 mM $CaCl_2$ in D-PBS during vesicle fusion. A 2 mM solution of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ at a 1:1 ratio in D-PBS was used for electrochemical measurement. (a) CVs of the bare and DOPC-DOPS membrane-formed electrodes. The scan rate was set at 100 mV/s. (b) Nyquist plots corresponding to the electrodes in (a). Holding potential: 0.26 V, Frequency range: 10^{-1}–10^{5} Hz, Perturbation amplitude: 10 mV. The decrease in peak current and the heightened resistance signifies the stabilizing effect of Ca^{2+} ions in the formation of the DOPC-DOPS lipid membrane.

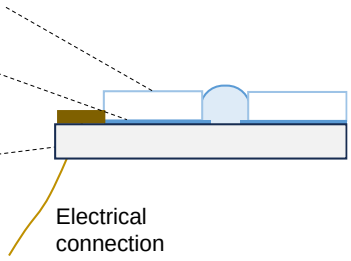
(a)



(b)



(c)



(d)

Critical components	Size
The size of the ITO coated glass slide	25 mm × 25 mm
The opening size of the ITO electrode	0.28 cm^2 (6 mm in diameter)
The opening size of the PDMS well	0.50 cm^2 (8 mm in diameter)
The size of the PDMS pillar (to create a lipid-free area in ECL imaging)	0.031 cm^2 (2 mm in diameter)

Figure S2
Electrochemiluminescence microscopy setup. (a) Image of the ECL setup under the microscope. The bright orange emission is attributed to the ECL of $[Ru(bpy)_3]^{2+}$/TPrA. (b) Image of the ITO electrode. Scale bar: 1 cm. (c) Schematic side view of the ITO electrode, presented not to scale. (d) Size of the components of the ECL electrode.

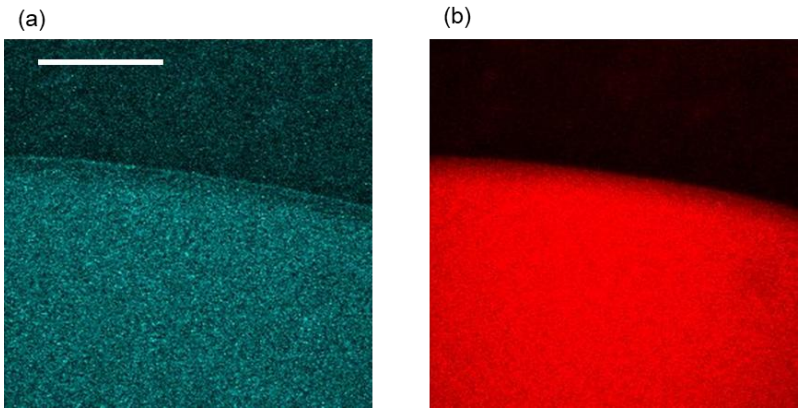


Figure S3
Fluorescence images of DOPC lipid membrane formed electrode. The fluorescence of $[Ru(bpy)_3]^{2+}$ was observed by focusing on the lipid membrane labelled with NBD-PE. (a) Fluorescence image of DOPC lipid membrane containing NBD-PE. NBD was excited with a laser at λ_{Ex} = 458 nm and the emission was observed through a filter at 475-575 nm. Scale bar: 100 µm. (b) Fluorescence image of the same membrane from (a) with the solution containing 400 µM $[Ru(bpy)_3]^{2+}$. $[Ru(bpy)_3]^{2+}$ was excited with a laser at λ_{Ex} = 458 nm and the emission was observed through a filter at 645-745 nm. The boundary between the insulating tape (black part) and the lipid membrane was blurred by the fluorescence of $[Ru(bpy)_3]^{2+}$, which diffuses freely in the solution, and it was not possible to observe the surface of the lipid membrane. A confocal laser scanning microscope (FV1000 Olympus, Japan) was used for the observation.

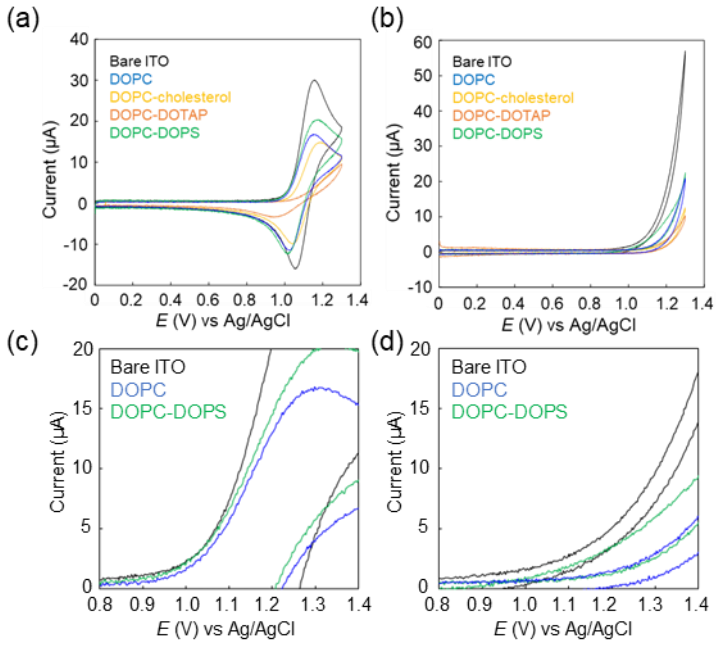


Figure S4
CVs on electrodes with lipid membranes in (a) 400 μM $[Ru(bpy)_3]^{2+}$ solution and (b) 25 mM TPrA solution. Scan rate: 100 mV/s. (c, d) Magnified CV curves of bare ITO, DOPC, and DOPC-DOPS membrane formed electrode from (a) and (b).

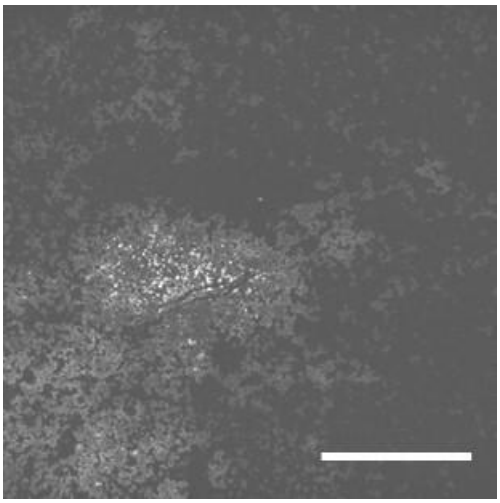


Figure S5
ECL image of DOPC-DOTAP membrane detached from the electrode. A voltage of 1.2 V was applied for 5 s. The exposure time for the image capture was 0.5 s. Scale bar: 100 µm.

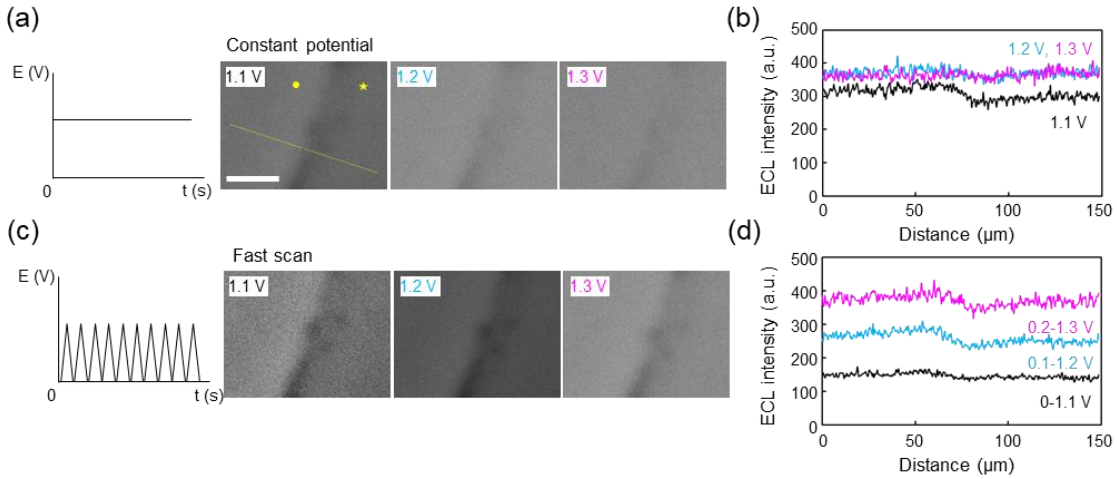


Figure S6
ECL images of DOPC-cholesterol membrane under different potential applications. The area with lipid membranes is marked with ★, whereas the area without lipid membranes is marked with ●. (a) A schematic and the ECL images under constant potential of 1.1, 1.2, or 1.3 V (Exposure time: 20 s). The boundary between the lipid-free area and the lipid membrane area became blurred at higher voltages due to continuous diffusion of the ECL molecules. (b) The ECL intensity profiles along the yellow line in (a). (c) A schematic and the ECL images under triangular waves between 0.0-1.1, 0.1-1.2, or 0.3-1.3 V. Scan speed was set 2 V/s. Exposure time: 20 s. Even at the higher voltages, the border remained clear while the ECL intensity remained high. (d) The ECL intensity profiles along the yellow line in (a). Differences in the ECL intensity between two areas were clearer when the triangular waves were applied. Scale bar: 100 μm.