Supporting information for:

A Liposome-based Energy Conversion System for Accelerating the Multi-enzyme Reactions

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1. Materials

ADH (EC 1.1.1.1) and DI (EC 1.8.1.4) were purchased from Sigma (St. Louis, MO, USA) and Amano Enzyme (Nagoya, Japan), respectively. NADH reduced disodium salt and egg lecithin were purchased from Sigma and Wako (Osaka, Japan), respectively. Cy2 and Cy3 were from GE Healthcare (Piscataway, NJ, USA). All other chemicals were of reagent grade quality.

2. Preparation of biocatalytic elements entrapped liposome

Egg lecithin was added at 1% (w/v) into 10 mM phosphate buffer (pH 7.0), and suspended using a bath-type sonicator for 5min. This suspension was homogenized into liposome using a spray blender TLBM901 (BIO MEDIA, Tokyo, Japan). The liposome was centrifuged by using a centrifuge MX-100 (TOMY, Tokyo, Japan) at 13000 rpm for 10 min. Supernatant was discarded, and then 50 μL of each ADH (25 mg/mL), DI (5 mg/mL), NADH (50 mM), and phosphate buffer (10 mM, pH7.0) solutions was added, and the mixture was subjected to freeze-and-thaw treatment 3 times. The non-entrapped biocatalytic elements were removed by gel permeation chromatography (GPC) with a Sepharose 4B column (1 x 20 cm) by using a Biologic Duoflow system (BIORAD, CA, USA). 100 μL of the liposome suspension was loaded on the GPC column and eluted with 10 mM phosphate buffer (pH 7.0) at a flow rate of 0.3 mL/min and every 1 mL fraction was collected. Biocatalytic elements entrapped liposome was collected and stored at 4 ^{o}C.

3. Fluorescence monitoring for detecting leakage of labeled ADH, labeled DI, and NADH from liposome and its fluorescence observation

According to the instruction of these fluorescent probes, ADH and DI were separately dissolved at 1 mg/mL in a sodium carbonate buffer (pH9.3), and each 1 mL solution of the ADH and DI was mixed with the Cy2 and Cy3 dyes in kit vial, respectively. The reaction solutions were incubated at room temperature for 30 min and mixed at every 10 minutes. Each 200 μL solution of fluorescence substance, Cy2 labeled ADH (Cy2-ADH), Cy3 labeled DI (Cy3-DI), or 50 mM NADH, was mixed with the

centrifuged liposome and followed by freeze-and-thaw treatment 3 times. GPC condition is the same as the normal procedure written in "section 2".

Release of fluorescence probe such as calcein from liposome was often detected by using fluorescence spectrophotometer.[1] Fluorescence measurements were performed to detect leakage of fluorescent probe, Cy2-ADH, Cy3-DI, and NADH for revealing the physical stability of the liposome to retain biocatalytic elements. Since the quenching effect between the probes is reduced when leaking them from liposome, we can detect the physical stability from the fluorescent change. Fluorescence spectrophotometer is an F-4500 (Hitachi, JAPAN) with a 150W Xe lamp and a 1 cm-quartz cell. All measurements were carried out with a slit of 5 nm in excitation and 20 nm in emission. Cy2, Cy3, and NADH were detected at 450 nm of excitation and 505 nm of emission, 510 nm of excitation and 570 nm of emission, 340 nm of excitation and 460 nm of emission, respectively. Figure S1 (a) shows fluorescence monitoring for Cy3-DI entrapped into liposome with an injection of ethanol and Triton X100 in order. Triton X100 has been known as the surfactant to solubilize liposome and used for release of Cy3-DI from liposome. Since the fluorescence intensity did not change before injection of ethanol, Cy3-DI was exactly entrapped into liposome. After ethanol was injected to a final concentration of 100 mM in the liposome solution, the intensity also unchanged, indicating that the liposome is physically stable at least in 100 mM ethanol. Once Triton X100 was injected to a final concentration of 0.3%, the intensity increased, implying that the liposome was unstable against Triton X100 and released Cy3-DI. These properties indicate that the liposome is strong enough for time and for 100 mM ethanol. Figures S1 (b) and (c) show fluorescence monitoring for Cy2-ADH and NADH entrapped into liposome, respectively. These profiles are very similar to those from the treatment of Cy3-DI. Since the liposome is physically stable enough to retain ADH, DI, and NADH under above conditions, we can say that enzymatic reactions occur only within liposome.

Fluorescence images of liposome in the main text (Figure 1) were taken by using an optical fluorescence microscope (Nikon, Tokyo, Japan) both with a 50 W mercury lamp and with a set of filters for fluorescence measurements of NADH, Cy2, and Cy3. The excitation lamp was a 100 W high-

pressure mercury lamp. A filter block with the excitation filter (EX 380–420 nm), the dichroic mirror (DM 430 nm) and the barrier filter (BA 450 nm) were used for the detection of NADH. EX 450–490 nm, DM 505 nm, and BA 520 nm were used for detection of Cy2. EX 510–560 nm, DM 575 nm, and BA 590 nm were used for detection of Cy3. The fluorescence emission was collected by using a Nikon S-Fluor long working distance objective 10 mm, magnification 20 times; numerical aperture 0.75.

4. Bulk electrolysis of Q_0 with and without liposome

Electrochemical reaction rate of Q_0 on a carbon felt electrode and Q_0 permeation through liposome might be the factors governing catalytic current intensity with the liposome system. Therefore, we examined the electrochemical reaction rate of Q_0 and the effect of Q_0 permeation through liposome by using bulk electrolysis technique. Chronoamperometry was carried out for the reduction of 1 mM Q_0 at -0.2 V vs. Ag/AgCl. Other conditions of electrochemical measurements are the same as the main text. Dotted line in Figure S2 shows current-time curve of Q_0 reduction without liposome under stirring. Current-time curve at the beginning of measurement, which reflects the reaction rate of Q_0 on the electrode, is higher than the catalytic current generated from liposome shown in Figure 2(a). Therefore, we can conclude that Q_0 reaction rate on the electrode is not rate determining step in this study. Solid line in Figure S2 shows current-time curve of Q_0 reduction with liposome. To enhance the effect of the Q_0 permeation through liposome on the bulk electrolysis, concentrated liposome was used in the measurements. Total entrapped volume inside liposome was about 30 μL in 100 μL of bulk solution. Since both current-time curves with and without liposome are almost the same, Q_0 permeation through liposome has no effect on electrolysis and is fast enough to reach the equilibrium inside and outside of liposome. Therefore, the permeation rate of Q_0 through liposome could be neglected. In short, Q_0 concentrations in the inner phase of the liposome and in the bulk phase are determined to be identical and set at 1 mM in the following simulation.

5. Quantification of ADH, DI, and NADH within liposome

The content of ADH and DI within liposome were determined from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using an XV pantera system (DRC Co. Ltd., Tokyo, Japan) on 15% T, 3% C gels. The samples were denatured with a SDS-reducing buffer (0.375 M Tris–HCl, 12 % (w/v) SDS, 30 % (w/v) sucrose, 0.0125 % (w/v) bromophenol blue), heated at 95 °C for 5 min, cooled, and centrifuged before applying to SDS-PAGE. Denatured samples were subjected to electrophoresis at 240 V for 15 min. Protein bands were visualized by Coomassie brilliant blue staining. The intensities of ADH and DI were visualized and quantified by a GDS-7900 image analyzer (UVD, CA, USA).[2]

The content of NADH within liposome was determined by high performance liquid chromatography (HPLC). We used an Alliance system (Waters, MA, USA) equipped with our own preparation column packed with BioGel P6 resin (BIO-RAD, CA, USA) at a size of 5 mm x 50 mm. Samples were diluted 10 times with ethanol, and 10 μl of diluted samples were injected into the column and eluted with 10 mM PBS buffer adjusted to pH 7.0 at a flow rate of 0.2 ml/min under room temperature. NADH was detected using a UV detector at 280 nm.

6. Kinetics

ADH has been known as an NAD-dependent oxidoreductase catalyzing the oxidation of ethanol to acetaldehyde, though the ADH reaction is reversible.[3] Ordered Bi-Bi mechanism was assumed for the reversible reaction of ADH as well as most of NAD-dependent oxidoreductases. Steady-state kinetics of the ethanol oxidation ($v_{\mathrm{ADH}}^{\mathrm{OX}}$) and the acetaldehyde reduction ($v_{\mathrm{ADH}}^{\mathrm{RED}}$) are given by

$$v_{\mathrm{ADH}}^{\mathrm{OX}} = \frac{v_{\mathrm{max,f}}}{1 + \frac{K_{\mathrm{M1}}}{[\mathrm{EtOH}]} + \frac{K_{\mathrm{M2}}}{[\mathrm{NAD}]} + \frac{K_{\mathrm{M1}} \cdot K_{\mathrm{M2}}}{[\mathrm{EtOH}][\mathrm{NAD}]}}$$

$$v_{\mathrm{ADH}}^{\mathrm{RED}} = \frac{v_{\mathrm{max,r}}}{1 + \frac{K_{\mathrm{M3}}}{[\mathrm{CH_3CHO}]} + \frac{K_{\mathrm{M4}}}{[\mathrm{NADH}]} + \frac{K_{\mathrm{M3}} \cdot K_{\mathrm{M4}}}{[\mathrm{CH_3CHO}][\mathrm{NADH}]}}$$

where $v_{max,f}$ ($=k_{cat}^{OX}[ADH]$) and $v_{max,r}$ ($=k_{cat}^{RED}[ADH]$) are the maximum velocity of ethanol oxidation and acetaldehyde reduction of ADH, respectively, [ADH] being the local concentration of ADH within liposome. In the ethanol oxidative reaction, k_{cat}^{OX} is the catalytic constant of oxidation, and K_{M1} and K_{M2} are the Michaelis constants for ethanol and NAD, respectively. On the other hand, in the acetaldehyde reductive reaction, k_{cat}^{RED} is the catalytic constant of reduction, and K_{M3} and K_{M4} are Michaelis constants for acetaldehyde and NADH, respectively. The steady-state kinetic parameters of ADH were determined by measuring the rate of NADH oxidation and NAD^+ reduction at 340 nm with UV-visible spectrophotometer performed by Shimadzu Multi-Spec 1500. Local concentration of ADH within the liposome, [ADH], was calculated on the basis of ADH content and internal volume of liposome, which were estimated from SDS-PAGE and phospholipid assay, respectively. Phospholipid assay of Wako Test C evaluated that concentration of phosopholipid comprising the liposome was 5.5×10^{-3} μmol in 100 μL bulk solution. Total internal volume of the liposome was reported to 30 μl per μmol phospholipid.[4] Therefore, the total internal volume of the liposome used in Figure 2 was 0.17 μL in 100 μL bulk solution. The kinetic parameters were evaluated as follows; $v_{max,f} = 9.6\ s^{-1}$, $v_{max,r} = 96\ s^{-1}$, K_{M1} = 181 mM, K_{M2} = 0.083 mM, K_{M3} = 1.0 mM, and K_{M4} = 0.1 mM.

DI catalyzes the oxidation of NADH with several electron mediators such as quinones,[5] flavins,[6] and metal complexes.[7] Simple ping-pong mechanism was assumed for the DI reaction in the simulation. The steady-state kinetics of the DI (v_{DI}) is expressed by

$$v_{DI} = \frac{v_{max,DI}}{1 + \frac{K_{M5}}{[NADH]} + \frac{K_{M6}}{[Q_0]}}$$

where $v_{max,DI}$ ($=k_{cat}^{DI}[DI]$) is the maximum velocity of the DI reaction, and k_{cat}^{DI} and [DI] are the catalytic constant and the local concentration of DI within liposome, respectively. K_{M5} and K_{M6} are the Michaelis constants of DI for NADH and Q_0, respectively. The steady-state kinetic parameters of DI were determined by measuring the rate of Q_0 reduction at 500 nm with UV-visible spectrophotometer

performed on a Shimadzu Multi-Spec 1500. The local concentration of the DI, [DI], was calculated with the DI content and the internal volume of liposome as well as ADH. These kinetic parameters were evaluated as follows; $v_{max,DI} = 12\ s^{-1}$, $K_{M5} = 0.79$ mM, and $K_{M6} = 1.1$ mM.

7. Simulation

The time differentiation for concentration of each chemical is described as

$$\frac{d}{dt}[Eb(t)] = -\frac{V_l}{V_b}k_1\left([Eb(t)] - [El(t)]\right)$$

$$\frac{d}{dt}[El(t)] = k_1\left([Eb(t)] - [El(t)]\right) - v_{ADH}^{OX}(t)\frac{[El(t)]}{[El(t)] + [Adl(t)]} + v_{ADH}^{RED}(t)\frac{[Adl(t)]}{[El(t)] + [Adl(t)]}$$

$$\frac{d}{dt}[Adl(t)] = v_{ADH}^{OX}(t)\frac{[El(t)]}{[El(t)] + [Adl(t)]} - v_{ADH}^{RED}(t)\frac{[Adl(t)]}{[El(t)] + [Adl(t)]} - k_2\left([Adl(t)] - [Adb(t)]\right)$$

$$\frac{d}{dt}[Adb(t)] = \frac{V_l}{V_b}k_2\left([Adl(t)] - [Adb(t)]\right)$$

$$\frac{d}{dt}[NADH(t)] = v_{ADH}^{OX}\frac{[El(t)]}{[El(t)] + [Adl(t)]} - v_{ADH}^{RED}\frac{[Adl(t)]}{[El(t)] + [Adl(t)]} - v_{DI}$$

$$\frac{d}{dt}[NAD(t)] = -v_{ADH}^{OX}\frac{[El(t)]}{[El(t)] + [Adl(t)]} + v_{ADH}^{RED}\frac{[Adl(t)]}{[El(t)] + [Adl(t)]} + v_{DI}$$

where [El(t)], [Adl(t)], [NAD^+(t)], and [NADH(t)] are concentration of ethanol, acetaldehyde, NADH and NAD^+ inside liposome at time (t), respectively. [Eb(t)], and [Adb(t)] are concentration of ethanol and acetaldehyde outside liposome at time (t), respectively. Since the amount of ADH catalyzing forward and reverse reactions was assumed to be proportional to the ratio of [El(t)] and [Adl(t)], net reaction rate of ethanol oxidation and acetaldehyde reduction are expressed as $v_{ADH}^{OX}\frac{[El(t)]}{[El(t)] + [Ald(t)]}$, and $v_{ADH}^{RED}\frac{[Adl(t)]}{[El(t)] + [Adl(t)]}$, respectively. Flux of ethanol and acetaldehyde through liposome is assumed to be proportional to the difference in concentration between inside and outside liposome. Permeation rate of ethanol through liposome, k_1, was set to be 750 s^{-1} by considering the following equation

$k_1 = P / d$

where P and d are permeability of ethanol and thickness of lipid bilayer, which are reported to 3.0×10^{-4} cm·s^{-1} and 4 nm, respectively.[4, 8] Permeation rate of acetaldehyde through liposome, k_2, was assumed to be the same as k_1. V_l and V_b, the internal volume of liposome and bulk volume in the measurements, were 0.17 μL and 100 μL, respectively, which are also described in "section 6". Therefore, internal volume factor (V_l/V_b) was 1/600. Initial concentration of NADH within liposome was estimated at 1.68 mM by using HPLC.

Since the permeation rate of Q_0 through liposome is fast enough to be neglected in calculation, the catalytic current generated from liposome can be described as a function of the enzymatic DI reaction rate and the internal volume of liposome (V_l). Catalytic current (I) generated from liposome is expressed by

$$I = nFV_l v_{DI} = nF \frac{V_l v_{\max,\mathrm{DI}}}{1 + \dfrac{K_{\mathrm{M5}}}{[\mathrm{NADH}]} + \dfrac{K_{\mathrm{M6}}}{[\mathrm{Q_0}]}}$$

Where n and F are number of electrons of Q_0 ($n = 2$) and Faraday constant, respectively.

In the simulation for the liposome system in which ADH has no reductase activity, the calculation was carried out at $v_{\mathrm{ADH}}^{\mathrm{RED}} = 0$. Therefore, time differentiation for concentration of each chemical is described as

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{Eb}(t)] = -\frac{V_l}{V_b} k_1 \left([\mathrm{Eb}(t)] - [\mathrm{El}(t)]\right)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{El}(t)] = k_1 \left([\mathrm{Eb}(t)] - [\mathrm{El}(t)]\right) - v_{\mathrm{ADH}}^{\mathrm{OX}}(t)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{Adl}(t)] = v_{\mathrm{ADH}}^{\mathrm{OX}}(t) - k_2 \left([\mathrm{Adl}(t)] - [\mathrm{Adb}(t)]\right)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{Adb}(t)] = \frac{V_l}{V_b} k_2 \left([\mathrm{Adl}(t)] - [\mathrm{Adb}(t)]\right)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{NADH}(t)] = v_{\mathrm{ADH}}^{\mathrm{OX}} - v_{\mathrm{DI}}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{NAD}(t)] = -v_{\mathrm{ADH}}^{\mathrm{OX}} + v_{\mathrm{DI}}$$

In the simulation for non-liposome system, internal volume factor (V_b/V_l), and permeation rate of ethanol and acetaldehyde were ignored because the enzymatic reactions proceed in the homogeneous condition. Time differentiation for the concentration of each chemical is described as

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{Eb}(t)] = -v_{\mathrm{ADH}}^{\mathrm{OX}}(t)\frac{[\mathrm{Eb}(t)]}{[\mathrm{Eb}(t)]+[\mathrm{Adb}(t)]} + v_{\mathrm{ADH}}^{\mathrm{RED}}(t)\frac{[\mathrm{Adb}(t)]}{[\mathrm{Eb}(t)]+[\mathrm{Adb}(t)]}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{Adb}(t)] = v_{\mathrm{ADH}}^{\mathrm{OX}}(t)\frac{[\mathrm{Eb}(t)]}{[\mathrm{Eb}(t)]+[\mathrm{Adb}(t)]} - v_{\mathrm{ADH}}^{\mathrm{RED}}(t)\frac{[\mathrm{Adb}(t)]}{[\mathrm{Eb}(t)]+[\mathrm{Adb}(t)]}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{NADH}(t)] = v_{\mathrm{ADH}}^{\mathrm{OX}}\frac{[\mathrm{Eb}(t)]}{[\mathrm{Eb}(t)]+[\mathrm{Adb}(t)]} - v_{\mathrm{ADH}}^{\mathrm{RED}}\frac{[\mathrm{Adb}(t)]}{[\mathrm{Eb}(t)]+[\mathrm{Adb}(t)]} - v_{\mathrm{DI}}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{NAD}(t)] = -v_{\mathrm{ADH}}^{\mathrm{OX}}\frac{[\mathrm{Eb}(t)]}{[\mathrm{Eb}(t)]+[\mathrm{Adb}(t)]} + v_{\mathrm{ADH}}^{\mathrm{RED}}\frac{[\mathrm{Adb}(t)]}{[\mathrm{Eb}(t)]+[\mathrm{Adb}(t)]} + v_{\mathrm{DI}}$$

In addition, $v_{\mathrm{max,\,f}}$, $v_{\mathrm{max,\,r}}$, $v_{\mathrm{max,\,DI}}$, and initial concentration of [NADH] were set as 1/600 (= V_l/V_b) of the liposome system, because [ADH], [DI], and [NADH] are diluted in the homogeneous condition compared with liposome system. Catalytic current is proportional to the products of the enzymatic DI reaction rate and the bulk volume (V_b)

$$I = nFV_{\mathrm{b}}v_{DI} = nF\frac{V_{\mathrm{b}}v_{\mathrm{max,DI}}}{1+\dfrac{K_{\mathrm{M5}}}{[\mathrm{NADH}]}+\dfrac{K_{\mathrm{M6}}}{[\mathrm{Q}_0]}}$$

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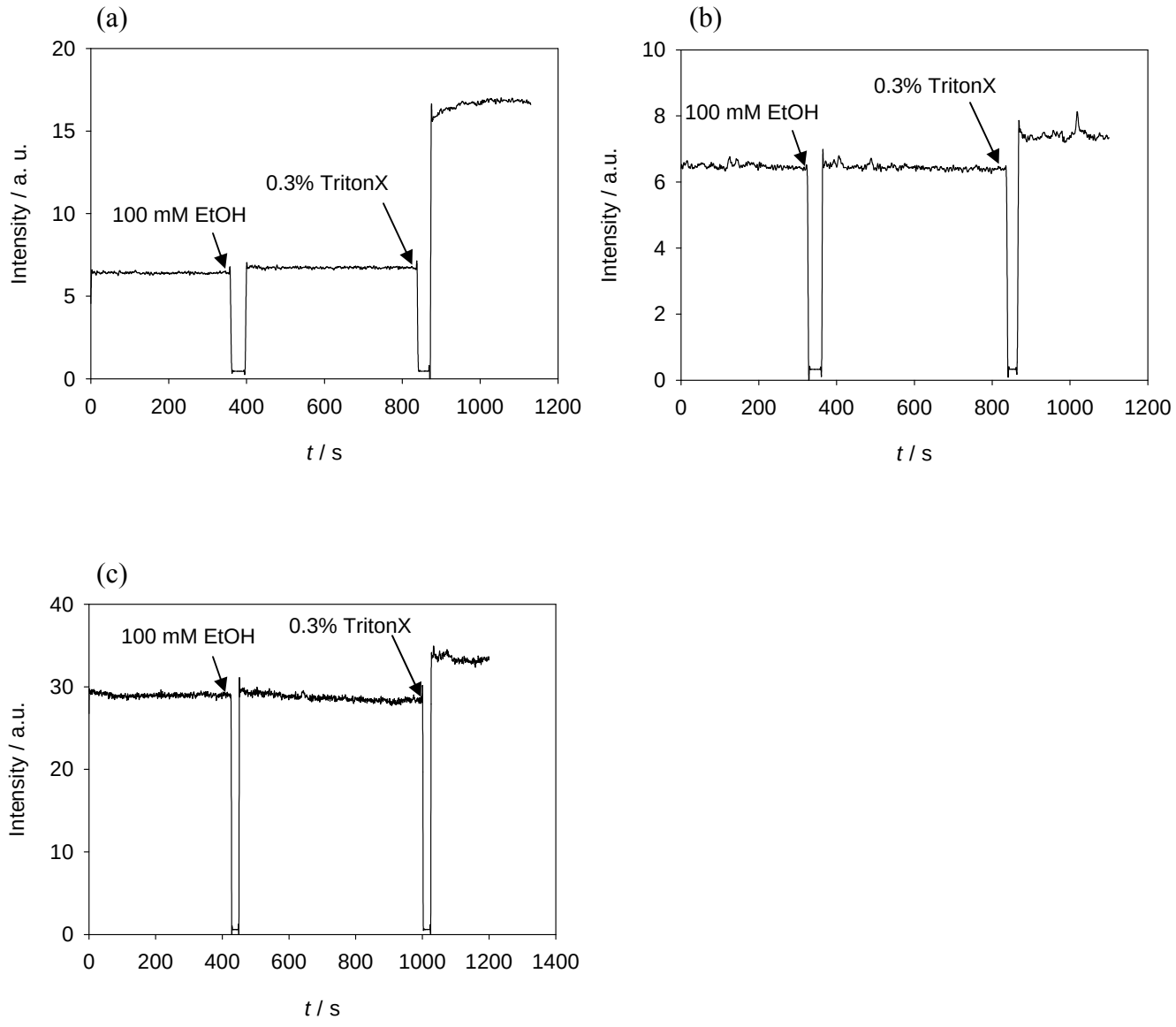

Figure S1. Fluorescence monitoring for leakage of (a) Cy2-DI, (b)Cy3-ADH, and (c) NADH from liposome with an injection of ethanol to a final concentration of 100mM and Triton X100 to a final concentration of 0.3 % in order. Liposome was diluted 200 times with 10 mM PBS before measurement.

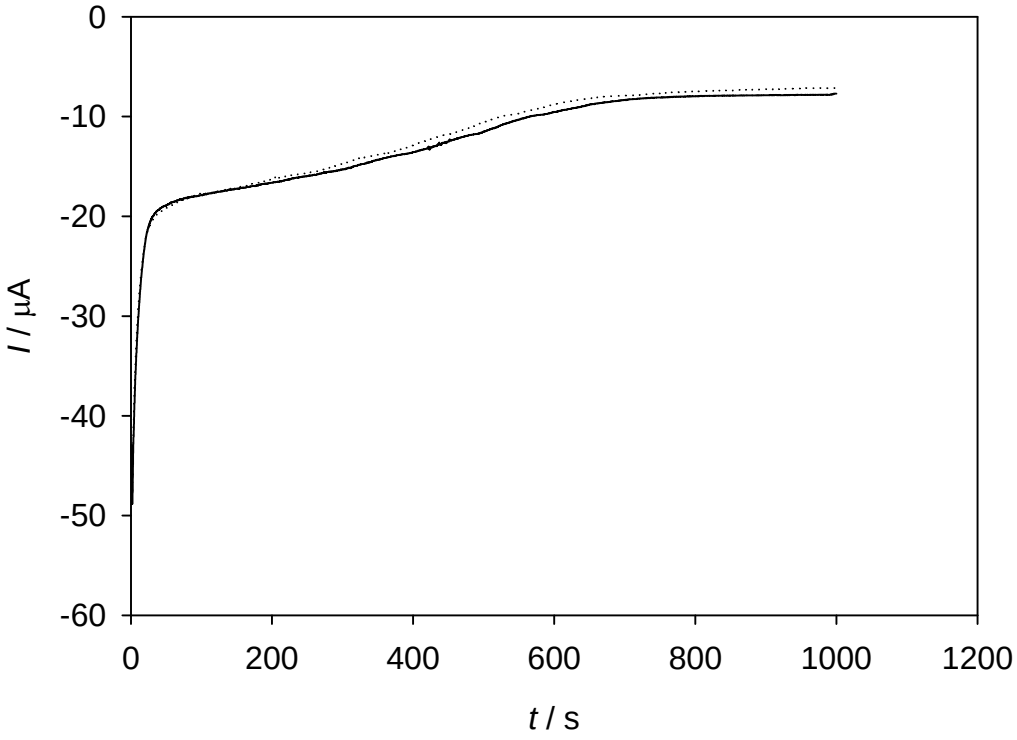

Figure S2. Current-time curve of 1 mM Q_0 reduction under stirring. Solid line and dotted line show with and without liposome in solution, respectively.