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Supplementary Information

Bioelectrocatalytic oxidation of glucose with antibiotic channel-containing liposomes

Shuji Fujita,[a] Ryuhei Matsumoto,[a] Kenichi Ogawa,[a] Hideki Sakai,[a] Akihiro Maesaka,[a] Yuichi Tokita,*[a] Seiya Tsujimura,[b] Osamu Shirai[b] and Kenji Kano*[b]

[a] Advanced Materials Laboratories, Sony Corporation,
Atsugi-shi, Kanagawa, 243-0021 (Japan)
E-mail: Yuichi.Tokita@jp.sony.com

[b] Division of Applied Life Sciences, Graduate School of Agriculture,
Kyoto University, Sakyo-ku, Kyoto, 606-8224 (Japan)
E-mail: kano.kenji.5z@kyoto-u.ac.jp

SEM images of liposome *in solution*

In a growing body of liposome studies for drug delivery systems, microreactors, and sensing devices, their structural information is useful to investigate not only stabilities and uniformities but responses to reagents and their behaviours during reactions.[1] Thus, we expected that clear imaging of liposome structure at nanometer scale, especially in solution, provides important information to assess the electrochemical reactivity with relevant structural response against additives. Optical microscopies including confocal microscopy have limited resolutions of micrometer size even though it is available to samples in solution. On the other hand, a scanning electron microscopy (SEM) is widely used to observe the structure of objects at a resolution of nano- to micrometer with its advantage of easy and high throughput handling. To obtain clear images, SEM usually requires a highly vacuumed condition, which leads liposomes to burst due to evaporation of aqueous phase. Therefore, the imaging in solution is quite difficult. Recently, ionic liquids have been reported to be applicable for electron microscopies for soft materials in solution under vacuumed conditions because ionic liquids exhibit both very low vapour pressure (stable solution under high vacuum) and high electric conductivity (preventing charge up).[2]
In this study, we also investigated structural differences between the liposomes with and without AmB. By use of an ionic liquid, we conducted the direct SEM observation of liposome with assemblage of the AmB channel *in solution* at nanometer resolution. We mixed equal volumes of the liposome suspension in 0.1 M phosphate buffer and an ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate (BMI-BF_4), and dropped the mixture on a silicon wafer followed by imaging it by SEM at room temperature under 0.1 Pa. As shown in Figure S2A, we successfully obtained a clear image of liposomes in the solution at a resolution of tens to hundreds of nanometer. The objects had circular shape with the rim brighter than their center. This brightness is thought to originate from the edge effect due to the enhanced secondary electron collection on the edges of structural objects, indicating that these objects actually have a spherical body of liposome.[3] We believe that this result is remarkable because of capturing the sharp image of liposome *in solution* at tens of nanometer resolution without substantial noise. We applied this method to the liposomes with AmB. As shown in Figure S2B, we observed a *star shape* structure in each vesicle. The bright region between the periphery of liposome and the dark star region seems to originate from the edge effect because the brightness in the rim is

dependent on an accelerating voltage (Figure S3),[4] hence liposome capsules were suggested to have a body with depressed surface (see Figure S4). Furthermore, the arcs of object outlines between adjacent vertexes of the star shape were often observed as a linear shape, also supporting that the surface of the liposome was depressed (see also Figure S4C). Since these star shapes were only observed in the liposome sample added with AmB, this depression of surface would be caused by inflow of the external solution into the interior of liposome or by the outflow of the buffer in liposome to the outside of liposome through the AmB channel. The outflow is considered to be driven by gradient in the concentration or imbalance of osmosis between the exterior and the interior of capsule. The depressed stress for the liposomes in Figure S2B was estimated to *ca.* 1.0×10^2 N/cm^2 using the previously reported elastic modulus for a membrane consisted of lecithin and cholesterol. [5] Surprisingly, such a mechanical stress to the membrane did not rupture the liposome capsule, indicating a strong plastic property of the membrane of the liposome with the AmB channel. Although, the liposomes were suspended in the mixture of the ionic liquid and the buffer solution in this observation, these structural images indicate the assemblage of AmB channel in the membrane.

Experimental Section

Materials: GDH (EC 1.1.1.47) and DI (EC 1.8.1.4) were purchased from Toyobo (Osaka, Japan) and Amano Enzyme (Nagoya, Japan), respectively. NAD^+ and Q_0 were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma Aldrich (St. Louis, MO, US), respectively. Cholesterol and egg lecithin were purchased from Wako (Osaka, Japan). BMI-BF_4 was purchased from Tokyo Chemical Industry (Tokyo, Japan). All other chemicals were of reagent grade quality.

Preparation of liposome encapsulating biocatalytic elements: In a flask, 6.7 mg of egg lecithin and 3.3 mg of cholesterol were dissolved in 20 mL ethanol. The lipid film was formed by drying the ethanol thoroughly with rotary-evaporation under vacuum in water bath at 40 °C for more than 1 hour, and subsequently suspended in 500 μL of the solution containing 2.5 mg/mL of GDH, 0.5 mg/mL of Di, and 5 mM NAD^+, using a bath-type sonicator for 1 min with scraping the film. This liposome suspension was washed with 500 μL of 0.1 M phosphate buffer, pH 7.0 for three times. At each washing treatment, the suspension was centrifuged at 3000 ×*g* for 4 min after vortex, and then the supernatant was discarded. The pellet was dissolved in 2.5 mL of 0.1 M phosphate buffer and kept at 4 °C shortly before the electrochemical measurements.

Leakage of NAD from liposome: Liposome with or without AmB containing the NAD was suspended in 0.1 M phosphate buffer, and incubated at room temperature for 3 hours with vortex at each 30 min. The suspension was then centrifuged at 3000 ×g for 4 min. The absorbance of supernatant was monitored at 280 mm to detect elution of NAD.

Electrochemical measurement: In the anode compartment, we stacked four sheets of carbon felt as electrode, which were contacted with Pt wire for current collection. The anode compartment was filled with 100 μL of 0.1 M phosphate buffer, pH 7.0, ionic strength 0.3, and connected with a salt bridge to the cathode compartment with a reference electrode and a counter electrode in saturated KCl. We added 10 μL of the liposome, 10 μL of 10mM Q_0, and subsequently added glucose solution to be 100 mM. We then added AmB dissolved in DMSO to be 0.5% (w/w) against the total lipids or added the same volume of DMSO as vehicle for a control experiment. We also added 10 μL of 3% triton X-100 to rupture the liposome.

Size-exclusion chromatography: We prepared liposomes containing Cy3-modified enzymes as the same procedure as above, and then applied 100 μL of liposome suspension to Sepharose 4B particles packed in 10 mm × 200mm column. Using 10mM phosphate

buffer, 1mL of fractions were collected with a fast protein liquid chromatography system (Bio-Rad, BioLogic DuoFlow). Collected fractions were added with 10%v/v of 3% triton X-100 to rupture the liposome, and then their fluorescence derived from the Cy3-modified enzymes were measured at 570 nm by excitation at 552 nm.

Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE): We applied suspension of liposome containing enzymes, and collected fractions of the size-excluded chromatography. The fractions were added with SDS-loading dye and boiled for 10 min, followed by electrophoresis in polyacrylamide gel. The gel was silver-stained with Bio-Rad Silver Stain and then imaged.

Scanning electron microscopy: We mixed the equal volumes of liposome and BMI-BF_4, and dropped 10 μL of the mixture on a silicon wafer. The wafer was set on the stage in the chamber of SEM (Helios NanoLab 400s, FEI), and then imaged with 4 mm of working distance under 0.1 Pa at room temperature. The accelerating voltage was set at 2 kV because the accelerating voltage more than 3 kV often destroyed the object or rendered the object to sink out of focal plane quickly. Since the sample was solution, the object were often moving by flow, therefore we needed to wait for the flow stopped or slow enough, checking by several short exposures.

Calculation Details

Parameters for the enzymatic activities and the flux of glucose were calculated as below.

The amount of enzyme

Encapsulation efficiency of catalytic components into liposome was estimated from the size-exclusion chromatography and SDS-PAGE as shown in Figure S1. The encapsulated fraction of enzymes into liposome was estimated 36% of the input.

k'_{cat}

For estimation of k'_{cat}, the amount of catalyzed glucose was calculated from the catalytic current (12 μA), Faraday constant (96485 C mol^{-1}), ant the number of electron upon oxidation of glucose (2). K'_{cat} was calculated from the amount of catalyzed glucose (6.2×10^{-11} mol sec^{-1}) and the amount of GDH (1.6×10^{-11} mol).

S_I^{total}

We estimated an average of radius of liposome as 1.3×10^{-7} m from SEM images. To

estimate the number of liposome, we calculated the number of lipids in a single liposome (1.1×10^{6}) from a surface area of liposome calculated from the radius (2.1×10^{-9} cm^2) and a known area of head group of lipid (4.0×10^{-15} cm^2).[6] Then, we estimated the number of liposome (3.5×10^{10}) from average molecular weight (MW) of lipid (647 g mol^{-1}) that was calculated from 67% of MW of lecithin and 33% of one of cholesterol and a total weight of lipids in the experiment. S_l^{total} was calculated as 7.4×10 cm^2 from the number of liposome and the surface area of single liposome.

r_{AmB}

For estimation of r_{AmB}, we estimated the surface ccupied by the pores of AmB as 5.0×10^{-15} cm^2 from the number of AmB channel (1.6×10^{13}) and the reported radius of AmB channel ($4.0 \times 10\text{-}^{8}$ cm). The number of AmB channel was calculated by assuming that the AmB channel consists of eight AmB molecules. We calculated r_{AmB} (0.11%) by dividing the area of the pore by the total surface area of liposomes (S_l^{total}).

References

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5. (a) The stress was calculated on the basis of Hooke's law using following factors; the reported elastic modulus, 0.6 N/m^2; the average change of depressed distance between the centers of outline arc and depressed arc, 100 nm; and the average radius of depressed region between adjacent vertexes, 130 nm; (b) M. A. Idiart and Y. Levin, *Phys. Rev. E.*, 2004, **69**, 061922; (c) E. Evans and W. Rawicz, *Phys. Rev. Lett.*, 1990, **64**, 2094-2097.
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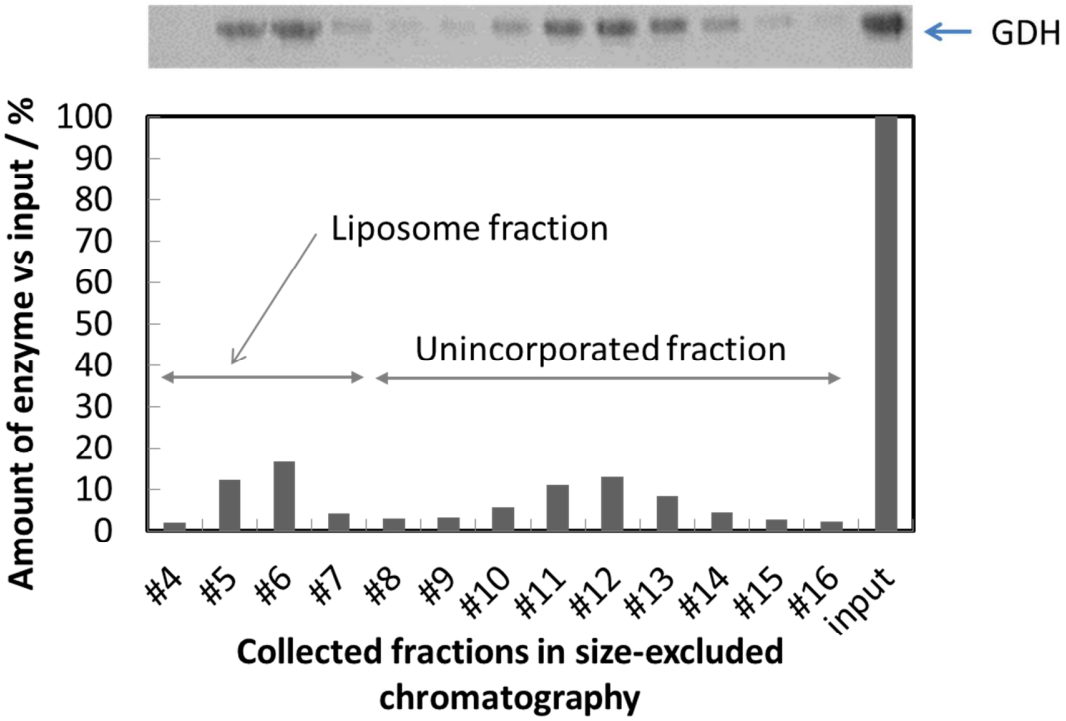

Figure S1. Fraction of enzymes incorporated into liposome by size-exlusion chromatography and SDS-PAGE. From both the chromatogram and densitometry of the gel image, we estimated 36% of enzymes incorporated into liposome.

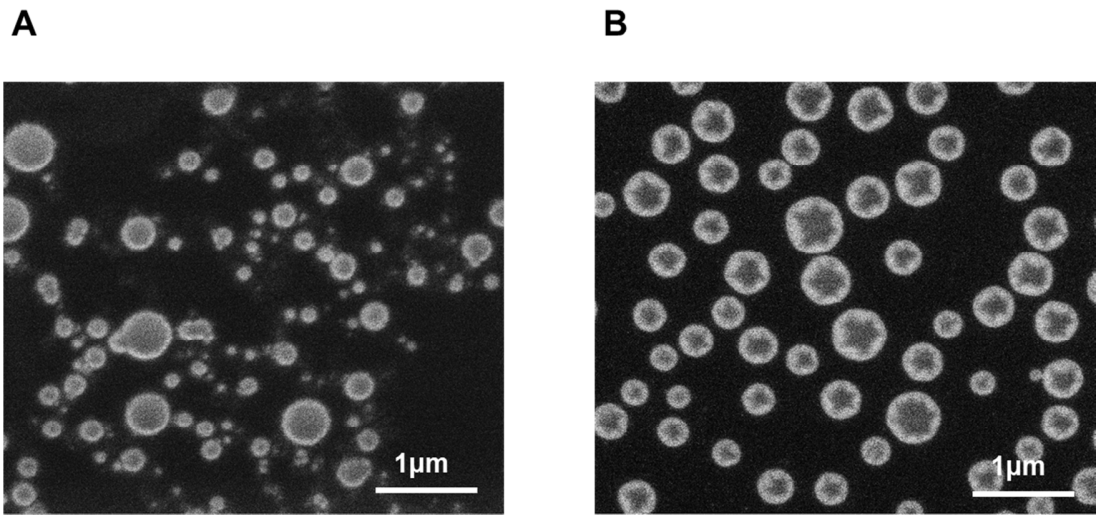

Figure S2. SEM images of the liposome in a mixture of phosphate buffer / BMI-BF_4 (v/v = 1/1) in the presence (A) and absence (B) of AmB

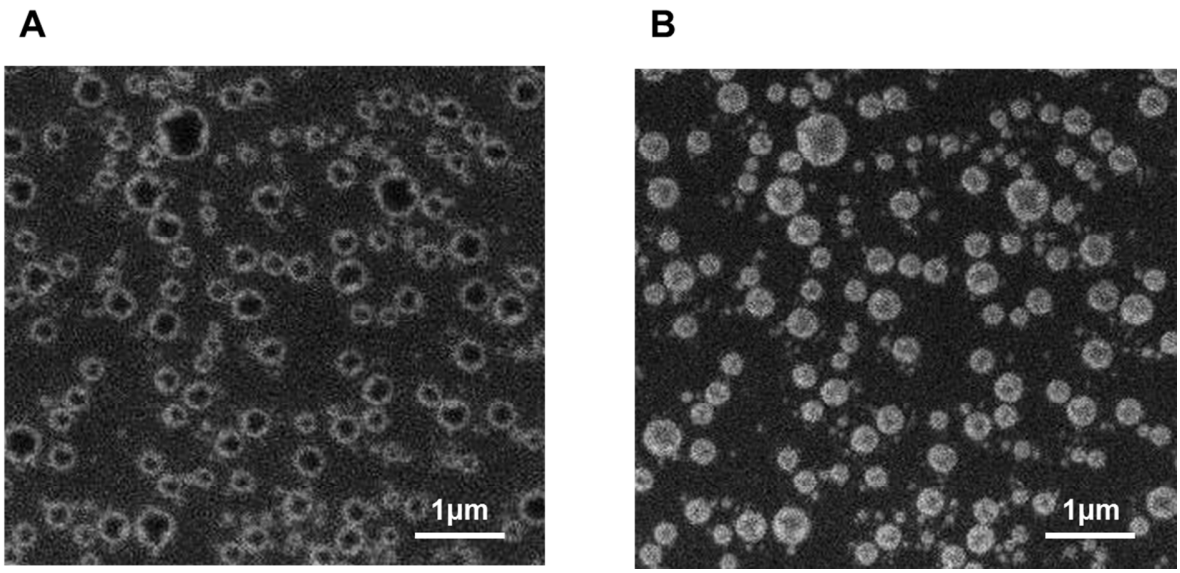

Figure S3. The bright region at the rim of liposomes in SEM image is dependent on accelerating voltage. (A) and (B) show the images at 1 kV and 2 kV of accelerating voltage, respectively. The image at 2 kV shows the bright regions thicker than that at 1 kV.

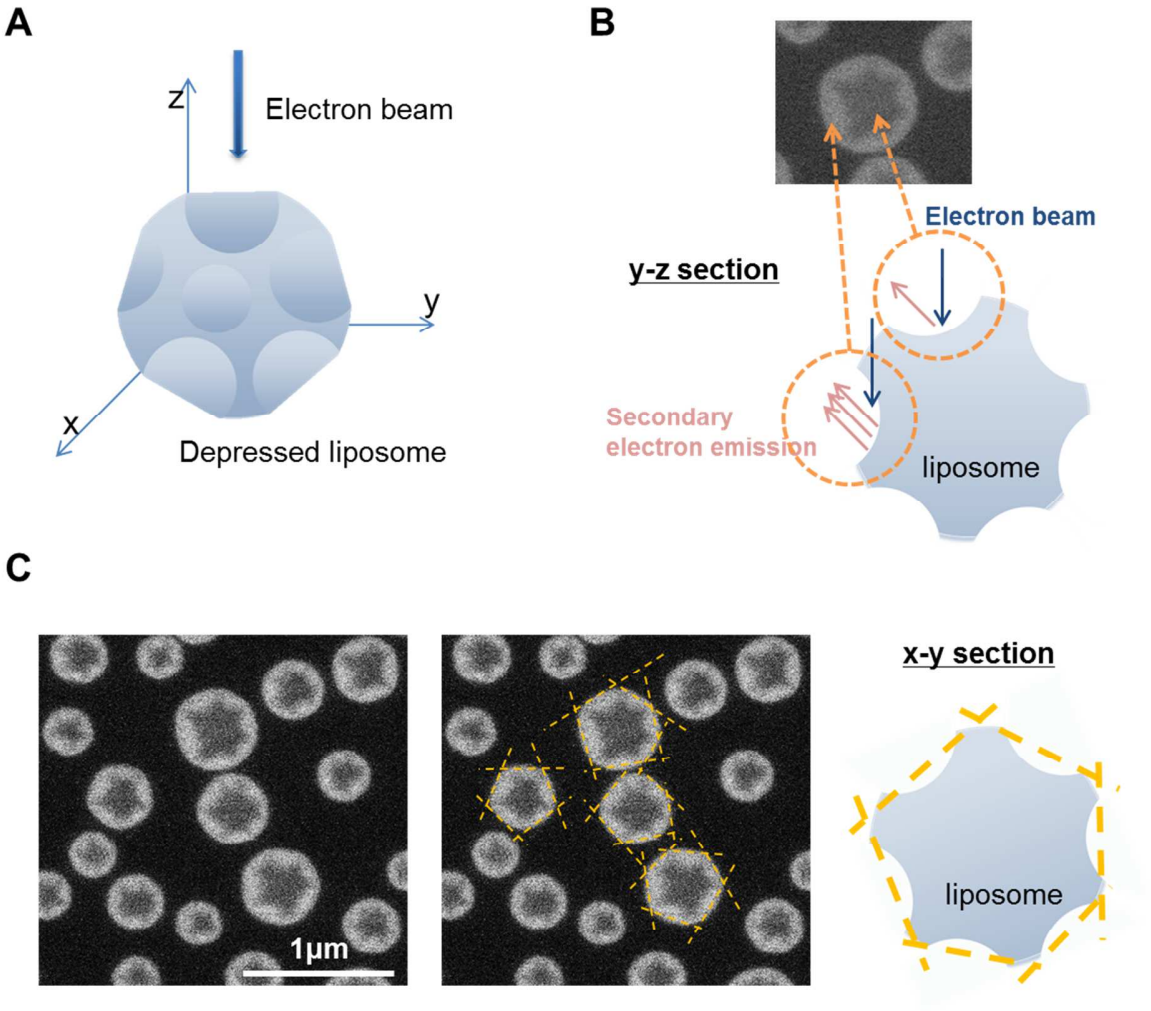

Figure S4. A model of liposome with depressed surface. (A) A schematic view of liposome with coordinate axis where the electron beam is irradiated along z axis. We proposed this structure model of liposome based on (B) the edge effect and (C) the outline shape of object. The simplified cross section of liposome in (B) or (C) is drawn in parallel or vertically along electron beam, respectively. (B) The surface angled parallel to the electron beam generates more secondary electron emissions, which turn the peripheral region in the object brighter, than the surface vertical to the electron beam. In (C), yellow lines indicate the linearly appearing arc of the object outline.