Membrane Integrated liposome Synthesized by a Liposome Fusion-Induced Membrane Exchange

*Wei-Peng Li, Xizi Long, Chiho Kataoka -Hamai, and Akihiro Okamoto**

Dr. X.-Z. Long, Prof. W.-P. Li, Prof. A. Okamoto
International Center for Materials Nanoarchitectonics, National Institute for Materials Science, Tsukuba, Ibaraki, 305-0044, Japan
E-mail: okamoto.akihiro@nims.go.jp

Prof. A. Okamoto
Graduate School of Chemical Sciences and Engineering, Hokkaido University, Hokkaido 060-8628, Japan

Prof. W.-P. Li
Department of Medicinal and Applied Chemistry, Kaohsiung Medical University, Kaohsiung, 807, Taiwan

Dr. C. Kataoka
Research Center for Functional Materials, National Institute for Materials Science, Tsukuba,
Ibaraki 305-0044, Japan

Keywords: liposome, outer-membrane vesicles, extracellular electron transport, cytochrome, *Shewanella oneidensis* MR-1, *Escherichia coli*

Biogenic extracellular vesicles (EVs) from mammalian cells and bacteria are assembled by lipid bilayer membrane with carried biologically active cargos such as proteins and mRNA, which received enormous attention due to their various potential applications, including immune therapy, drug delivery system, catalysis, liquid biopsy, microbial fuel cells, and so on. However, scanty EVs produced by biogenesis limited their applicability in the actual condition, and therefore new technologies to enlarge the

production of EVs must be developed and remain the challenge. In this study, we created a novel method named LIME (liposome fusion-induced membrane exchange) to acquire a large quantity of biologically active vesicles, in which the excess lipid components fused into the cell's membrane, thus promoting the process of EVs liberation. This method was first verified in gram-negative bacteria, *Shewanella oneidensis* MR-1 with *c*-type cytochrome complex (Cyts) on the outer-membrane and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine contained liposomes as the lipid donor were used. Interestingly, the significant difference in spectroscopy and heme staining between original liposomes and active membrane-integrated liposomes (MILs) revealed that the electrochemically active Cyts migrate from MR-1 outer-membrane to the liposome successfully. Moreover, MILs with Cyts enabled enhancing the current production from *Escherichia coli* K-12, demonstrating that the electron transfer activity of Cyts was preserved after the LIME process, and MILs showed massive potential as drug carriers, vaccine, and a tool for strains-crossed membrane proteins migration. Our approach indicates an all-new direction to produce artificial EVs with specific proteins and functions, which will significantly benefit the future development of EVs.

1. Introduction

Cell secreted extracellular vesicles loading enzymes and genes inside the hollow cavity and attaching the membrane proteins in the phospholipid bilayer shell reveals the particular capability of intercellular communication to initiate various biofunctions. Therefore, novel bio-applications such as liquid biopsy and immunotherapy involved

biological nanoparticles action are extensively explored recent years.[1,2,5,6] Outer-membrane vesicle (OMV) released from bacteria is one type of secreted extracellular vesicles where the membrane proteins on the surface of OMV play the critical role as a transporter, receptor, enzyme, or anchor as on the original cell membrane.[3] Therefore, OMVs have great potential to be a bio material in various applications like vaccine, bio-sensor, drug delivery, and bio-catalysis.[4] [7] [8] However, since the producing efficiency of OMVs is mainly depended on the intrinsic cells characteristics decided by the genome, it is challenging to utilize OMVs as a practical material. For instance, under a common incubation condition, gram-negative bacteria, not at all strains, could actively produce OMVs under the regulations by gene and environment or the passively generate OMVs through external factor-induced membrane bubbling from damaged cell.[12]. This biogenesis of OMVs results in nonviable productivity, high cost, and long culture time. Genetic programming to up-regulate gene expression is a controllable technique to promote OMVs production but it is applicable with gene sequence defined culturable host bacteria only.[13] Therefore, developing a feasible and gene-engineering free method to efficiently produce OM-integrated vesicles is necessary toward practicing applications.

Liposome, a nanoparticle made of the phospholipid bilayer with excellent biocompatibility, reveals a unique capability of coalescence into the cell membrane through the fusion process.[14] Due to the high fluidity of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), the DOPE-contained liposome has provided with more superior performance for membrane fusion compared to the DOPE free liposome.[16] We, therefore, examined if DOPE-contained liposome nanoparticles could fuse into bacteria and then release membrane-integrated liposomes (MIL) in order to eliminate excessive lipid substances from cell membrane (Figure 1a), by which a new artificial EVs production method termed liposome fusion-induced membrane exchange (LIME) was created. *Gram-negative* bacteria, *Shewanella oneidensis* MR-1(*S. oneidensis* MR-1), was used ascribed to their photo- and electro-chemically detectable outer-membrane bound cytochrome complex. [17,18] The electron transfer activity of the cytochromes on MIL was examined by the evaluation of current generation to non-native host of *Escherichia coli* (*E. coli*) with MIL. Generally, based on this LIME method, artificially produced MIL with native membrane proteins and preserved catalytic performance exhibited potential versatile bio-applications such as vaccine, bio-catalysis, immunotherapy.

2. Results and discussion

2.1. Incubating liposome with *S. oneidensis* MR-1 producing MIL

Liposome composed of 80% 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 20% DOPE was prepared *via* a standard extrusion method. The prepared uniform spherical shape liposome showed a size distribution between 65 - 100 nm (Figure S1). We then fused the liposome with *S. oneidensis* MR-1 culture (OD_{600} =1 in defined medium with lactate) at 30 °C for 20 hours with shaking (180 rpm). Compare with the cell pellet without adding liposome, the presence of liposome resulted a significant red color turning to white (Figure S2). Given the red color was assigned to *c*-type cytochrome in MR-1,[19] this loss would be associated with the release of membrane protein from cell membrane to solution. We quantified the total protein in the supernatant and found that the concentration increased from 60 μg/mL to 120 μg/mL (Figure S3). Meanwhile, the release of *c*-type cytochrome was captured by comparing the diffusion transmission UV-visible spectroscopy loading with integrating sphere. Stronger absorption from cell body at Soret band (410 nm) and Q-band at (540 nm) were observed without adding liposome while less absorbance from cell body was detected when adding liposome. In contrast, supernatant exposed to the liposome revealed significantly higher compared to that of cells without liposome (Figure 1b). The releasing of proteins here, including *c*-type cytochrome, from cell to the supernatant would be caused by the MIL producing. Therefore, we further visualized the culture of incubation by scanning electron microscope (SEM). Intact cell envelope and spherical nanoparticles were observed (Figure S4), suggesting the producing and secretion of MIL by cell.

The MIL was concentrared by the ultracentrifugation from the supernatant. The particle size distributions were obtained by zetaview nanoparticle tracking analyzer (Figure 1c). The average diameter of MIL in the supernatant and the resuspended solution keep constant at 160 nm (with full width at half maximum 132 nm). Bright red color pellet was obtained with liposome fusion while no visible pellet was harvested from the sample without liposomes co-incubation (Figure 1d), indicating that the fusion of liposome with MR-1 cells remarkably increased the production of MIL. In order to identify the combination of cytochromes with MIL, the MIL sample was stained by 3,3'-diaminobenzidine (DAB) for HR-TEM.[22] As shown in Figure 1e, MIL dots showed black stanning color with size around 100 nm, indicating the integration of membrane protein with MIL successfully. Given the resulted liposome contain high concentration of *c*-type cytochrome, our data suggest that MIL would be with the active membrane Cyts protein.

2.2. Identification and characterization of *c*-Cyts protein complex in the MIL

Next, we examined whether the c-Cyts complex (MtrCAB-OmcA) moved from the OM of *S. oneidensis* MR-1 to MIL. The proteins on MIL were further verified by

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (**Figure 2**a). After the Coomassie brilliant blue (CBB) staining, a series of protein bands similar to the OM components reported in MR-1 were found, indicating these outer-membrane proteins of the *S. oneidensis* MR-1 indeed moved to the MIL. Through heme staining, the related protein bands visualized on the gel, demonstrated these representative proteins of MtrC, MtrA, CymA, and OmcA remaining on the MIL. These outer-membrane proteins are crucial for extracellular electron transport (EET) of *S. oneidensis* MR-1.[17,18] Given the CymA does not localize in OM, the formation of MIL may wrapped inside the MIL when fused with MR-1 cell.

To confirm each *c*-Cyt protein was not denatured on MIL, we performed circular dichroism (CD) spectroscopy. A previous study demonstrated that decaheme proteins, MtrC, MtrA, and OmcA, has strong CD signal originated from inter heme exciton coupling among ten hemes.[20] The CD spectroscopy used to analyze MIL containing supernatant incubation of MR-1 with the liposomes (Figure 2b). The CD spectra showed two particular signals at 392 and 417 nm, which is previously assigned to the membrane proteins of MtrCAB-OmcA complex in an intact cell and purified protein.[21] Upon normalizing the signal intensity by heme concentration, the $\Delta\varepsilon$ was comparable with an intact cell of MR-1, indicating that strong inter-heme coupling were maintained in these identified proteins. Its supernatant showed weak signals at the same wavelength due to the residual cells in solution after one-time centrifugation (Figure S5). Accordingly, the CD signals of MR-1 incubated with liposomes significantly decreased to indicate the *c*-type cytochrome proteins transport to MIL (Figure S6). By comparing the CD signal of the cells exposed liposome and MIL, approximately 51.7 % of MtrCAB complexes moved from cell to the liposome. Furthermore, the redox activity was also maintained in these proteins. Figure 2c showed both characteristic peaks of MIL red-shift with around 7 nm under the anaerobic condition to indicate these proteins still have the redox capability even they leak out from cells. The redox state-dependence signal shift of heme proteins was also obtained by UV-vis measurement (Figure S7), demonstrating that both redox activity and inter-heme interactions are maintained even in MIL. Taken together, these data strongly suggest that LIME method efficiently move OM proteins from cell to liposome without activity loss.

2.3. Preserved protein function after the MIL merging into *E. coli*

It was known that the MtrCAB protein complex function as an electric conduit to transport electrons from the cell interior to the exterior solid electrode surface. A recent study reported that the addition of OMVs collected from *Geobacter* sp. contributed to the current producing.[8] In order to investigate the capability of electron transport of protein MtrCAB-OmcA integrated in MIL, we added MIL to *E. coli*. The wild-type *E.*

coli showed lower current producing capacity in the presence of 10 mM glucose with ITO working electrode poised at +200 mV vs Ag/AgCl. After the adding MIL to *E. coli*, 3-fold current amplification at 15 hours than without MIL was observed as expected (Figure 3a) while the presence of MIL only did not show any current production, suggesting that MtrCAB-OmcA complex function in MIL as with native host.

We further confirmed that cytochrome on the MIL was incorporated into the *E. coli* by spectroscopic and electrophoresis method. Upon 2 hours' co-incubation of *E. coli* and MIL, we collected only cells by low-speed centrifugation. The UV-vis spectroscopy of the pellet resuspension suggested that MIL donated cytochrome to *E. coli* after incubation (Figure 3b). Moreover, the specific protein bands such as MtrC and OmcA was detected on the gel of co-incubated *E. coli*, whereas no band for heme protein was obtained in native *E. coli* cells (Figure 3c). This time CymA and MtrA was not observed in *E. coli*'s membrane fraction suggesting the incorporation process would release most of the CymA and MtrA to the supernatant again. We also conducted CD spectroscopy to examine the intra-heme exciton coupling of MIL fused *E. coli.* While no characteristic peak was found from intact *E. coli* in the CD spectrum, the MIL fused *E. coli* revealed a peak at around 410 nm (oxidative condition) (Figure S8). No visible CD signal was measured in the supernatant, demonstrating most of MIL fused into *E. coli* cells and strong multi-heme exciton coupling of cytochromes remained. In general, MIL are able to fuse into the *E. coli*, toward the enhancement in the EET effect.

2.4. Direct lipid tracking for studying LIME mechanism

In order to tract the fusion process of the liposome into the bacteria directly, fluorescent marking method was applied. The 1 % TR-DOPC as a fluorescence probe was mix in liposome to construct the TR-liposome, enabling visualizing the fusion of liposome with bacteria. The fluorescence spectrum reveals a broad emission peak of the TR-liposomes with a maximum emission wavelength at 620 nm under the excitation light at 590 nm, which signified the combination of fluorescence probe and liposome in the preparation of TR-liposome (Figure S9). Once the TR-liposome could be fused with the membrane, the TR-merged MR-1 will have the fluorescent feature (Figure S10). As expected, the fluorescence spectrum showed the emission feature of the TR-merged *S. oneidensis* MR-1 and no emission from crude cells (Figure 4a-b). Taking by the observation in the dark-field fluorescence images, 54.8% of the *S. oneidensis* MR-1 cells exhibited evident emission after incubation with TR-liposomes (**Figure 4**a). No fluorescence signals were observed from cells without TR-liposomes. These results demonstrated that our liposome is able to merge with Gram-negative microbes during the period of liposome-existing incubation. The releasing of MIL from the cell

membrane was proved by staining *S. oneidensis* MR-1 cells with the FM™ 1-43 dye. Figure 4c showed that FM-stained cells exhibited the apparent fluorescence emission to see the contours of the cells clearly. No change of the fluorescence feature in the cells was observed after regular incubation for 20 hours. After the fusion, a significantly different image showing the disappearance of these rod-contour signals from cell body while many fluorescence smaller round shape spots were found after LIME treatment which strongly inferred that outer membrane of MR-1 released from cells to these biological nanoparticles, namely the MIL here.

We have already discovered the transfer of proteins in the LIME process from bacteria to MIL. The lipidomic composition changing analysis would further confirm the transfer of the membrane lipid bilayer part to splice the comprehensive mechanism of LIME. The MR-1 cells originally comprised of 92.87% of PE and tiny amount of PC (0.011%) (Figure 4d). After the fusion with liposome, the ratio of PC and PE in MR-1 cells changed to 1.9% and 84.89%, respectively. The increasing of ratio of PC into MR-1 indicated liposome with 80% DOPC was fused with MR-1 cell. The MIL with PC of 8.52% and PE of 77.91%, more close to liposome, were obtained to evidence the membrane lipid bilayer's reorganization during fusion process. Further analysis was carried out according to the difference in carboxylic acids of same lipid head. The liposome was synthesized by DOPC and DOPE both with C36 carboxylic chain. However, there is C36 carboxylic chain in no original *S. oneidensis* MR-1 without fusion process but C34:1 of 88.34 %, C34:2 of 11.64 % (Figure S11a). After the LIME process, the C34:1/C34:2 as 0.41%/0.25% in PC dramatically decreased in the fused MR-1 cell while a high content of C36:1/C36:2 as 27.13%/68.14% was detected, illustrating that the exogenous lipid inserted into the cell membrane. In MIL, C34:1/C34:2 and C36:1/C36:2 has been found to be 0.36%/0.15% and 28.84%/67.65%, respectively. These ratios are very close to fused MR-1, demonstrating that the membrane source of MIL come from the LIME process. A similar result in the PE type analysis has been found to indicate a significantly high ratio of C36:1 and C36:2 on fused cells and MIL than the primary cells (Figure S11b). Taking by the lipidomic analysis, an unequivocal process of membrane exchange involved lipid migration and reorganization between the liposome and *S. oneidensis* MR-1 has been evidenced.

3. Conclusion

In the present wok, a new method as LIME for harvest of biologically active membrane hybrid vesicles was established. Based on the fusion of liposome with Gram-negative bacteria MR-1, drastically promotion of vesicles production was achieved. Moreover, the MIL harvest from MR-1 cell still exhibited fusion ability to host *E. coli*

cells and the proteins derived from MR-1 remained active for electron transfer, therefore, showing the great potential for real applications. Although the present study only conducted LIME process to two Gram-negative bacteria, *S. oneidensis* MR-1 and *E. coli*, the potential application of our method to other culture microbes or even with eukaryote cells was attractive considering that the producing efficiency of vesicles or exosome was mainly depended on the intrinsic cells characteristics decided by the genome. The LIME method is a novel technique to open a prosperous avenue, which not only shows the feasibility to produce a wide variety of biological vesicles equipped with the particular membrane protein from the interested cell but also reveals the potential in cross-species functional outer-membrane protein transport.

4. Experimental Section/Methods

4.1. Materials: All reagents were analytical purity and used without further purification. Sodium lactate solution ($CH_3CH(OH)COONa$, 70%), ammonium chloride (NH_4Cl, 99.5%), calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$, 95%), sodium bicarbonate ($NaHCO_3$, 99.5%), sodium chloride (NaCl, 99.5%), and magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$, 98%) were bought from Wako. 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES, $C_8H_{18}N_2O_4S$, 99%) was purchased from Dojindo. LB broth (Luria-Bertani, miller), and yeast extract were obtained from BD. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids, Inc. N-(Texas Red sulfonyl) -1,2-dihexadecanoyl-snglycero-3-phosphoethanolamine, triethylammonium salt (Texas Red®-DHPE) was purchased from Biotium. Chloroform ($CHCl_3$, 99%), sodium phosphate dibasic (Na_2HPO_4, 99%), and sodium phosphate monobasic (NaH_2PO_4, 99%) were bought from Sigma-Aldrich. FM™ 1-43 Dye (N-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino) Styryl) Pyridinium Dibromide) was obtained from Invitrogen. Deionized water was obtained by using a Millipore direct-Q water purification system.

4.2. Preparation of DM medium with 10 mM lactate (DM-L): These materials contained 1.25 g of $NaHCO_3$, 0.04 g of $CaCl_2$, 0.5 g of NH_4Cl, 0.1 g of $MgCl_2$, 5 g of NaCl, 3.6 g of HEPES, and 0.25 g of yeast extract were added in 0.5 L of ultrapure water. Then, the solution was autoclaved at 122 ℃ for 25 min to obtain the sterile DM medium. The 0.8 g filtered sodium lactate solution (70%) was added in sterile DM medium to harvest the DM-L medium.

4.2. Preparation of liposome: The extrusion method was applied to prepare the liposomes. The extruder was obtained from Avanti Polar Lipids, Inc. The 100 mg of DOPC and DOPE powder was dispersed in 5 mL of chloroform as lipid stock solutions, respectively. 0.252 mL DOPC and 0.059 mL DOPE were mixed together in a glass bottle, and then the mixture was dried through N_2 gas purging to form the lipid film. Afterward, the 0.8 mL of PBS buffer was added in the bottle, and the sample was fizzed by liquid nitrogen until forming the ice phase. And then, the sample was melted through immersing in a warm bath. The cooling-melting process was repeated several times until the lipid film completely dissolves into the buffer. The polycarbonate membrane with 100 nm pore size was set inside the extractor. And then, the lipid solution filled up the syringe and injected into the extractor for extruding operation at least ten times. Afterward, the liposomes were obtained and stored in refrigerator for future use.

For the preparation of TR dye-loaded liposomes, an adjustive of 1 % Texas Red®-DHPE was added into the lipid mixture and followed same fabrication process.

4.4. Cell culture of S. oneidensis MR-1 and E. coli.: The bacteria were sub-cultured in 15 mL of sterile LB solution for aerobic incubation at 30 °C . After 20 hr, the culture solution was centrifuged at 6000 rpm for 10 min, and then the cell's pellet was dispersed by DM-L. The cells were washed by DM-L at least two times before use in further experiments. UV-vis was used to measure the optical density at 600 nm of bacteria.

4.5. Liposome-induced membrane exchange process for harvesting of MIL: 1 mL of liposomes (20 mg) were added in 20 mL of *S. oneidensis* MR-1 of 1 O.D., and then the sample was sharked in 30 °C incubation for 20 hours. After that, the solution was centrifuged at 6000 rpm for 5 min to obtain the supernatant and it was centrifuged again to remove almost bacteria. Afterward, the supernatant was filtered by the sterile filters with 0.45 um of porous size to remove residual bacteria completely. Then, the solution was centrifuged at 50000 rpm for 4 hr through ultracentrifugation (5000rpm, 2hours) to get the MIL for further characterization and use.

4.6. Electrochemical measurement: The sealed reactor with the three electrodes system was used for related electrochemical measurements. The 5 mL DM-L medium was added into the reactor, then the oxygen in this close system was removed by N_2 purging for 20 min. Afterward, the reactor was connected with the potentiostat at room temperature for further electrochemical analysis. The electrode was poised at a potential of +0.2 V for starting measurement. After 30 min, the 0.1 mL of *E. coli.* suspension was injected into the reactor with a final concentration of 0.8 optical density (OD_{600}). After another 60 min, the 100 μL of MIL (8 mg) or DM-L was added in the reactors

correlated to their experimental group. For the group of MIL alone, the same experiment process was followed, but no bacteria were provided in the reactor.

4.7. Fluorescence imaging for observation of fusion and liberation processes: For the observation of fusion, TR dye-loaded liposome (TR-liposome) were applied with *S. oneidensis* MR-1. After incubation for 20 hours, the sample was centrifuged at 6000 ppm for 5 min to obtain the cell pellet, and re-dispersed in the fresh DM-L. The washing process was conducted at least two times to remove free TR-liposomes. And then, the imaging was observed through a fluorescence microscope with excitation at 590 nm and emission at the range of 620-700 nm.

For the observation of MIL liberation, FM™ 1-43 Dye was used to stain the MR-1 cells in a standard protocol of dye staining. The dye-stained cells were co-incubation with liposomes at 30 °C for 20 hr. Afterward, the sample was dropped on the glass slide for further observation *via* a microscope with excitation at 590 nm and emission detection at a range of 620-700 nm.

Acknowledgements

W.-P. Li and X.-Z. Long contributed equally to this work.

This work was financially supported by a Grant-in-Aid for Research from the Japan Society for the Promotion of Science KAKENHI (Grant No. 17H04969); PRIME, the Japan Agency for Medical Research and Development (19gm6010002h0004); JST, PRESTO (Grant No. JPMJPR19H1), Japan; and the postdoctoral program from Japan Society for the Promotion of Science (Grant No. P20105).

References

[1] C. Schwechheimer, M. J. Kuehn, Outer-membrane Vesicles from Gram-negative Bacteria: Biogenesis and Functions. *Nat. Rev. Microbiol.* **2015**, *13*, 605-619.

[2] A. T. Jan, Outer Membrane Vesicles (OMVs) of Gram-negative Bacteria: A Perspective Update. *Front. Microbiol.* **2017**, *8*, 1053.

[3] K. E. Bonnington, and M. J. Kuehn, Protein Selection and Export via Outer Membrane Vesicles. *Biochim. Biophys. Acta* **2014**, *8*, 1612-1619.

[4] G. Qing, N. Gong, X. Chen, J. Chen, H. Zhang, Y. Wang, R. Wang, S. Zhang, Z. Zhang, X. Zhao, Y. Luo, X.-J. Liang, Natural and Engineered Bacterial Outer Membrane Vesicles. *Biophys. Rep.* **2019**, *5*, 184-198.

[5] J. C. Contreras-Naranjo, H.-J. Wu, V. M. Ugaz, Microfluidics for Exosome Isolation and Analysis: Enabling Liquid Biopsy for Personalized Medicine. *Lab Chip* **2017**, *17*, 3558-3577.

[6] N. L. Syn, L. Wang, E. K.-H. Chow, C. T. Lim, B.-C. Goh, Exosomes in Cancer Nanomedicine and Immunotherapy: Prospects and Challenges. *Trends biotechnol.* **2017**, *35*, 665-676.

[7] P. Martins, D. Machado, T. H. Theizen, J. P. O. Guarnieri, B. G. Bernardes, G. P. Gomide, M. A. F. Corat, C. Abbehausen, J. L. P. Módena, C. F. O. R. Melo, K. N. Morishita, R. R. Catharino, C. W. Arns, M. Lancellott, Outer Membrane Vesicles from Neisseria Meningitidis (Proteossome) Used for Nanostructured Zika Virus Vaccine Production. *Scientific Reports* **2018**, *8*, 8290.

[8] X. Liu, X. Jing, Y. Ye, J. Zhan, J. Ye, S. Zhou, Bacterial Vesicles Mediate Extracellular Electron Transfer. *Environ. Sci. Technol. Lett.* **2019**, *7*, 27-34.

[9] M. Toyofuku, N. Nomura, L. Eberl, Types and Origins of Bacterial Membrane Vesicles. *Nat. Rev. Microbiol.* **2019**, *17*, 13-24.

[10] S. Hirayama, R. Nakao, Glycine Significantly Enhances Bacterial Membrane Vesicle Production: a Powerful Approach for Isolation of LPS-reduced Membrane Vesicles of Probiotic *Escherichia coli*. *Microb. Biotechnol.* **2020**, *13*, 1-17.

[11] I. A. MacDonald, M. J. Kuehn, Stress-Induced Outer Membrane Vesicle Production by *Pseudomonas aeruginosa*. *J. Bacteriol.* **2013**, *195*, 2971-2981.

[12] Microbial BiotechnologyM. Toyofuku, Bacterial Communication through Membrane Vesicles. *Biosci. Biotechnol. Biochem.* **2019**, *83*, 1599-1605.

[13] V. Premjani, D. Tilley, S. Gruenheid, H. L. Moual, J. A. Samis. Enterohemorrhagic *Escherichia coli* OmpT Regulates Outer Membrane Vesicle Biogenesis. *FEMS Microbiol. Lett.* **2014**, *355*, 185-192.

[14] Z. Wang, Y. Ma, H. Khalil, R. Wang, T. Lu, W. Zhao,Y. Zhang, J. Chen, T. Chen, Fusion Between Fluid Liposomes and Intact Bacteria: Study of Driving Parameters and *In Vitro* Bactericidal Efficacy. *Int J Nanomed.* **2016**, *11*, 4025-4036.

[15] C. R. Safinya, K. K. Ewert, Liposomes Derived from Molecular Vases. *Nature* **2012**, *489*, 372-374.

[16] A. E. Gad, G. D. Eytan, Chlorophylls as Probes for Membrane Fusion Polymyxin B-induced Fusion of Liposomes. *Biochim. Biophys. Acta* **1983**, *727*, 170-176.

[17] L. Shi, H. Dong, G. Reguera, H. Beyenal, A. Lu, J. Liu, H.-Q. Yu, J. K. Fredrickson, Extracellular Electron Transfer Mechanisms Between Microorganisms and Minerals. *Nat. Rev. Microbiol.* **2016**, *14*, 651-662.

[18] K. H. Nealson, A. R. Rowe, Electromicrobiology: Realities, Grand Challenges, Goals and Predictions. *Microb. Biotechnol.* **2016**, *9*, 595-600.

[19] T. L. Poulos, Heme Enzyme Structure and Function. *Chem. Rev.* **2014**, *114*, 3919-3962.

[20] Y. Tokunou, P. Chinotaikul, S. Hattori, T. A. Clarke, L. Shi, K. Hashimoto, K. Ishii, A. Okamoto, Whole-cell Circular Dichroism Difference Spectroscopy Reveals an *In Vivo*-specific Deca-heme Conformation in Bacterial Surface Cytochromes. *Chem. Commun.* **2018**, *54*, 13933-13936.

[21] Y. Tokunou, A. Okamoto, Geometrical Changes in the Hemes of Bacterial Surface c-Type Cytochromes Reveal Flexibility in Their Binding Affinity with Minerals. *Langmuir* **2019**, *35*, 7529-7537.

[22] A. M. Seligman, M. J. Karnovsky, H. L. Wasserkrug, J. S. Hanker, Nondroplet Ultrastructural Demonstration of Cytochrome Oxidase Activity with a Polymerizing Osmiophilic Reagent, Diaminobenzidine (DAB). *J. Cell Biol.* **1968**, *38*, 1-14.

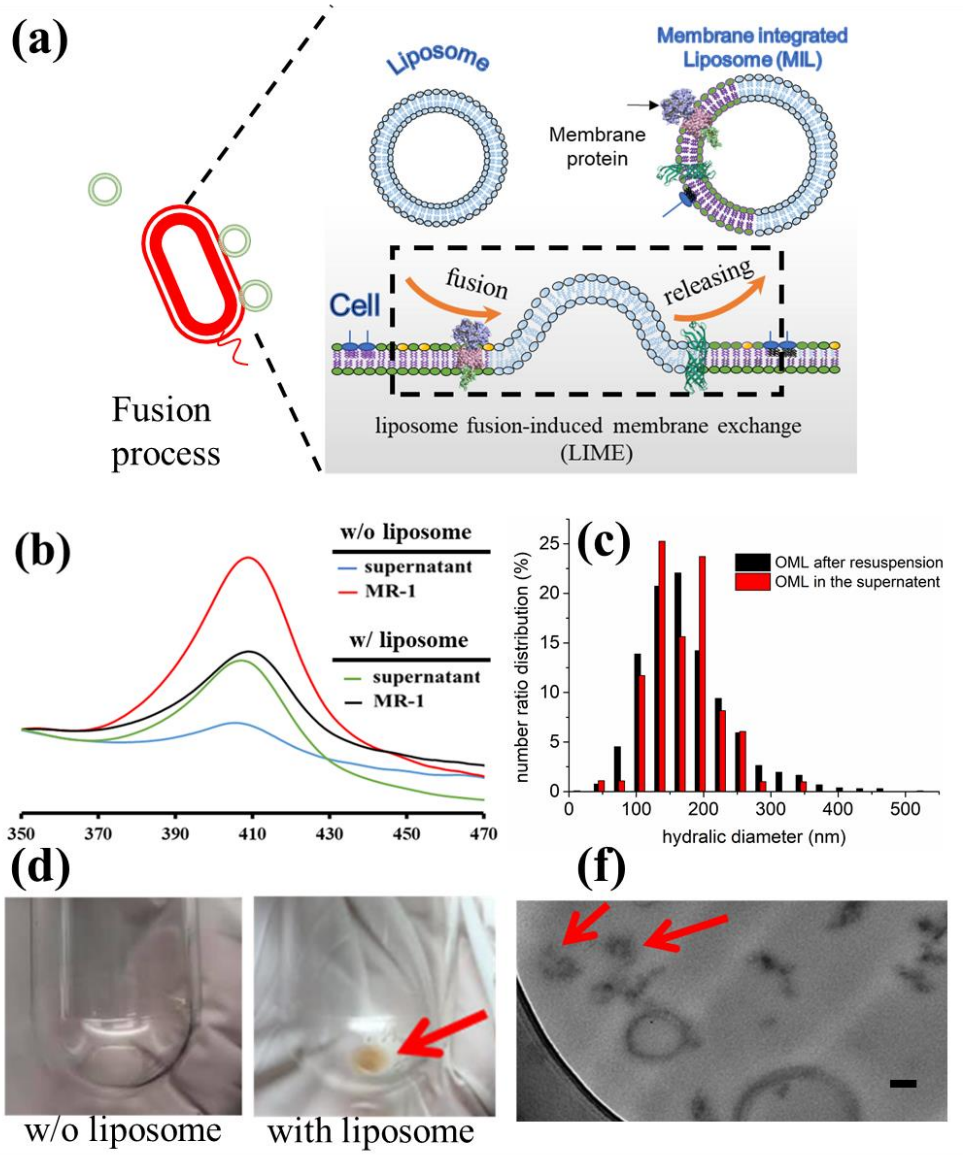


Figure 1. The producing of outermembrane integrated liposome (MIL) by liposome fusion-induced membrane exchange (LIME) process. (a) The schematic of LIME process by the fusion between liposome and *S. oneidensis* MR-1 and then releasing of MIL. (b) The diffusion transmission UV-vis spectra of *S. oneidensis* MR-1 cell and supernatant after incubating with liposome. Supernatant and cells were separated by the centrifugation in 6000 rpm for 10 minutes. (c) The size distribution of MIL in the supernatant and the resuspended solution. The MIL in supernatant was directly injected into Zetaview analyzer. The resuspended MIL sample was obtained after ultra-centrifugation (210,000 g, 2h). (d) The photograph of pellet collected with and without fusion with liposome. (Pellet was labeled by red arrow). No pellet was observed w/o liposome dosage. (f) The HR-TEM image of MIL dots with DAB staining. The scale bar is 100 nm.

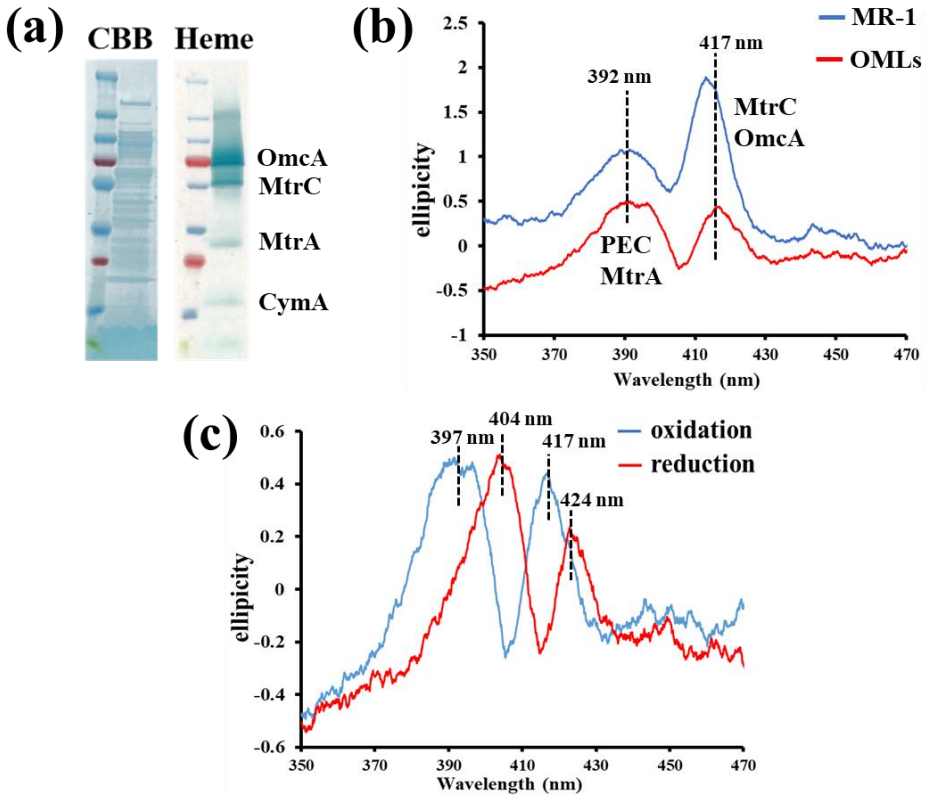


Figure 2. Identification of the heme proteins in MIL. (a) Protein profiles of cellular fraction isolated from MIL, stained with the Coomassie brilliant blue (CBB) and heme-reactive 3,3′,5,5′-tetramethylbenzidine-H_2O_2 (heme staining). (b) Circular Dichroism (CD) spectra of *S. oneidensis* MR-1 and MIL dispersed in defined medium (DM). Samples are collected after 20 hours' incubation. (c) The CD spectra of *S. oneidensis* MR-1 supernatant under oxidative and reductive condition. The supernatant was reduced by adding sodium dithionite (50 mg/3 mL).

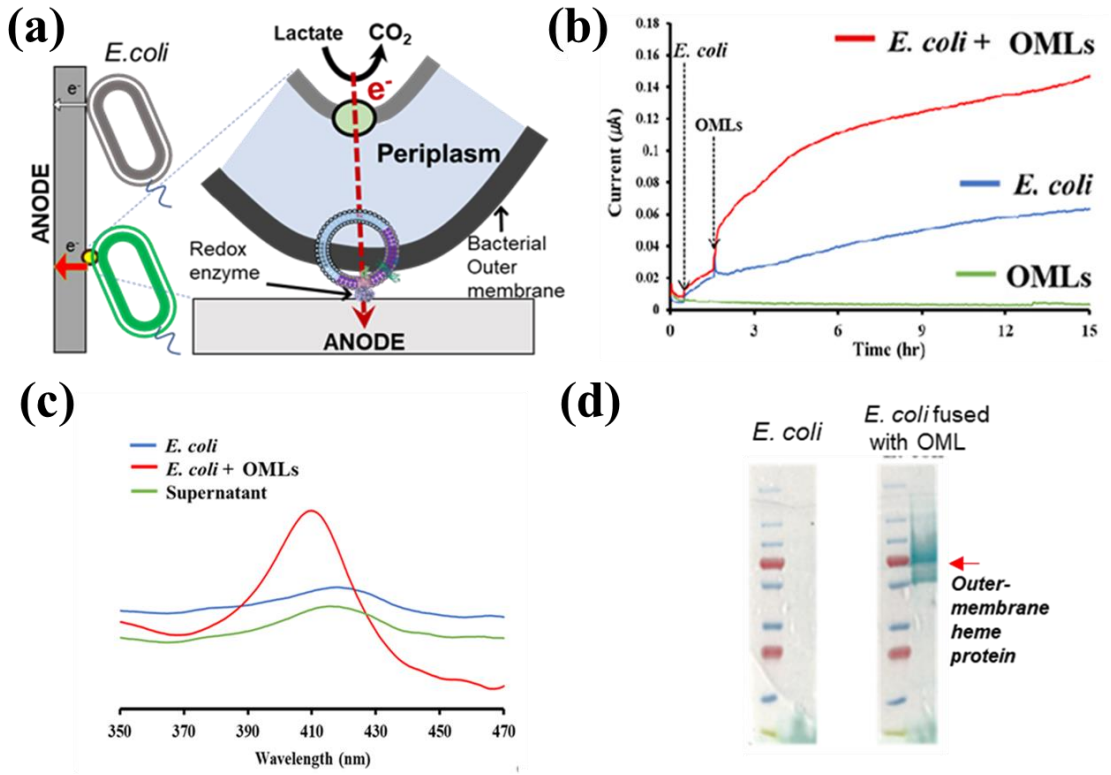


Figure 3. The MIL increasing electron transfer capacity of *E. coli*. (a) The schematic of enhancing of *E. coli*'s electron transfer to electrode by fusing with MIL. (b) The single-potential amperograms of *E. coli*, *E. coli* + MIL and MIL alone at 0.2 V versus the saturated Ag/AgCl reference electrode at 30 °C. The *E. coli* and MIL were added into the electrochemical reactors at 0.5 hr and 1.5 hr, respectively. MIL was collected from 20 mL of *S. oneidensis* MR-1 supernatant after LIME process. (c) The UV-vis spectra of *E. coli*, MIL-fused *E. coli*, and its supernatant. (d) Heme staining of *E. coli* and MIL-fused *E. coli*.

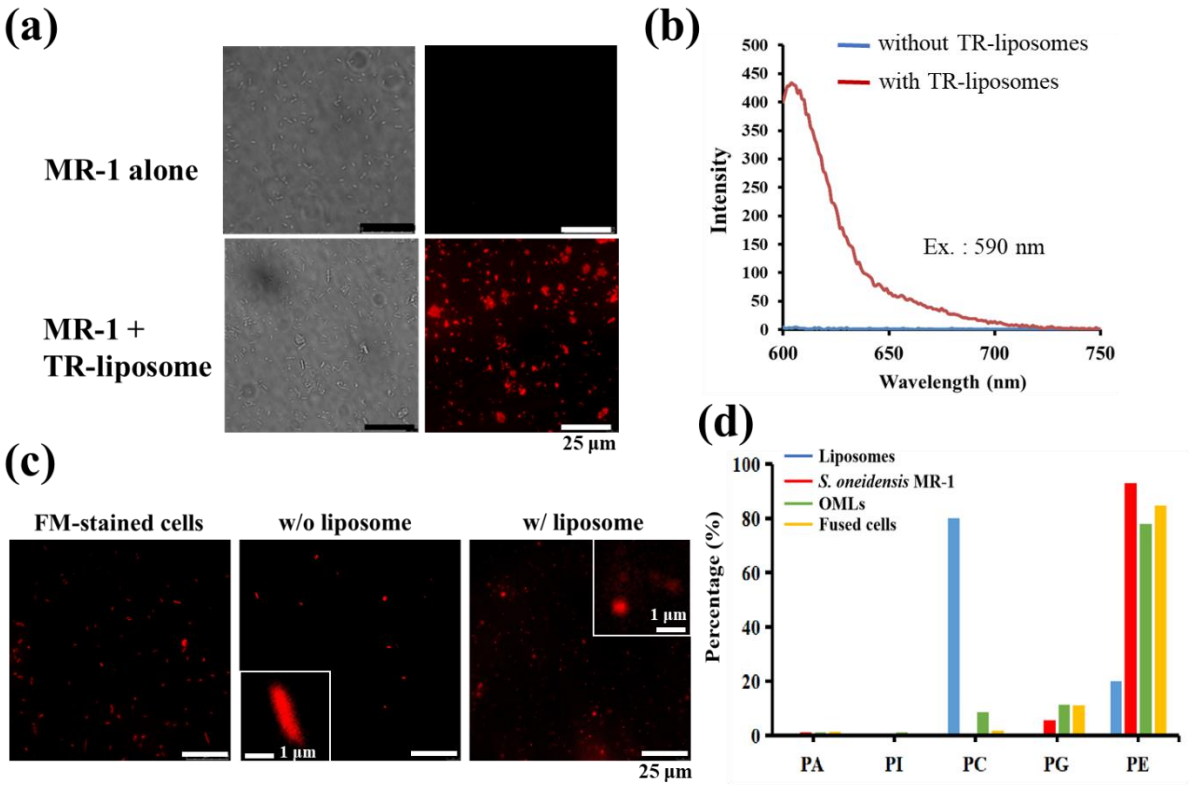


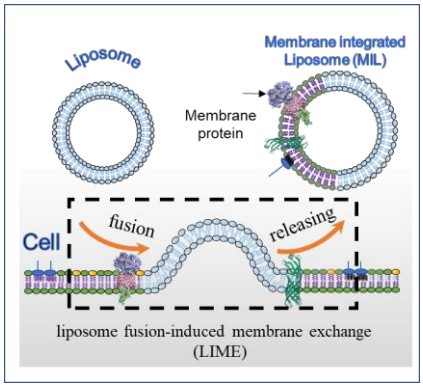
Figure 4. Exploration of LIME mechanism through lipidomic analysis. (a) The bright field and fluorescence images of MR-1 and TR-inserted MR-1. The scale bars were all set as 25 μm for these images. (b) The fluorescence spectra of MR-1 and TR-inserted MR-1. (c) The fluorescence images of FM-stained MR-1 cells before and after the incubation with or without liposome. The inserts on w/o liposome and w/ liposome showed their high magnification images. (d) Lipidomic analysis revealed the percentage of lipid type in liposome, *S. oneidensis* MR-1, MIL, and fused cells.

Novel artificial biological vesicles were produced through an all-new approach of liposome-induced membrane exchange, which was achieved by supplying excessive DOPE-contained liposome in the incubation with *S. oneidensis* MR-1. These outer-membrane-integrated liposomes (MIL) equipped the natural cytochromes involving electron transport. They were fused with *E. coli* to expose the functional protein, resulting in a dramatically enhanced electricity production.

Wei-Peng Li, Xi-Zi Long, Chiho Kataoka -Hamai, and Akihiro Okamoto*

Membrane Integrated liposome Synthesized by a Liposome Fusion-Induced Membrane Exchange

ToC figure



Supporting Information

Membrane Integrated liposome Synthesized by a Liposome Fusion-Induced Membrane Exchange

*Wei-Peng Li, Xi-Zi Long, Chiho Kataoka -Hamai, and Akihiro Okamoto**

Dr. X.-Z. Long, Prof. W.-P. Li, Prof. A. Okamoto
International Center for Materials Nanoarchitectonics, National Institute for Materials Science, Tsukuba, Ibaraki, 305-0044, Japan
E-mail: okamoto.akihiro@nims.go.jp

Prof. A. Okamoto
Graduate School of Chemical Sciences and Engineering, Hokkaido University, Hokkaido 060-8628, Japan

Prof. W.-P. Li
Department of Medicinal and Applied Chemistry, Kaohsiung Medical University, Kaohsiung, 807, Taiwan

Dr. C. Kataoka
Research Center for Functional Materials, National Institute for Materials Science, Tsukuba,
Ibaraki 305-0044, Japan

X.-Z. Long and W.-P. Li contributed equally to this work.

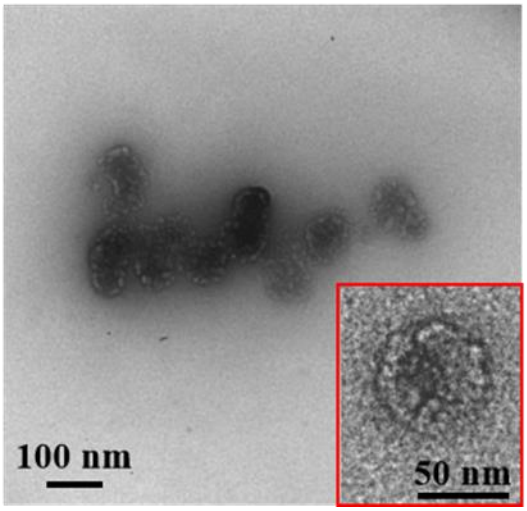


Figure S1. The HR-TEM image of raw liposome. The inset shows the high magnification of single particle image.

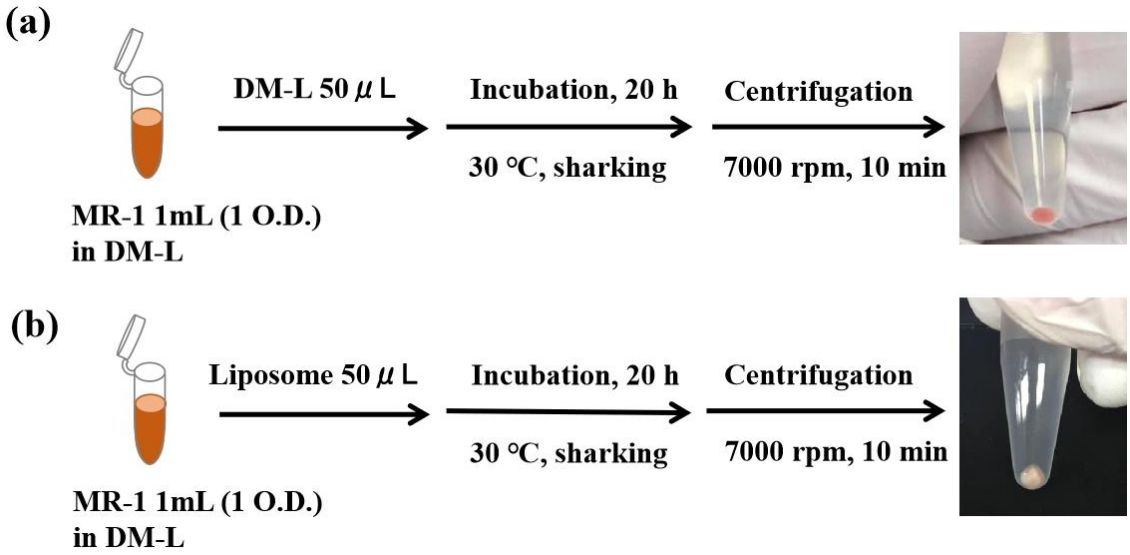


Figure S2. The illustration showed the experimental procedure of LIME. The cell pellet obtained after incubation and centrifugation was shown in the photos.

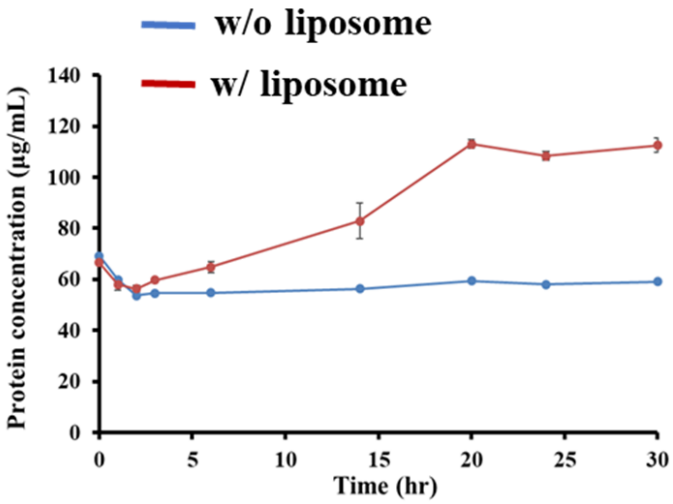


Figure S3. The dynamic of MIL producing under the w/o and w/ liposome incubation. Protein assays were used to quantify the protein concentration in the supernatant at different incubation times.

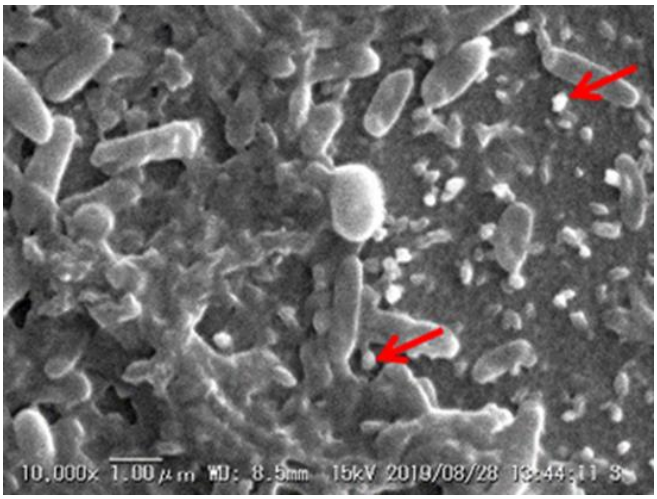


Figure S4. The SEM image of *S. oneidensis* MR-1 dried on the glass after 20 hours incubation with liposome. The red arrows indicate the spherical vesicles evidencing the MIL production.

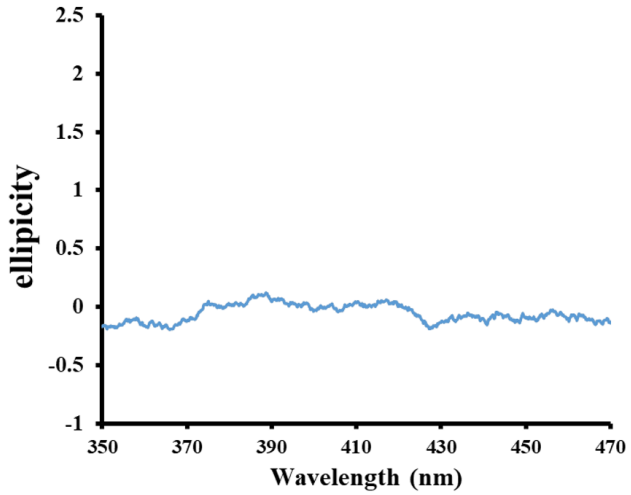


Figure S5. The CD spectrum of supernatant from the culture of *S. oneidensis* MR-1 after the incubation without liposome for 20 hours.

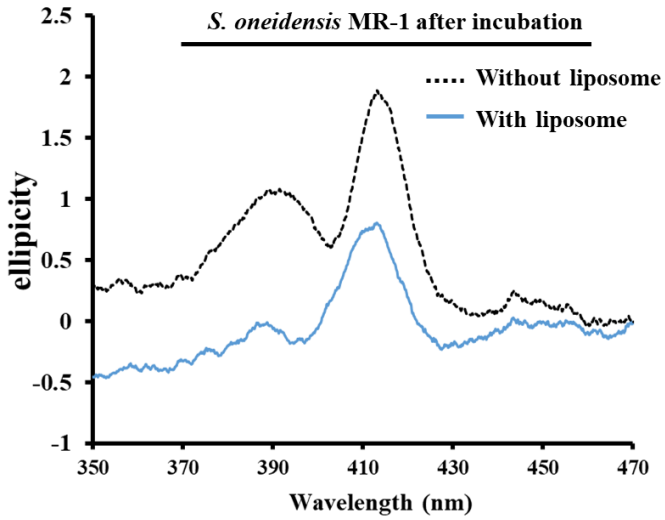


Figure S6. The CD spectra of *S. oneidensis* MR-1 after the incubation for 20 hours with and without liposome.

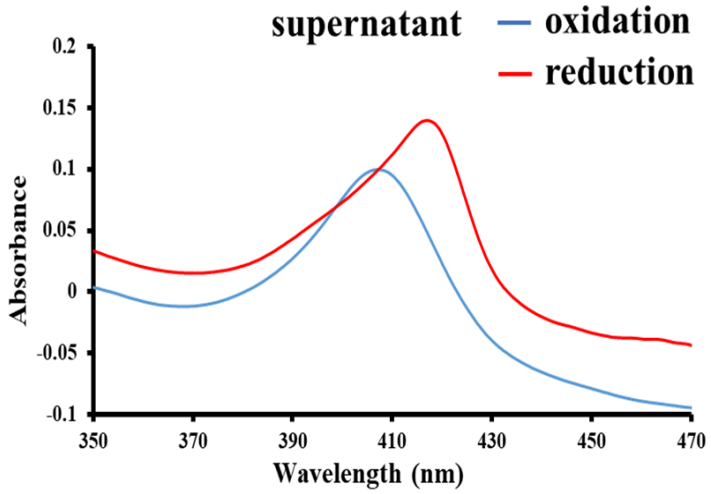


Figure S7. The UV-vis spectra of the supernatant with liposome-contained incubation under the oxidative or reductive condition.

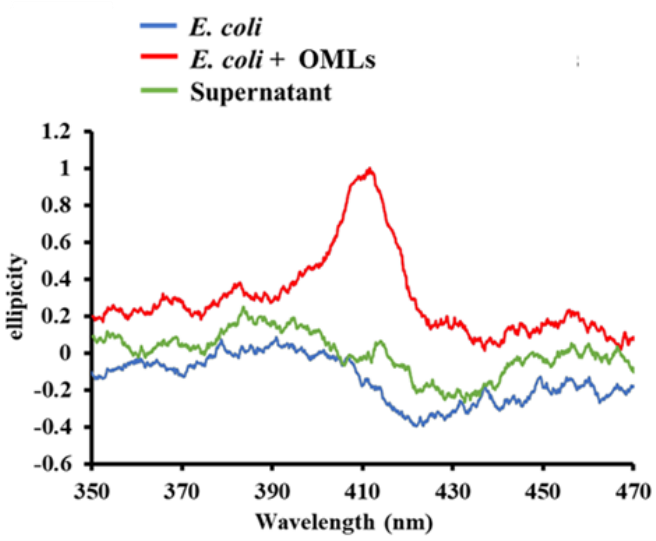


Figure S8. The CD spectrum of *E. coli*, MIL-fused *E. coli*, and its supernatant.

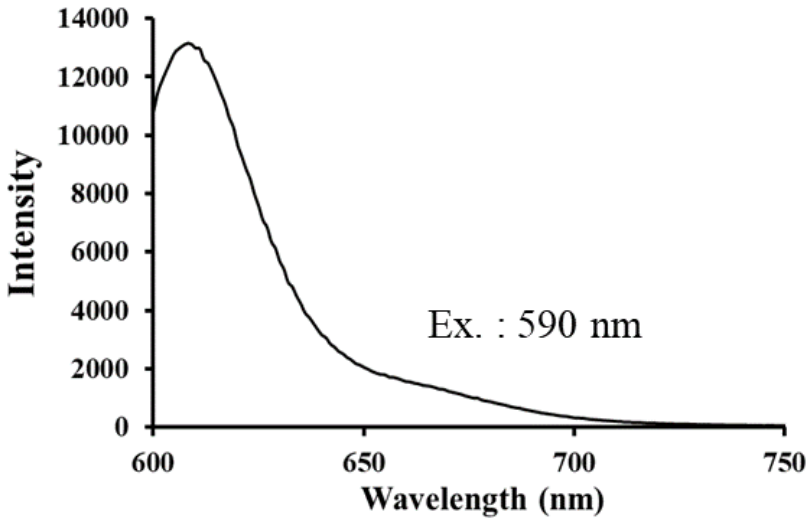


Figure S9. The fluorescence spectrum showed the emission feature of TR-liposome under the excitation at 590 nm.

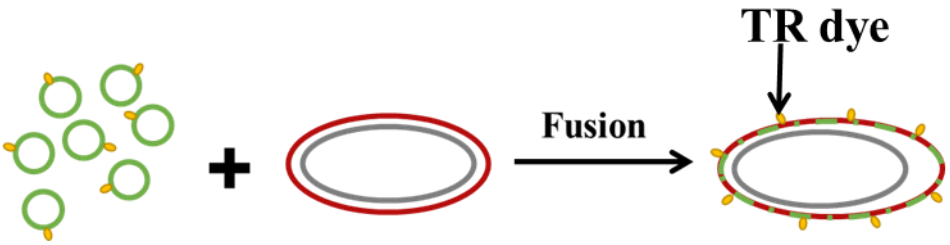


Figure S10. The illustration reveals a fusion process between TR-liposome and *S. oneidensis* MR-1, and the final product of TR-inserted cells.

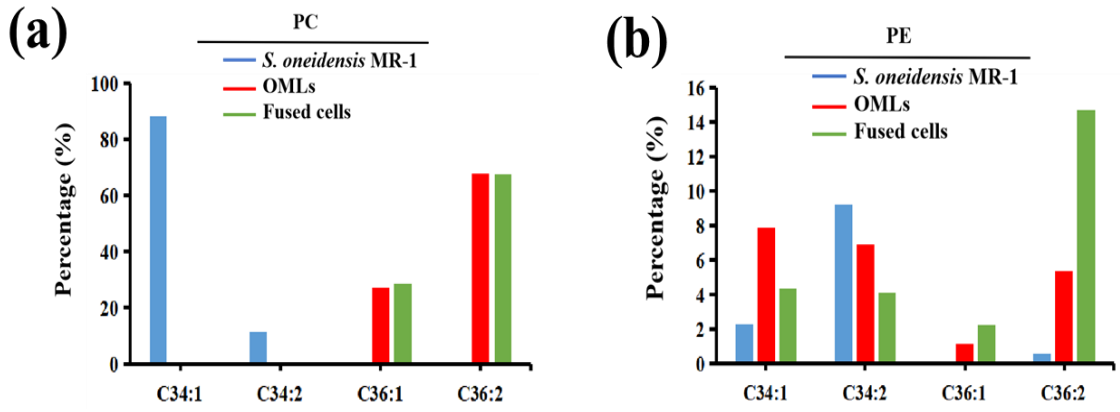


Figure S11. (a, b) Comparing the ratio of lipid types (PC and PE) in *S. oneidensis* MR-1, MIL, and fused cells.

a) Identical percentage of C36:1 and C36:2 indicate that MIL are released from Fused cell surface.

However, in (b), C34:1 and C34:2 are abundant only in MIL, while PC indicated lipids are well mixed in the cell membrane, suggesting that these two lipids may be the key player for releasing MIL from cell surface. Is there any, report of OMV in bacteria which contains C34:1 or C34:2 more? The literature will support our observation in mole