Lipocalin 2, synthesized using a cell-free protein synthesis system and encapsulated into liposomes, inhibits the adhesion of *Porphyromonas gingivalis* to human oral epithelial cells

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Abstract

Background and objective: Lipocalin 2 (LCN2), a glycoprotein expressed in epithelial cells and leukocytes, has an antibacterial effect and plays a role in innate immunity. The delivery of LCN2 encapsulated in liposomes to oral epithelium may be useful to prevent oral infectious diseases. This study aimed to investigate the inhibitory effect of LCN2, artificially synthesized using a cell-free protein synthesis (CFPS) system, on the adhesion of *Porphyromonas gingivalis* to oral epithelial cells in order to approach oral healthcare using LCN2.

Methods: LCN 2 was synthesized using a CFPS system and assayed by Western blotting, mass spectrometry and enzyme-linked immunosorbent assay (ELISA). The bilayer liposomes were prepared by the spontaneous transfer method using 1,2-dioleoyl-sn-glycero-3 phosphocholine (DOPC), 3-sn-phosphatidylcholine from Egg Yolk (Egg-PC), and 1,2-dioleoyl-sn-glycero-3 phosphoethanolamine (DOPE). The cellular and medium fractions derived from the culture of oral epithelial cells with liposome-encapsulated LCN2 were assayed by Western blotting and ELISA. The effect of the synthesized LCN2 on adhesion of the labeled *P. gingivalis* to oral epithelial cells was investigated as an evaluation of its antibacterial activity.

Results: The synthesized LCN2 protein was identified by Western blotting; its amino acid

sequence was similar to that of recombinant LCN2 protein. The additions of DOPE and octa-arginine in the outer lipid-layer components of liposome significantly increased the delivery of liposomes to epithelial cells. When oral epithelial cells were cultured with the synthesized and liposome-encapsulated LCN2, LCN2 was identified in the cellular and medium fractions by Western blotting and its concentration in the cellular fraction from the culture with the synthesized LCN2 was significantly higher than that of a template DNA-free protein. The synthesized LCN2 and liposome-encapsulated LCN2 significantly inhibited the adhesion of *P. gingivalis* to oral epithelial cells compared with template DNA-free protein.

Conclusion: LCN2 was artificially synthesized by a CFPS system, encapsulated in liposomes and delivered to oral epithelial cells, and demonstrated an antibacterial action against *P. gingivalis*. This approach may become a useful model for oral healthcare.

1 | INTRODUCTION

Oral healthcare plays an important role in preventing systemic diseases as well as oral diseases such as periodontal diseases, dental caries and infectious mucosal diseases.[1,2] Tooth brushing is an integral part of oral healthcare, especially among elderly individuals, that prevent infectious oral and systemic diseases.[3] Plaque control via tooth brushing is difficult for elderly individuals with physical handicaps and for those whose hands are disabled. Although chemical components in mouth rinses, toothpaste, and gels are partially useful in maintaining oral health, the suitable use of these dental materials is complicated for elderly individuals. Antibacterial drugs/antibiotics and anti-inflammatory drugs are at times used for treatments of periodontal diseases, however, the overuse of these drugs is not suitable as elderly individuals often take multiple medicines for other systemic diseases.[4]

Antimicrobial peptides (AMPs), including lipocalin 2 (LCN2), β-defensin 2 (BD-2) and secretory leukocyte protease inhibitor, are expressed in oral epithelial cells and play an important role in innate immunity of the oral cavity.[5,6] LCN2 is a secreted glycoprotein with a molecular weight of 24-25 kDa, and produced by leukocytes, macrophages and epithelial cells.[6,7] The secreted LCN2 binds to its receptor (24p3R) that is expressed in neutrophils, fibroblasts, retinal and ovarian epithelial cells, and shows

multiple physiological functions such as antibacterial activity, modulation of inflammatory responses and cell migration.[6,8-12] LCN2 binds to bacterial siderophores and depletes iron/heme, a condition required for bacterial growth; thus, it suppressed the growth of *Escherichia coli*,[13,14] and combated against *Klebsiella pneumoniae* in mice liver,[15] suggesting that LCN2 has a bacteriostatic action and plays a role in protection against infectious diseases.

Several cell-free protein synthesis (CFPS) systems have been derived from *E. coli*, wheat embryos, rabbit reticulocytes, yeast and insect cells, and contain the whole cell lysates from several cells.[16-19] In contrast, the PURE system is a reconstituted CFPS system and contains T7 RNA polymerase, nucleoside triphosphates (NTPs), aminoacyl-tRNA synthetases, amino acids for transcription, ribosome, several initiation factors, elongation factors, release factors, ribosome recycling factor for translation and enzymes system to regenerate ATP and GTP for energy regeneration.[20] The PURE system contains the proteins and ribosomes that are individually purified from *E. coli* at a high level, but not the whole cell lysates, and the reactive solution without specific factors can be prepared using the PURE system.[21] The synthesized proteins are scarcely degraded because the PURE system does not contain nucleases, proteases, and the factors that are not associated with protein synthesis.[21] Several proteins, including green fluorescent

107 protein, β-galactosidase and immunoglobulin-G, G-protein on lipid bilayer and
108 granulocyte-macrophage colony-stimulating factor, have been synthesized using several
109 CFPS systems.[22-24] However, there were few reports that AMPs were synthesized using
110 CFPS systems, and LCN2 has never been synthesized using any CFPS system in the past.

111 Liposomes are vesicles of lipid bilayers with hydrophilic and hydrophobic parts
112 and are classified according to their vesicle size and construction of uni-lamellar and
113 multi-lamellar.[25,26] They can be prepared using the methods such as spontaneous transfer,
114 centrifugation, electro-formation and microfluidics,[27,28] and encapsulate peptides/
115 proteins, RNA, DNA and several chemical drugs.[26,29] Some liposomes have parts that are
116 bound to cells on their vesicle membrane and have constructions that are similar to
117 cellular membrane.[30,31] Due to these characteristics, liposomes have been studied as a
118 drug delivery system (DDS) for treatments of diseases such as tumors, infectious diseases,
119 rheumatoid arthritis, cystic fibrosis and periodontal diseases.[32,33] Peptides/proteins,
120 including albumin, epidermal growth factor, calcitonin and insulin have been
121 encapsulated into liposomes and delivered orally.[26] Giant liposomes with a diameter of
122 approximately 1-100 μm are similar to biological cells,[25] and prepared using two
123 phospholipids with mineral oil at the oil/water interface by spontaneous transfer
124 method.[28] CFPS systems and DNA plasmid coding for connexin-43 enhanced green

fluorescent protein (Cx43-EGFP) or microRNAs for yellow fluorescent protein (YFP) were encapsulated into giant liposomes, and Cx43-EGFP and YFP proteins were synthesized in those liposomes,[29,34] suggesting that specific proteins are synthesized by CFPS system in giant liposomes.

We have been studying AMPs that are produced by human oral epithelial cells,[5] and aim to create a foundation for oral healthcare using AMPs. In the present study, we investigated LCN2 synthesis using the CFPS system and efficient delivery of LCN2 encapsulated into giant liposomes to oral epithelial cells and its inhibitory effect on the adhesion of *P. gingivalis* to oral epithelial cells.

2 | MATERIALS AND METHODS

2.1 | Materials

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3 phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3 phosphoethanolamine-N-lissamine rhodamine B sulfonyl (Rhodamine-DOPE) were purchased from Avanti Polar Lipid, Inc. (Alabaster, AL). Mineral oil and 3-sn-Phosphatidylcholine from Egg Yolk (Egg-PC) were purchased from FUJIFILM Wako PURE Chemical Co. (Osaka, Japan). Lissamine™ Rhodamine B 1,2-Diphytanoyl-sn-glycero-3-phosphoethanolamine,

143 Triethylammonium salt (Rhodamine DHPE) and (N-(7-Nitrobenz-2-Oxa-1,3-diazol-4-
144 yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, Triethylammonium salt
145 (NBD-PE) were purchased from Thermo Fisher Scientific Japan (Tokyo, Japan).
146 CytoTell^{TM} UltraGreen was purchased from AAT Bioquest Inc. (Sunnyvale, CA, USA).
147
148 **2.2 | LCN2 synthesis using CFPS system**
149 LCN2 peptide was synthesized according to the instruction manual for PUREfrex$^{®}$2.1
150 (GeneFrontier, Chiba, Japan). In brief, the template DNA prepared for LCN2 protein
151 synthesis, cysteine, glutathione, amino acids, NTPs, tRNA, ribosome and translation
152 factors in the kit were mixed at a rate indicated in the instruction manual, and incubated
153 at 37°C for 6 h. The template DNA for LCN2 protein synthesis was prepared by two PCR
154 procedures. The total cDNA was synthesized by a reverse transcription reaction from
155 human bone marrow RNA (TaKaRa Bio Inc., Shiga, Japan) using DNase I Amplification
156 Grade (Invitrogen, Life Technologies Corp./Thermo Fisher Scientific, Carlsbad, CA,
157 USA) and PrimerScript® II 1st strand cDNA Synthesis Kit (TaKaRa Bio). The first PCR
158 was performed using cDNA, the forward primer, reverse primer for LCN2 and
159 PrimeSTAR Max DNA Polymerase (TaKaRa Bio) at 98°C for 10 s, 56°C for 5 s and 72°C
160 for 5 s for 35 cycles. The product of first PCR was purified using QIAquick Gel Extraction

Kit (QIAGEN) to prepare a template DNA for the second PCR reaction. The second PCR was performed using this template DNA, the forward primer containing T7 promoter lesion and ribosome binding site in an up-stream of initiation codon for LCN2, the reverse primer and PrimeSTAR Max DNA Polymerase (TaKaRa Bio) at 98°C for 10 s, 56°C for 5 s and 72°C for 5 s for 35 cycles. The second PCR product was purified using QIAquick Gel Extraction Kit and used as the template DNA for LCN2 protein synthesis. The sequences of the forward and reverse primers used in the present study were as follows: the forward primer with AT-rich sequence: 5'-AAG GAG ATA TAC CA ATG AGT ACT AGT GAT TTA ATT CCA GCA CCA CCT CTG AGC-3', the reverse primer: 5'-GGA TTA GTT ATT CA TCA GCC GTC GAT ACA CTG GTC-3', and T7PRO-SD primer: 5'-GAA ATT AAT ACG ACT CAC TAT AGG GAG ACC ACA ACG GTT TCC CTC TAG AAA TAA TTT TGT TTA ACT TTA AGA AGG AGA TAT ACC A-3'. The total amount of synthesized proteins was determined using TaKaRa BCA Protein Assay Kit (TaKaRa Bio).

2.3 | Sodium dodecyl sulfate (SDS)-Polyacrylamide gel electrophoresis (PAGE) and Western blotting

The protein samples were prepared via the syntheses of LCN2 with or without template

179 DNA using PURExpress®2.1 kit. The synthesized LCN2 (total protein, 70 μg), template
180 DNA-free protein (total protein, 70 μg) and recombinant LCN2 protein (2 μg, Abcam,
181 ab243270, Cambridge, MA, USA) were electrophoretically separated on SDS-
182 polyacrylamide gel (4-20%, Bio-Rad, CA, USA), and stained by Coomassie Brilliant
183 Blue (Quick-CBB PLUS, FUJIFILM Wako Pure Chemical) for 1 h and de-stained by
184 distilled water for 1 h.

185 In Western blotting of LCN2,[12] the synthesized LCN2 protein (total protein, 1 μg),
186 template DNA-free protein (total protein 1 μg) and recombinant LCN2 protein (15 ng)
187 were electrophoretically separated on SDS-polyacrylamide gel (4-20% or 15%) and
188 transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon®-P, Merck
189 Millipore Ltd., Cork, ILR), or using Trans-Blot Turbo Transfer System® (Bio-Rad). The
190 PVDF membrane with LCN2 protein was blocked in Tris-buffered saline (TBS)
191 supplemented with Tween 20 (TBS-T) and 2% bovine serum albumin (BSA) for 1 h at
192 room temperature and was washed in TBS-T and immuno-reacted with anti-LCN2
193 antibody (1/2000 dilution, LSBio, TE Huissen, The Netherlands) in TBS-T-0.1%BSA
194 overnight at 4°C. After washing, the membrane was reacted with horseradish peroxidase
195 (HRP)-conjugated anti-rabbit IgG (1/10000 dilution, GeneTex) in TBS-T-0.1%BSA for 1
196 h at room temperature. An immune signal was developed on a membrane by ECL™

197 Western Blotting Detection Reagents (GE Healthcare) and visualized using Image Quant
198 LAS 500 (GE Healthcare). In another experiment, liposome-encapsulated LCN2 protein
199 was analyzed by Western blotting. Furthermore, human oral epithelial cells (TR146 cell)
200 were cultured with liposome-encapsulated LCN2 protein, and its cell lysates (cellular
201 fraction) were dissolved in RIPA Lysis Buffer containing protease inhibitor cocktail and
202 sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and incubated
203 on ice for 15 min. The medium fraction was collected and mixed with
204 phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich). The cellular and medium
205 fractions were separated using SDS-PAGE and transferred to PVDF membranes, and
206 analyzed by Western blotting for LCN2.
207
208 **2.4 | Mass spectrometry analysis**
209 The synthesized LCN2 protein (total protein 820 μg) and recombinant human LCN2
210 protein (5 μg, Abcam) were electrophoretically separated on SDS-polyacrylamide gel
211 (15%) and stained using Quick-CBB PLUS (FUJIFILM Wako Pure Chemical). The gel
212 fragments of LCN2 in the synthesized protein sample and recombinant LCN2 protein
213 were cut and digested with trypsin (4 μg/mL) at 37°C overnight according to a previous
214 method.[35] The digested samples were injected into UltiMate 3000 RSLCnano system with

215 Acclaim PepMap RSLC Nano Column (75 mM x 15 cm, Thermo Fisher Scientific Inc.)
216 and separated under a gradient solvent flow between 0.1% formic acid and 80%
217 acetonitrile/0.1% formic acid for 60 min at a flow rate of 300 nl/min. The samples were
218 analyzed using Orbitrap Elite (Thermo Fisher Scientific Inc.), and data were analyzed
219 using Mascot (Matrix Science).
220
221 **2.5 | Liposome preparation**
222 Liposomes were prepared by a spontaneous transfer method with DOPC and Egg-PC
223 according to the modified method reported by Hamada *et al.*.[36] In brief, 1 mM DOPC and
224 0.454 mM Egg-PC were dissolved in chloroform/methanol (2:1 v/v), dried up by nitrogen
225 gas, and re-dissolved in mineral oil and sonicated at 50°C for 1 h. In a tube, 1 mM DOPC
226 was overlaid on 0.5 M glucose solution and incubated for 2 h at room temperature. The
227 volume ratio of DOPC and glucose solution was 0.7:1. In microscopic observation of
228 liposomes, NBD-PE (0.63 mM, Thermo Fisher Scientific) was added to the components
229 of outer lipid-layer with DOPC, and rhodamine-DHPE (0.23 mM) was present in the
230 components of inner lipid-layer with Egg-PC. In another experiment, DOPE with or
231 without rhodamine-DOPE (0.77 mM) and stearyl octa-arginine (STR-R8, 0.1 mM, Life
232 Tein, NJ, USA), a cell-penetrating peptide (CPP), were added to the components of outer

lipid-layer with DOPC. 0.454 mM Egg-PC and 0.5 M sucrose solution were mixed and emulsified, and its volume ratio was approximately 19:1. Water-in-oil droplets were prepared from Egg-PC and sucrose solution and overlaid on the DOPC, DOPE and glucose solution phase to prepare liposomes enclosed by phospholipid bilayers. The prepared liposomes were observed using an inverted fluorescence microscope (ECLIPSE Ti-U, Nikon, Tokyo, Japan) at 485/535 nm (excitation/emission) and 560/580 nm (excitation/emission).

2.6 | Delivery assay of liposomes to oral epithelial cells

Human oral epithelial cell line (TR146 cell) was seeded in a 96-well black plate (Sumitomo Bakelite, Tokyo, Japan) and an 8-well chamber slide (NUNC, Rochester, NY, USA) at a cell density of 0.9 x 10^4 cells/cm^2 and cultured in Ham's F-12 medium (FUJIFILM Wako Chemical) supplemented with 10% fetal bovine serum and penicillin and streptomycin for 5 days. The delivery assay of liposomes was performed according to the modified method reported by Takara *et al.*.[37] Briefly, the cells were incubated with the liposome that contained DOPC, DOPE with or without rhodamine-DOPE (1 mol%), and Egg-PC, or STR-R8 (0.1 mM) for 3 h and then washed in phosphate-buffered saline (PBS) and observed using a phase-contrast and fluorescence microscopies (Nikon) at

560/580 nm (excitation/emission). The fluorescence intensity derived from rhodamine-DOPE in liposomes was measured using a fluorescence micro-plate reader (TECAN Infinite® M200Pro, Switzerland) at 555/575 nm (excitation/emission) to evaluate a delivery of liposomes to oral epithelial cells.

2.7 | Encapsulation of LCN2 into liposomes

The synthesized LCN2 protein was concentrated by a lyophilization, dissolved in 0.5 M sucrose solution, and mixed well with Egg-PC to prepare an emulsion for 30 s. The water-in-oil droplets containing LCN2 were overlaid on the 1st-phase that was composed of DOPC, DOPE and glucose solution, and incubated at 4°C for 30 min according to the modified method reported by Hamada *et al.*.[36] The liposome-encapsulated LCN2 was collected by an ultra-filtration (Amicon Ultra-0.5 Centrifuge Filter Devices, 3K) and used to evaluate an encapsulation of LCN2 into liposomes, a delivery to oral epithelial cells, and an effect on adhesion of *P. gingivalis* to oral epithelial cells.

2.8 | Enzyme-linked immunosorbent assay (ELISA)

The amount of LCN2 protein synthesized was determined using Human Lipocalin-2/NGAL Immunoassay (R&D Systems, Minneapolis, MN, USA) in accordance with the

manufacturer's instructions. In another experiment, oral epithelial cells were cultured with liposomes with or without the synthesized LCN2 protein for 12 h. After removing the medium and washing the cells in PBS, the cell lysates were prepared using RIPA Lysis Buffer containing protease inhibitor cocktail and sodium orthovanadate (Santa Cruz Biotechnology). The amount of LCN2 in the cell lysates was determined using LCN2 ELISA kit.

2.9 | Adhesion assay of *P. gingivalis* to oral epithelial cells

The adhesion assay of *P. gingivalis* to oral epithelial cells was performed according to the modified version of the method used by Zanaboni *et al.*.[38], and the concentrations of the synthesized LCN2, the liposome-encapsulated LCN2 and recombinant LCN2 were decided on the basis of results of preliminary experiments (Supplemental data 1). Briefly, epithelial cells were seeded at a cell density of 2 x 10^4 cells/cm^2 in a 96-well black plate (Sumitomo Bakelite) and cultured for 5 days, followed by culture with the synthesized LCN2 protein (LCN2 concentration:100 ng/mL, total protein concentration:3.56 μg/mL), template DNA-free protein (total protein concentration:3.56 μg/mL) and recombinant LCN2 (10 ng/mL) for 16 h. *P. gingivalis* (ATCC33277) was anaerobically cultured in a brain heart infusion (BHI, BD Bioscience, Franklin, Lakes, NJ) broth containing hemin

(5 μg/mL) and menadione (1 μg/mL) for 24 h and washed in PBS, and labeled with Cyto Tell UltraGreen (AAT Bioquest Inc., Sunnyvale, CA, USA) for 15 minutes at 37°C in the dark. The labeled *P. gingivalis* was washed and seeded at 2.5 x 10^6/well at 100 of a multiplicity of infection (MOI) and incubated with the cells pre-treated with the synthesized LCN2 protein, template DNA-free protein and recombinant LCN2 for 2 h at 37°C. After washing the cells and bacteria in PBS, a fluorescence intensity of the bottom surface of well was determined using a fluorescence microplate reader (TECAN) at 485/535 nm (excitation/emission) to evaluate an adhesion of bacteria to oral epithelial cells. In another experiment, oral epithelial cells and *P. gingivalis* were individually pre-cultured with the liposome-encapsulated LCN2 protein (LCN2 concentration:300 ng/mL, total protein concentration:10.7 μg/mL), template DNA-free protein (total protein concentration:10.7 μg/mL) and only liposomes (Control) for 16 h. Subsequently, the pre-treated cells and bacteria were co-incubated at 37°C for 2 h. After washing the cells and bacteria in PBS, a fluorescence intensity of the culture well was determined using a fluorescence microplate reader (TECAN) at 485/535 nm (excitation/emission).

3 | RESULTS

3.1 | Identification of the synthesized LCN2 and mass spectrometry analysis

305 The proteins were synthesized using the reaction mixture with and without a template
306 DNA of LCN2 using a CFPS system. Total protein concentration of the two synthesized
307 protein samples were 7.22 and 7.02 mg/mL, respectively. 70 μg of the synthesized
308 proteins were analyzed by SDS-PAGE. Both samples contained some proteins, and the
309 specific band with approximately 23 kDa molecular weight was detected in the protein
310 sample synthesized with a template DNA of LCN2, and the position of this band was
311 similar to that of a recombinant LCN2 protein (Figure 1A). However, this protein band
312 with 23 kDa molecular weight was not detected in the sample without the template DNA.
313 When the synthesized proteins were analyzed by Western blotting using a specific anti-
314 LCN2 antibody, the immuno-response bands were detected in the protein samples that
315 were synthesized with the template DNA of LCN2 and recombinant LCN2 protein, but
316 not in the sample synthesized without the template DNA (Figure 1B).
317 The fractions of synthesized LCN2 protein and recombinant LCN2 protein were
318 investigated by a mass spectrometry analysis. LCN2 protein was detected in the
319 synthesized LCN2 and recombinant LCN2 samples. The Mascot score and the number of
320 detected peptides in the synthesized LCN2 sample were 1677 and 141, respectively, and
321 those of recombinant LCN2 were 5632 and 1059, respectively (Table 1). The coverage of
322 amino acid sequences of the synthesized LCN2 protein and recombinant LCN2 protein

323 over the amino acid sequence of LCN2 protein on the SwissProt data base was 74.7% and
324 82.3%, respectively.
325
326 **3.2 | Characteristic of the prepared liposomes and delivery of liposomes to oral**
327 **epithelial cells**
328 Liposomes with phospholipid bilayer were prepared from a first water-in-oil phase of
329 DOPC-glucose and a second water-in-oil phase of Egg-PC-sucrose by a spontaneous
330 transfer method (Figure 2A). These liposomes had various sizes that their diameters
331 ranged from approximately 1-180 μm (Figure 2B). When NBD-PE (green color) was
332 added to the outer lipid-layer component and rhodamine-DHPE (red color) was added to
333 the inner lipid-layer component, the outer layer was stained green, and the inner layer was
334 stained red (Figures 2C and 2D), suggesting that these liposomes had a bilayer structure
335 (Figure 2E).
336 When oral epithelial cells were cultured with liposomes containing rhodamine-
337 DOPE (Figure 3A), fluorescent dots were observed on the culture of oral epithelial cells
338 (Figure 3B), however, no fluorescent dots were observed in the cell culture without
339 rhodamine-DOPE (Figure 3C and 3D), showing that liposomes were delivered to oral
340 epithelial cells. When DOPE (0-20 mol%) with or without rhodamine-DOPE (1 mol%)

was added to the outer lipid-layer components composed of 80-100 mol% DOPC, a delivery of liposomes to the cells increased in a dose-dependent manner between 1-20 mol%, and the activity of its delivery was significantly higher at 5, 10 and 20 mol% of DOPE (Figure 3E). The delivery of liposomes to oral epithelial cells increased to approximately 10 % when DOPE (10 mol%) was added to the outer lipid-layer component (90 mol% of DOPC). The delivery of liposomes was further increased when STR-R8 (0.1 mM), a cell-penetrating peptide, was added to the outer lipid-layer component and compared with that of the STR-R8-free outer lipid-layer (P<0.05, Figure 3F).

3.3 | Encapsulation of the synthesized LCN2 into liposomes and its delivery to oral epithelial cells

According to the protocol (Figure 4A), the synthesized LCN2 sample (Figure 4, Sample 1) was concentrated by a lyophilization (Sample 2) and encapsulated into liposomes (Sample 3). The liposomes with LCN2 protein were collected by an ultrafiltration (Sample 4 and 5), and the LCN2 protein of each sample fraction was analyzed by Western blotting. LCN2 protein was detected in the liposome-encapsulated LCN2 sample (Figure 4B, Sample 3) and the liposome fraction collected by an ultrafiltration (Sample 4),

however, it was not detected in the flow-through fraction of ultrafiltration (Sample 5). These results showed that the synthesized LCN2 protein was encapsulated into liposomes.

Oral epithelial cells were cultured with the liposome-encapsulated LCN2, and LCN2 protein in the cell culture was investigated by Western blotting and ELISA (Figure 5A). Immuno-signal of LCN2 protein was detected in the cellular and medium fractions (Figure 5B, Sample 1-6), and this signal was similar to those of LCN2 in liposomes before cell culture (Sample 7) in the conditioned medium of oral epithelial cells (Sample 8) and recombinant LCN2 protein (Sample 9). LCN2 immuno-signals in the cellular and medium fractions from the culture with liposome-encapsulated LCN2 (Sample 3 and 6) were stronger than those of the controls that contained only liposomes without LCN2 protein (Sample 1 and 4) and liposome-encapsulated protein that was synthesized with template DNA-free protein (Samples 2 and 5). The amount of LCN2 protein in the cellular fraction was determined by ELISA to investigate an amount of synthesized LCN2 protein that was delivered to epithelial cells (Figure 5C). LCN2 concentration in the cellular fraction of culture with liposome-encapsulated LCN2 protein was 15.04 ng/mL, and those from liposomes without LCN2 and with a template DNA-free protein were 9.32 ng/mL and 9.01 ng/mL, respectively. There was a significant difference in LCN2 level in the cellular fractions between the LCN2-liposome and control liposome groups ($P<0.01$).

These results showed that the synthesized and LCN2 protein encapsulated into liposomes were delivered to oral epithelial cells.

3.4 | Inhibition of adhesion of *P. gingivalis* to oral epithelial cells by LCN2

On investigating the effect of the synthesized LCN2 protein on the adhesion of *P. gingivalis* to oral epithelial cells, the synthesized LCN2 (100 ng/mL) decreased the adhesion of *P. gingivalis* to oral epithelial cells by approximately 10% (Figure 6A). This was a significant inhibition compared with that of the control ($P<0.01$) and was similar to the level shown by a recombinant LCN2 protein. However, the synthesized sample that was derived from a template DNA-free protein did not significantly inhibit an adhesion of bacteria to oral epithelial cells. In the case of LCN2 protein encapsulated into liposomes, the synthesized LCN2 protein (300 ng/mL) in liposomes significantly inhibited an adhesion of *P. gingivalis* to oral epithelial cells in comparison with template DNA-free protein (Figure 6B). These results suggested that the synthesized LCN2 protein and its liposome-encapsulated LCN2 protein significantly inhibited an adhesion of *P. gingivalis* to oral epithelial cells.

4 | DISCUSSION

395 We aim to create a foundation for oral healthcare using antimicrobial peptides. In this
396 procedure, several steps including protein synthesis, preparation of liposome as a carrier,
397 delivery of the liposome-encapsulated peptide to oral epithelial cells and its effectiveness
398 as AMP were investigated. LCN2 is expressed in epithelial cells, leukocytes and
399 macrophages, and has antimicrobial activity by acting as a scavenger of siderophores.[8,9]
400 However, an effect of LCN2 on periodontopathic bacteria has not been reported so far.
401 Regarding the association between AMPs and periodontal diseases, there were several
402 reports that BD-2 showed antibacterial activity against *P. gingivalis* and *Aggregatibacter*
403 *actinomycetemcomitans*,[39] and calprotectin inhibited the binding and invasion of *P.*
404 *gingivalis* to oral epithelial cells.[40] Furthermore, cathelicidin (LL-37) showed
405 antimicrobial activity against periodontopathic bacteria such as *Fusobacterium*
406 *nucleatum* and *Prevotella intermedia*.[41] In the present study, we investigated the effect
407 of LCN2 on an adhesion of *P. gingivalis* to oral epithelial cells because this adhesive stage
408 is the beginning step of attack of periodontal diseases and showed that LCN2 inhibited
409 an adhesion of *P. gingivalis* to oral epithelial cells, suggesting that LCN2 as well as BD-
410 2, calprotectin and LL-37 have suppressive effects on periodontopathic bacteria and may
411 possibly contribute to a prevention of periodontal diseases. LCN2 has an inhibitory effect
412 on the growth of *E. coli* and *K. pneumoniae* by binding to bacterial siderophore and

413 depleting iron.[13-15,42] However, the mechanism of inhibitory effect of LCN2 on the
414 adhesion of *P. gingivalis* to oral epithelial cells was not clear because *P. gingivalis* does
415 not produce siderophores.[43] In our preliminary experiment, there was no inhibitory effect
416 on the adhesion of *P. gingivalis* to the cells when LCN2 was not contained in the co-
417 cultured medium of cells and labeled *P. gingivalis* after pre-treatment of cells with LCN2
418 (Supplemental data 2). In addition, LCN2 receptor, 24p3R, was not expressed in oral
419 epithelial cells (TR146), suggesting that LCN2 acted on *P. gingivalis*. *P. gingivalis* does
420 not produce siderophores, however, has some proteins to acquire iron and heme in
421 bacterial membrane. Those proteins including hemophore-like proteins (HmuY, HusA),
422 hemagglutinins, hemolysins, gingipains (Kgp, RgpA, RgpB), TonB-dependent outer-
423 membrane receptor (HmuR, HusB, IhtA) are associated with a mechanism to acquire
424 iron/heme in *P. gingivalis*.[43] *P. gingivalis* proliferated using iron that is derived from
425 transferrin degraded by Arg- and Lys-gingipain,[44] and adhered to erythrocytes using
426 hemagglutinins.[43] An inhibitory mechanism of LCN2 on *P. gingivalis*-adhesion was not
427 accurately elucidated in the present study and this investigation will be an interested
428 matter in future study.

429 LCN2 was synthesized using a CFPS system in the present study. The CFPS system
430 appears to be a useful method to prepare LCN2 protein for oral healthcare because cells

431 are not necessary for protein synthesis, and the synthesized protein is not prepared from
432 vectors in the genes of *E. coli*, yeast and virus as recombinant proteins. Particularly, the
433 PURE system appears to be suitable since all components in this system are more purified
434 rather than cell lysate and already reported,[21] and a composition of reaction mixture can
435 be controlled depend on a modification of protein synthesis, and this CFPS system does
436 not contain proteases and a contamination of RNase was low, and the synthesized LCN2
437 is difficult to degrade because the PURE system does not contain factors other than the
438 necessary factors for protein synthesis.[21,45] Furthermore, LCN2 synthesis using a CFPS
439 system may be easy compared to the preparation of a recombinant LCN2 protein in a
440 laboratory because the procedures of plasmid DNA preparation and *E.coli* culture are not
441 needed. The LCN2 synthesized using a PURExpress kit had almost the same molecular
442 weight (23 kDa) as that of a commercial recombinant LCN2, and its protein sequence
443 coverage in a mass spectrometry was also high and similar to that of a recombinant LCN2
444 protein. However, LCN2 protein fraction synthesized in the present study contained some
445 proteins from PURE system and was not purified, and we will have to further purify
446 LCN2 synthesized using a CFPS system to be utilized for oral healthcare in the future.
447 On the other hand, a biological LCN2 is a glycoprotein, however, the synthesized LCN2
448 protein in the present study might not be a mature LCN2 because the post-translational

449 glycosylation is not properly performed by PURE system. Although a recombinant
450 LCN2 as well as the synthesized LCN2 showed a similar inhibitory effect of *P. gingivalis*-
451 adhesion to oral epithelial cells, an influence of glycosylation on this inhibitory effect was
452 not investigated in the present study. We think that it is important to elucidate an
453 influence of glycosylation of the synthesized LCN2 protein in the future study.

454 Regarding the synthesis concentration, a mean concentration of the synthesized
455 LCN2 was approximately 233 μg/mL in seven experiments when a template DNA with
456 the AT-rich codon in N-terminal domain was used in LCN2 synthesis, and LCN2
457 concentration in the synthesized sample was determined using LCN2 ELISA kit. In
458 contrast, LCN2 protein concentration was very low (approximately 0.46 μg/mL) when a
459 template DNA derived from original gene sequence of LCN2 was used in a preliminary
460 experiment. This result suggested that the AT-rich codon in N-terminal domain in the
461 template DNA sequence remarkably increased the synthesis concentration of LCN2 in
462 the used PURE system. Many proteins have been synthesized using CFPS systems and
463 their concentration of the synthesized proteins ranged from under 10 μg/mL to over 2000
464 μg/mL.[46,47] For example, green fluorescent protein (GFP) was synthesized at 1600 μg/mL
465 using the wheat germ system,[17] and human bone morphogenic protein-2 was synthesized
466 at a concentration of 37 μg/mL using Chinese Hamster Ovary-based cell-free synthesis

467 system,[48] and human granulocyte-macrophage colony-stimulating factor was synthesized
468 at a maximum concentration of 700 μg/mL using *E. coli*-based open cell-free synthesis
469 system.[22] The mean concentration (233 μg/mL) of LCN2 synthesized in the present study
470 was not very high, however, this concentration was fully effective because 100 ng/mL of
471 the synthesized LCN2 significantly inhibited the adhesion of *P. gingivalis* to oral
472 epithelial cells.

473 Liposomes in the present study were prepared by a spontaneous transfer method
474 which was a simple method for liposome formation and did not require any specific
475 instruments.[28,36] The prepared liposomes varied in size with their diameters ranging from
476 1-180 μm and were defined as giant liposomes.[25] This size was similar to that of the
477 liposomes prepared by Yamada *et al.* (10-50 μm) and Liu *et al.* (20-120 μm).[28,34] Giant
478 liposomes are used as a DDS tool because various biological materials, including nucleic
479 acids, plasmids, proteins and drugs were encapsulated into the liposomes.[26,49,50] In the
480 present study, the liposomes were delivered to oral epithelial cells, and supposed to exist
481 on the cellular surface, and incorporated into the cells (Supplemental data 3), and the
482 addition of DOPE (10 mol%) to the liposomal components increased a delivery of
483 liposomes to oral epithelial cells. The interaction between cells and liposomes is regulated
484 by an uptake of liposomes into cells and the fusion between cellular membrane and

485 liposome membrane. These regulations are selected by an electronic charge and by
486 modifying liposomal surface and fluidity of liposome membrane.[51-53] DOPE is a cone-
487 type phospholipid with two unsaturated bonds and influences a fusogenic capacity
488 between liposome and cellular membrane.[54] It is a component of cationic liposomes and
489 improves a transfection efficiency of nucleic acids into cells.[55] An addition of DOPE to
490 the outer lipid components of liposome may elevate a cationic electric charge, and
491 increase a delivery of liposomes to oral epithelial cells. In the present study, STR-R8
492 slightly, but significantly increased a delivery of liposomes to oral epithelial cells.
493 Liposomal surface is modified by targeting materials such as adhesion peptides,
494 antibodies, CPPs and polyethylene glycol to increase a delivery efficiency of materials to
495 cells and tissues.[53,56] Octa-arginine is a poly-cationic peptides that flows into cells with a
496 low charge, and this mechanism is associated with endocytosis and direct translocation
497 across plasma membranes of octa-arginine peptides.[52] Octa-arginine peptide is a major
498 CPP that increases a cellular delivery efficiency of biomaterials including proteins,
499 peptides, nucleic acids and drugs by regulating endocytosis and direct translocation.[52,57]
500 We did not elucidate the detailed mechanism of internalization of STR-R8-conjugated
501 liposomes in the present study, but supposed that STR-R8 in the liposomes might up-
502 regulate a delivery activity of liposomes with LCN2 to oral epithelial cells by regulating

503 endocytosis or direct translocation across plasma membranes.

504 DDS has been used for a chemical treatment of periodontal diseases. Antibacterial
505 and anti-inflammatory medications such as minocycline, doxycycline, metronidazole and
506 chlorhexidine were included in carrier devices and transported to periodontal pockets and
507 tissues.[33,58-60] On the other hand, some gels/pastes, fibers, strips, capsules, particles and
508 liposomes are known as DDS devices,[33] and several carrier devices are used for
509 periodontal treatments.[59,60] Shi *et al.*[61] showed that resveratrol encapsulated in liposomes
510 ameliorated an experimental periodontitis in mice. When *A. actinomycetemcomitans*-
511 infected human oral keratinocytes were cultured with liposomes that had their surfaces
512 grafted with cyclodextrin, a cytotoxicity of keratinocytes and nitric oxide production were
513 decreased significantly.[62] These recent reports show that liposomes with chemical drugs
514 and biological materials can be used in the treatments of periodontal diseases. The present
515 study shows the possibility that liposome-encapsulated LCN2 may be useful for a
516 prevention of periodontal diseases, and contribute to establish a foundation for oral
517 healthcare in the future.

518

519 **5 | CONCLUSION**

520 LCN2, an antimicrobial peptide, was artificially synthesized using a CFPS system,

encapsulated into liposomes, and delivered to oral epithelial cells, which then inhibited the adhesion of *P. gingivalis* to oral epithelial cells. The present LCN2-encapsulated and liposomes-based drug delivery system may contribute to the preventive periodontal healthcare.

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CONFLICT OF INTEREST

None of the authors have any conflicts of interest related to this study.

AUTHOR CONTRIBUTIONS

Jun-ichi Kido; Planning study, performing almost experiments, writing a manuscript and summarizing study, Yuka Hiroshima; Planning study, performing bacterial adhesion assay, instructing experimental methods and summarizing study, Rie Kido; Performing

experiments (cell culture, ELISA, Western blotting), Kaya Yoshida; Observing liposomes and instructing experimental methods, Koji Naruishi and Yuji Inagaki; Supporting Western blotting procedure, Kazuaki Kajimoto and Masatoshi Kataoka; Instructing liposome preparation and delivery experiments, Yasuo Shinohara; Instructing cell-free protein synthesis and analysis of protein and evaluating result data, Hiromichi Yumoto; Advising study performance.

DATA AVAILAILITY STATEMENT

All data supporting the findings of this study are available from the corresponding author upon reasonable request.

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717
718

FIGURE LEGENDS

FIGURE 1 SDS-PAGE and Western blotting of the synthesized LCN2 protein. The proteins were synthesized with or without a template DNA sequence for LCN2 using the PURE system. The synthesized proteins and a recombinant LCN2 protein were analyzed by SDS-PAGE and CBB staining (A), and Western blotting with a specific antibody against LCN2 protein (B). These results were confirmed using three independent protein samples.

FIGURE 2 Liposome preparation and its observation. Liposomes were prepared using DOPC and Egg-PC by a spontaneous transfer method (A and B). Liposomes were prepared using DOPC and NBD-PE in the components of outer lipid-layer and Egg-PC and rhodamine-DHPE in the components of inner lipid-layer, and observed using a fluorescence microscope at 485/535 nm (C, green) and 560/580 nm (D, red) (excitation/emission) at a magnification of x 20. Microscopic images of the same field were merged (E). Scale bars: 100 μm.

FIGURE 3 Delivery of liposomes to oral epithelial cells. Oral epithelial cells were cultured for 5 days and then incubated for 3 h with liposomes with rhodamine-DOPE (A,

737 B) and without rhodamine-DOPE (C, D). After washing, the cells were observed using a
738 phase-contrast microscopy (A and C) and fluorescence microscopy (B and D) at 560/580
739 (excitation/emission) at a magnification of x 400. Scale bars: 20 μm. (E, F) Effects of
740 DOPE and STR-R8 on the delivery of liposomes to oral epithelial cells. The confluent
741 oral epithelial cells were incubated with liposome containing DOPE (0-20%, E) and STR-
742 R8 (0.1 mM, F) for 3 h. After washing, a fluorescence intensity of the cultured cell was
743 measured using a fluorescence plate reader at 555/575 nm (excitation/emission). The
744 mean fluorescence intensity of the Control in (E) and (F) were 39453 and 39579,
745 respectively, and the values were shown as percentage of the Control. Data are expressed
746 as mean ± SD of 4-8 samples. *P<0.05, **P<0.01.
747
748 **FIGURE 4** Encapsulation of LCN2 into liposomes. (A) shows the procedure of
749 encapsulation of the synthesized LCN2 into liposomes and collection. The synthesized
750 LCN2 samples were analyzed by Western blotting with 15% SDS gel (B). Sample 1:
751 synthesized LCN2, Sample 2: lyophilized Sample 1, Sample 3: Sample 2 encapsulated
752 into liposomes, Sample 4: ultra-filtrated Sample 3, Sample 5: flow-through of ultra-
753 filtrated Sample 3, Sample 6: recombinant LCN2. This result was confirmed using three
754 independent protein samples.

755

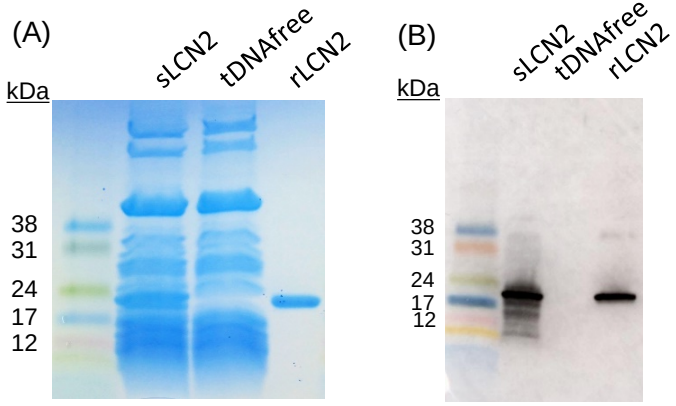
756 **FIGURE 5** Delivery of the liposome-encapsulated LCN2 to oral epithelial cells. (A)
757 shows the protocol of delivery of the liposome-encapsulated LCN2 to the cells, collection
758 and assays. (B) The samples, including protein-free (only liposome control), a template
759 DNA-free protein and the synthesized LCN2 protein, were analyzed by Western blotting
760 with 15% SDS gel. Sample 1: cellular fraction of protein-free, Sample 2: cellular
761 fraction of a template DNA-free protein, Sample 3: cellular fraction of the synthesized
762 LCN2 protein, Sample 4: medium fraction of protein-free, Sample 5: medium fraction of
763 the template DNA-free protein, Sample 6: medium fraction of the synthesized LCN2
764 protein, Sample 7: liposome-encapsulated LCN2 protein before addition to the cell
765 culture, Sample 8: the conditioned medium of TR146 cells, Sample 9: recombinant LCN2.
766 This result was confirmed using three independent protein samples. (C) LCN2
767 concentration in the cellular fractions derived from protein-free (Control), a template
768 DNA (tDNA)-free protein and synthesized LCN2 (sLCN2) protein were determined by
769 ELISA. Data are expressed as mean ± SD of 6-7 samples. $^{**}P<0.01$.

770

771 **FIGURE 6** Inhibitory effect of the synthesized LCN2 on the adhesion of *P. gingivalis*
772 to oral epithelial cells. (A) Oral epithelial cells at confluence were incubated with protein-

773 free (Control), the synthesized LCN2 (sLCN2, 100 ng/mL), a template DNA (tDNA)-free
774 protein and recombinant LCN2 (10 ng/mL) for 16 h. *P. gingivalis* cultured under
775 anaerobic condition for 24 h and then labeled with a fluorescent reagent. (B) Oral
776 epithelial cells were incubated with the samples that were encapsulated protein-free
777 (Control), the synthesized LCN2 (sLCN2, 300 ng/mL) and the template DNA (tDNA)-
778 free protein into liposomes for 16 h. *P. gingivalis* also was pre-treated with the
779 liposome-encapsulated samples for 16 h and then labeled with a fluorescent reagent. (A,
780 B) The pre-treated cells and labeled bacteria were co-incubated for 2 h. After washing the
781 culture, a fluorescent intensity on the culture was determined using a fluorescence plate
782 reader. The mean fluorescence intensity of the Control in (A) and (B) were 31806 and
783 36339, respectively. The values were shown as percentage of the Control. Data are
784 expressed as mean ± SD of 24 samples (A) and 16 samples (B). $^{**}P<0.01$.

Figure 1


(A)
sLCN2
tDNAfree
rLCN2
kDa
38
31
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17
12
(B)
sLCN2
tDNAfree
rLCN2
kDa
38
31
24
17
12

Table 1. Comparison of synthesized LCN2 and recombinant LCN2 in nano LC-MS/MS analysis

Sample name	Database accession	Mascot score	Number of detected peptide	Protein sequence coverage (%)
	SwissProt			
Synthesized sample	NGAL HUMAN	1677	141	74.7 [1)]
Recombinant LCN2	NGAL HUMAN	5632	1059	82.3 [2)]

NGAL: Neutrophil gelatinase-associated lipocalin/LCN2

1) Synthesized sample

MPLGLLWLGL ALLGALHAQA QDSTSDLIPA PPLSK**VPLQQ NFQDNQFQGK**
WYVVGLAGNA ILREDKDPQK MYATIYELKE DKSYNVTSVL FPKKK**CDYWI**
RTFVPGCQPG EFTLGNIKSY PGLTSYLVRV VSTNYNQHAM VFFKKVSQNR
EYFK**ITLYGR TKELTSELKE NFIR**FSK**SLG LPENHIVFPV PIDQCIDG**

2) Recombinant LCN2

MPLGLLWLGL ALLGALHAQA QDSTSDLIPA PPLSK**VPLQQ NFQDNQFQGK**
WYVVGLAGNA ILREDKDPQK MYATIYELKE DKSYNVTSVL FPKKKCDYWI
RTFVPGCQPG EFTLGNIKSY PGLTSYLVRV VSTNYNQHAM VFFKKVSQNR
EYFKITLYGR TKELTSELKE NFIRFSKSLG LPENHIVFPV PIDQCIDG

Underlined Bold characters show the peptides matched to the amino acid sequence of LCN2 on data base.

Figure 2

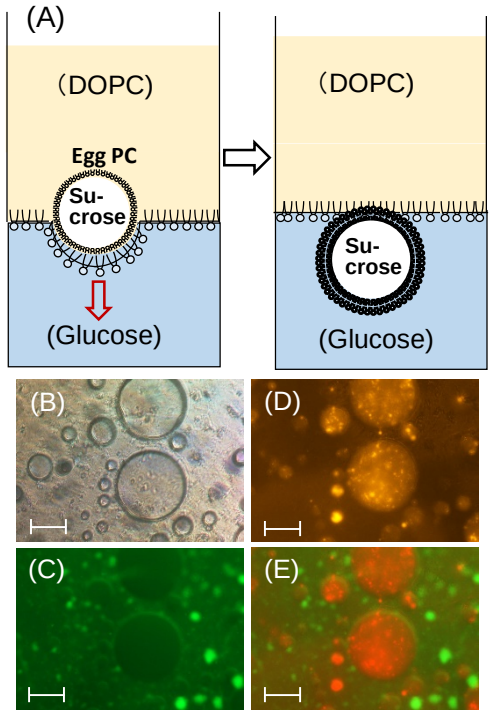
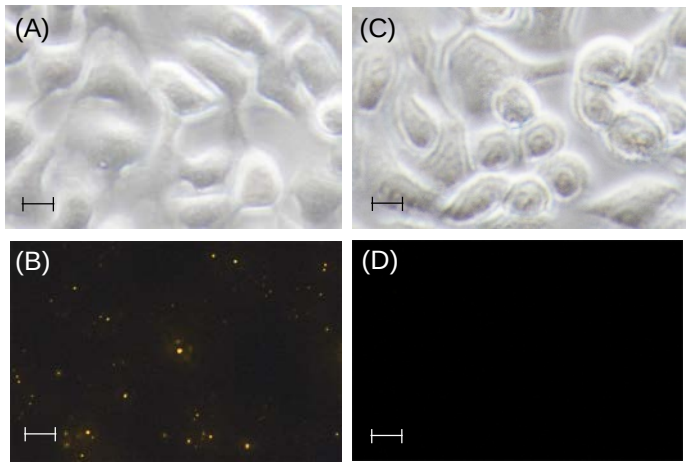
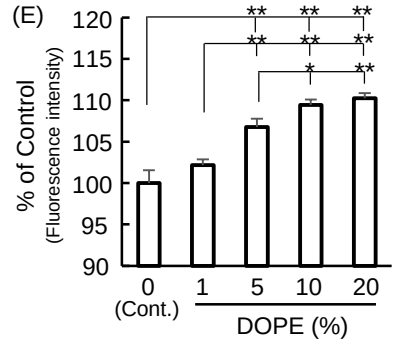


Figure 3


(A)
(C)
(B)
(D)


(E)
% of Control
(Fluorescence intensity)
120
115
110
105
100
95
90
**
**
**
**
**
*
**
0
(Cont.)
1
5
10
20
DOPE (%)

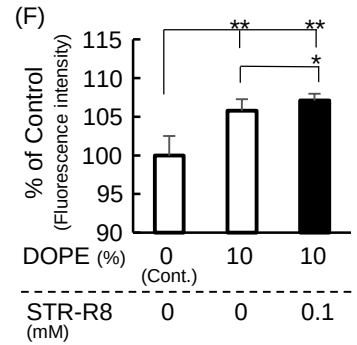

(F)
% of Control
(Fluorescence intensity)
115
110
105
100
95
90
**
**
*
DOPE (%)
0
(Cont.)
10
10
STR-R8
(mM)
0
0
0.1

Figure 4

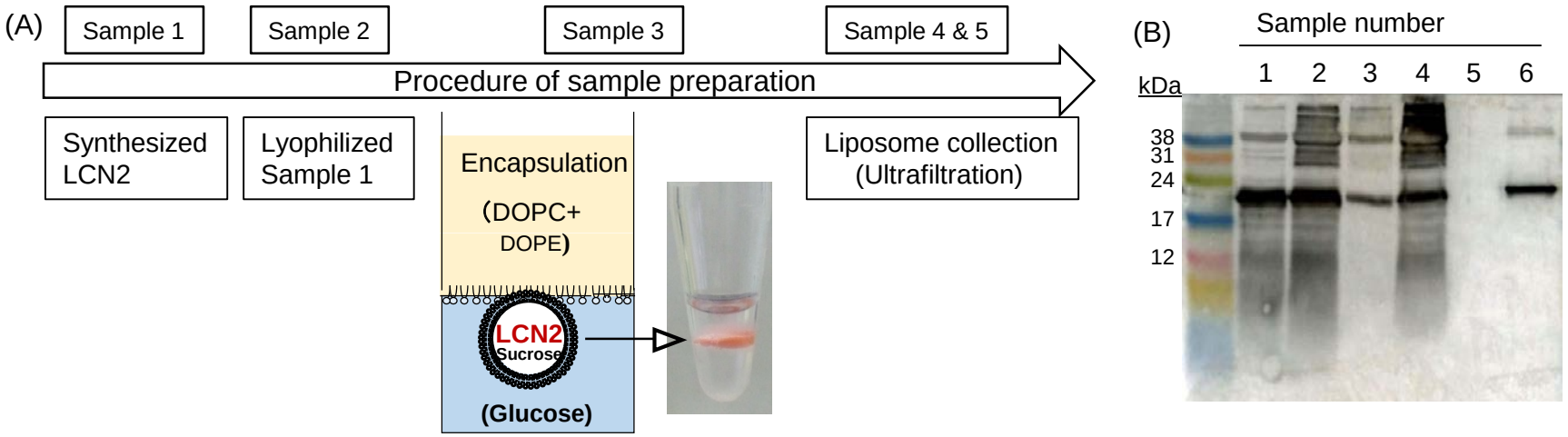
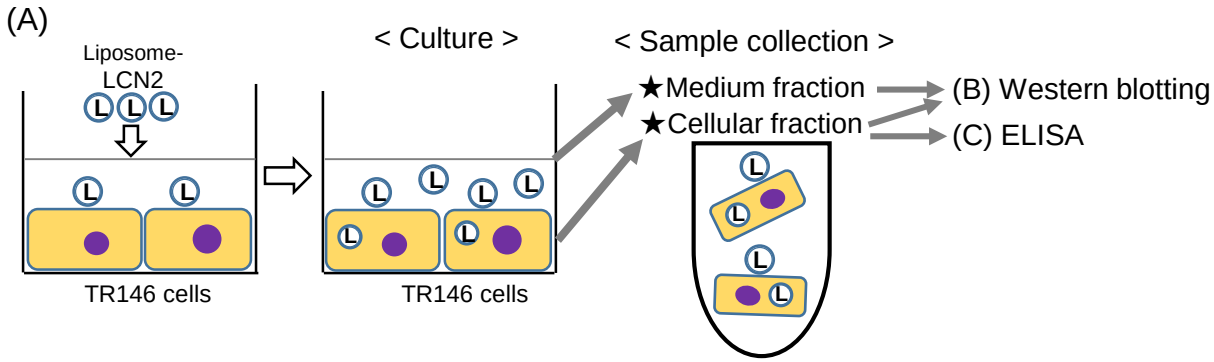
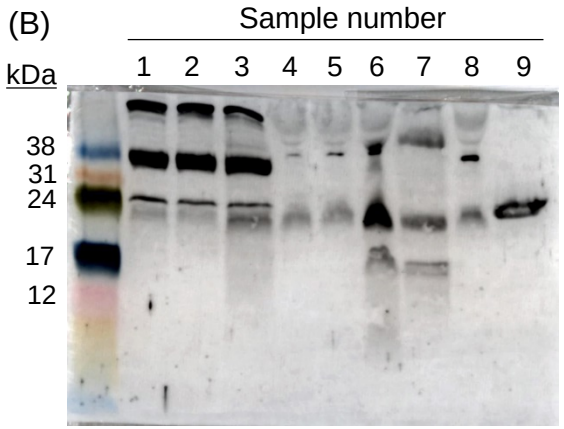

(A)
Sample 1
Sample 2
Sample 3
Sample 4 & 5
Procedure of sample preparation
Synthesized LCN2
Lyophilized Sample 1
Encapsulation
(DOPC+ DOPE)
LCN2
Sucrose
(Glucose)
Liposome collection (Ultrafiltration)
(B)
Sample number
1 2 3 4 5 6
kDa
38
31
24
17
12

Figure 5


(A)
Liposome-
LCN2
< Culture >
< Sample collection >
★Medium fraction
★Cellular fraction
(B) Western blotting
(C) ELISA
TR146 cells
TR146 cells


(B)
Sample number
1 2 3 4 5 6 7 8 9
kDa
38
31
24
17
12

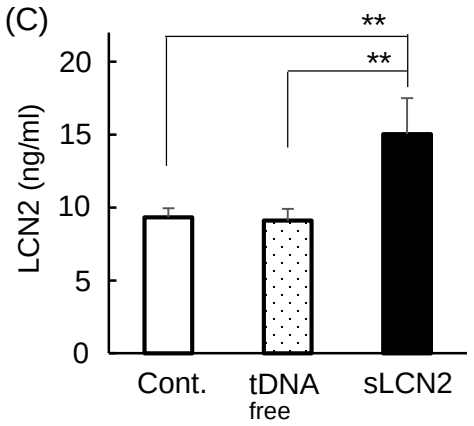
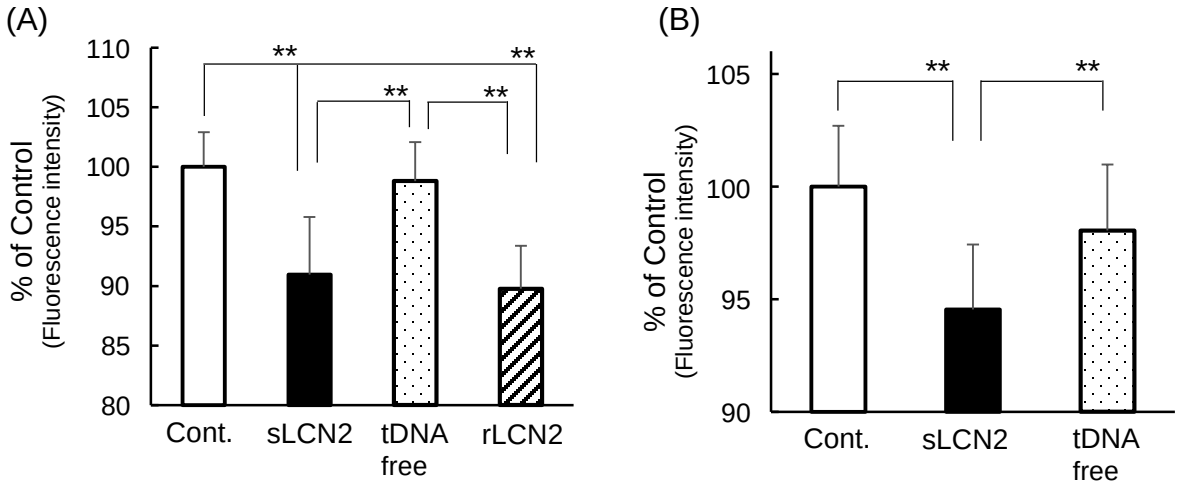

(C)
**
**
LCN2 (ng/ml)
20
15
10
5
0
Cont.
tDNA free
sLCN2

Figure 6


(A)
% of Control
(Fluorescence intensity)
110
105
100
95
90
85
80
**
**
**
**
Cont.
sLCN2
tDNA free
rLCN2
(B)
% of Control
(Fluorescence intensity)
105
100
95
90
**
**
Cont.
sLCN2
tDNA free

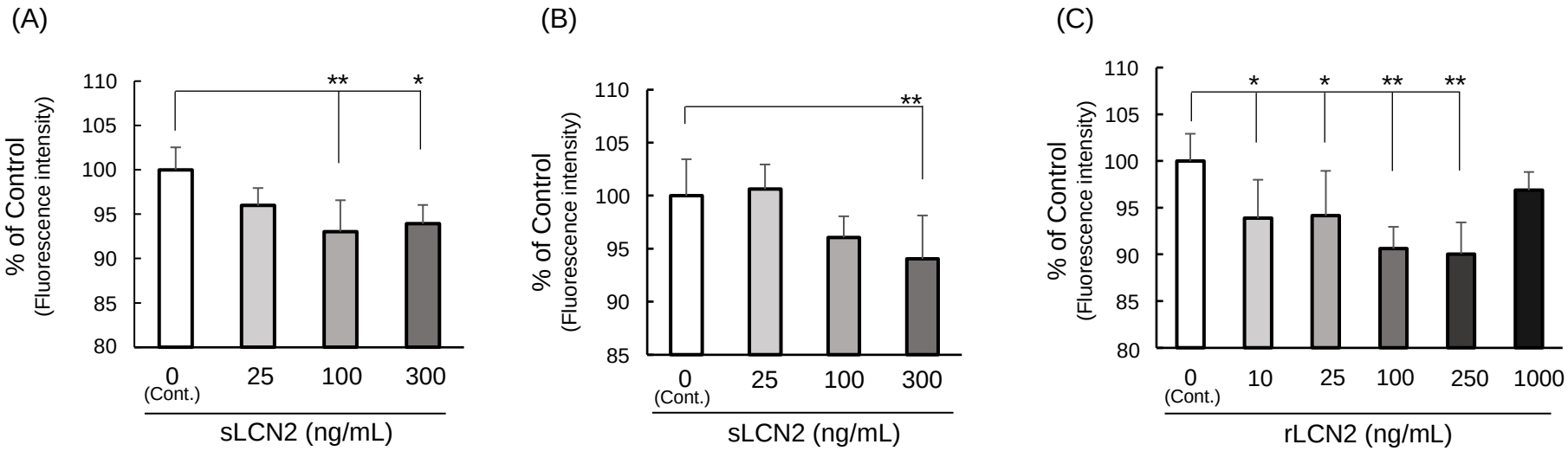


Figure S1. Effect of the synthesized LCN2 and recombinant LCN2 on the adhesion of *P. gingivalis* to oral epithelial cells.
Oral epithelial cells (TR146 cells) were cultured with the synthesized LCN2 (A, sLCN2; 0-300 ng/mL), liposome-encapsulated sLCN2 (B, 0-300 ng/mL) and recombinant LCN2 (C, rLCN2; 0-1000 ng/mL) for 16 h. *P. gingivalis* were cultured for 24 h and labeled with a Fluorescent reagent, and then co-incubated with the cells for 2 h. After washing the culture, a fluorescent intensity was determined using a fluorescence plate reader at 485/535 nm (excitation/emission). The values were shown as percentage of each Control. Data are expressed as mean ± SD of 4-6 samples (A), 7-8 samples (B, C). *P<0.05, **P<0.01.

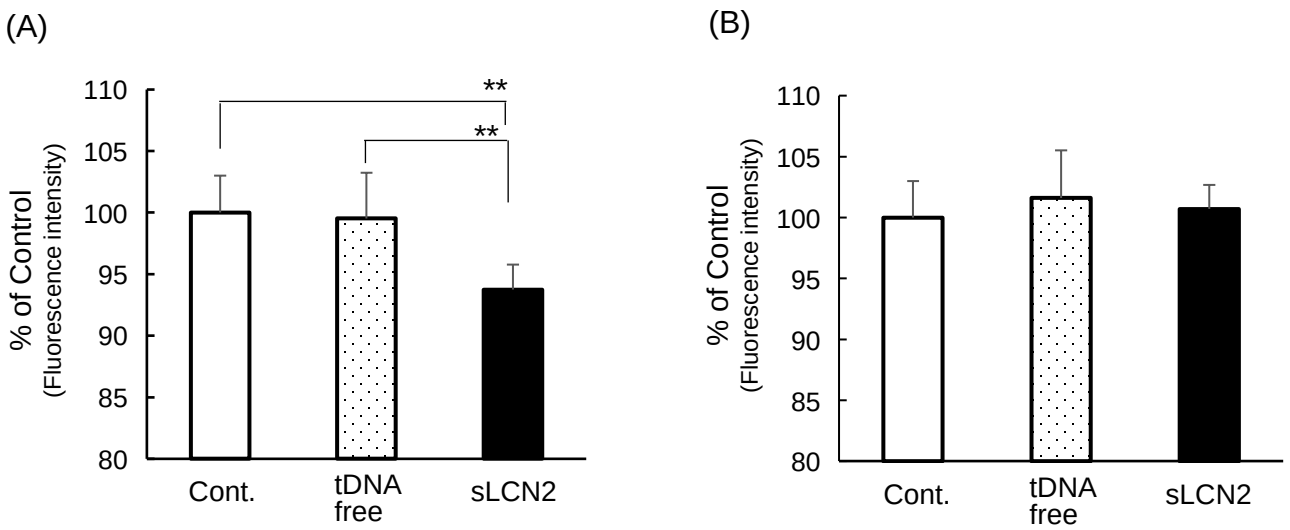


Figure S2. Effect of the synthesized LCN2 on the adhesion of *P. gingivalis* to oral epithelial cells.
Oral epithelial cells (TR146 cells) were cultured with the synthesized LCN2 (sLCN2; 100 ng/mL, total protein; 3.78 μg/mL), template DNA-free protein (tDNAfree; total protein; 3.78 μg/mL) or without proteins (Control) for 16 h. *P. gingivalis* were cultured for 24 h and labeled with a fluorescent reagent and then added to the culture of pre-treated oral epithelial cells and co-incubated for 2 h (A). In contrast, after the pre-treatment of epithelial cells with sLCN2, the cells were washed fully with PBS, and then co-incubated with the labeled *P. gingivalis* in sLCN2-free medium for 2 h (B). After washing cell-bacteria culture, a fluorescent intensity was determined using a fluorescence plate reader at 485/535 nm (excitation/emission). The values were shown as percentage of each Control. Data are expressed as mean ± SD of 7-8 samples. ***P*<0.01.

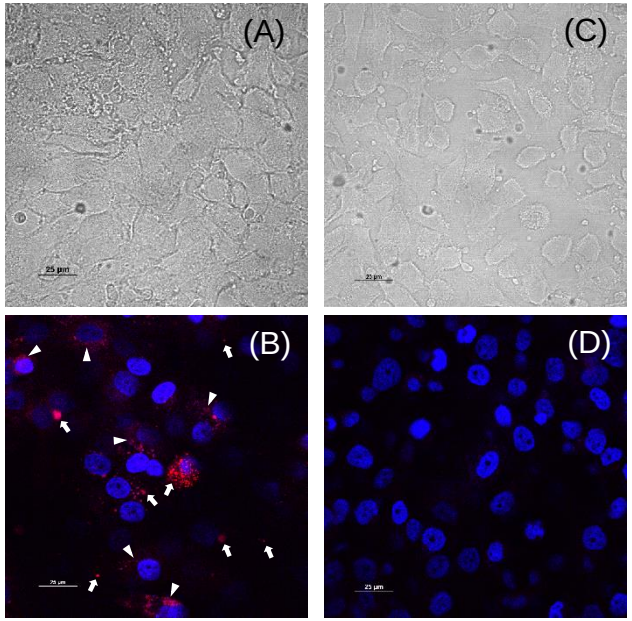


Figure S3. Observation of liposomes delivered to oral epithelial cells using a confocal laser scanning microscopy. Oral epithelial cells (TR146 cells) at sub-confluent were incubated with Rhodamine-DOPE (10 mol%)-DOPC(90 mol%) (A, B) and DOPC (100 mol%) (C, D) for 3 h. The cells was fixed in 10% formalin solution and cell nuclei were stained with Hoechst 33342. After washing the cells, the cell culture was observed at a magnification of 600-fold using a phase-contrast microscopy (A, C) and a confocal laser scanning microscopy (ECLIPSE Ti, Nikon) (B, D). White arrows showed the liposomes with Rhodamine inside the cells, and white arrowheads were supposed to be the liposomes on the cell surface. Scale bars represent 25 μm.