β-defensin 2 synthesized by a cell-free protein synthesis system and encapsulated in liposomes inhibits adhesion of *Porphyromonas gingivalis* to oral epithelial cells

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Keywords: β-defensin 2, Cell-free protein synthesis system, Liposome, Drug delivery system, *Porphyromonas gingivalis*

Abstract

β-defensin 2 (BD-2), an antimicrobial peptide (AMP), is expressed by oral epithelial cells and plays an important role in innate immunity of the oral cavity. Cell-free protein synthesis (CFPS) systems have been studied for the synthesis of various proteins, however, the synthesis of BD-2 by a CFPS system has not been extensively explored. Liposomes have been developed as tools for drug delivery. A delivery of liposome-encapsulated AMP to oral epithelium may be useful to prevent oral infectious diseases. In the present study, we investigated the antimicrobial activity of the BD-2 protein, artificially synthesized using a CFPS system and encapsulated in liposomes. BD-2 protein was artificially synthesized using template DNA and a reconstituted CFPS system and was identified by western blotting. Bilayer liposomes were prepared using 1,2-dioleoyl-sn-glycero-3-phospho-choline and 3-sn-phosphatidylcholine from egg yolk. The artificially synthesized BD-2 was encapsulated in liposomes, collected by ultrafiltration, and detected by western blotting. Human oral epithelial cells were cultured with the liposome-encapsulated BD-2 and the concentration of BD-2 in the cell lysate of the culture with the synthesized BD-2 was higher than that of the control cultures. The antimicrobial activity of the synthesized BD-2 was investigated by an adhesion assay of *Porphyromonas gingivalis* to oral epithelial cells. The artificially synthesized BD-2 and

its liposome significantly inhibited adhesion of *P. gingivalis* to oral epithelial cells. These results suggest that artificially synthesized BD-2 and liposome-encapsulated BD-2 shows antimicrobial activity and can potentially play a role in oral healthcare for periodontal diseases.

Introduction

Oral healthcare practices is important to prevent systemic and oral diseases such as periodontal diseases and dental caries [1, 2]. Plaque control by tooth brushing is an essential action for oral healthcare; however, proper tooth brushing techniques may be difficult for aged individuals with physical disabilities. Although some chemical drugs in carrier devices are commercially available and administered to periodontal pockets for the treatment of periodontal diseases [3], the use of biological methods with biomolecules for the prevention of periodontal diseases has not been extensively explored.

β-defensin 2 (BD-2), a cysteine-rich cationic peptide with three disulfide bonds and a molecular weight of 4-5 kDa, is one of the antimicrobial peptides (AMPs) that is expressed in oral epithelial cells and immune cells, shows antimicrobial activity, and plays an important role in innate immunity and biological defense [4-6]. BD-2 expression is low level in keratinocytes of healthy epithelium, but is increased by pro-inflammatory cytokines, bacteria and lipopolysaccharide [7] and this peptide shows broad spectrum antimicrobial activity against bacteria and fungi by forming pores and increasing the permeability of bacterial membranes [8-10]. The synthesized BD-2 demonstrated antibacterial activity against *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* [11], and BD-2 inhibited the growth of *P. gingivalis* and *Fusobacterium nucleatum* [12],

88 suggesting that BD-2 plays a significant role in innate immunity against periodontal
89 diseases.

90 Cell-free protein synthesis (CFPS) system is a platform technology used for
91 artificial synthesis of proteins without direct use of living cells and bacteria, and recently
92 many proteins including membrane proteins, proteins with cytotoxicity and
93 immunoglobulins have been synthesized using CFPS systems [13-16]. The CFPS system
94 is different from the preparing method of recombinant proteins and does not use plasmids
95 or virus vectors in the process of protein synthesis. The PURE system is a reconstituted
96 CFPS system that consisting of multiple factors, which is suitable for efficient and high-
97 purity protein synthesis because it contains factors that are associated with protein
98 synthesis, but not components associated with protein degradation [13, 17]. There are a
99 few reports on the synthesis of AMPs using CFPS systems. Chen *et al.* [18] showed a
100 fusion protein containing human BD-2 using an *E. coli* cell-free system in which the
101 plasmid containing the human BD-2 gene was needed, and the prepared fusion protein
102 was purified to obtain a pure BD-2 protein. However, their method was complicated, and
103 the purity of the prepared BD-2 solution was not very high.

104 Liposomes encapsulate peptides/proteins, nucleic acids and chemical drugs [19-21]
105 and are known as carrier devices for drug delivery system (DDS) in the treatments for

106 several diseases including various tumors, leukemia, infectious diseases, rheumatoid
107 arthritis and cystic fibrosis [22, 23]. Liposomes are vesicles composed of phospholipid
108 layers comprising hydrophilic and hydrophobic parts, having several lipid structures such
109 as mono-layer and bi-layers with a diameter of 0.1~100 μm [19, 24]. Yamada *et al.* [25]
110 prepared giant bi-layer liposomes with diameters of 10-50 μm using two phospholipids
111 by the spontaneous transfer method. The giant liposomes encapsulated DNA plasmids,
112 microRNAs, proteins, CFPS system and bacteria [20, 26, 27], suggesting that giant
113 liposomes can be useful as carrier devices for specific proteins synthesized by the CFPS
114 system.

115 The purpose of the present study was to investigate the antibacterial activity of
116 artificially synthesized BD-2 that was encapsulated in liposomes and delivered to oral
117 epithelial cells, against *P. gingivalis*, and the possibility that the present biological system
118 contributes to oral healthcare of periodontal diseases.

119

120 **Materials and Methods**

121 **Materials**

122 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3
123 phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipid, Inc. (Alabaster,

AL). 3-sn-Phosphatidylcholine from egg yolk (Egg-PC) and mineral oil were obtained from FUJIFILM Wako PURE Chemical Co. (Osaka, Japan). PURExfrex®2.1 was obtained from GeneFrontier Co. (Chiba, Japan) and CytoTell™ UltraGreen was purchased from AAT Bioquest Inc. (Sunnyvale, CA, USA).

BD-2 synthesis using CFPS system

The template DNA sequence for BD-2 protein synthesis was designed according to a modification of the DNA sequence derived from human DEFB4A mRNA (NM_004942.3), with the sequences of T7 promoter and ribosome binding site, and AT-rich codons. The DNA template design for BD-2 was supported by GeneFrontier Co., and its sequence was 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 ATG GGA ATT GGA GAT CCA GTA ACT TGT TTA AAA TCT GGC GCT ATT TGC CAC CCT GTT TTC TGT CCG CGC CGT TAC AAA CAG ATC GGT ACT TGC GGC CTG CCG GGT ACC AAG TGT TGC AAA AAA CCG TAA TGA ATA ACT AAT CC. The BD-2 protein was synthesized according to the instruction manual of PURExfrex®2.1 (GeneFrontier). Briefly, the template DNA for BD-2 protein synthesis, cysteine, glutathione, and three solutions including amino acids, NTPs, tRNA, ribosome,

translation factors and other factors in the kit and pure water were mixed at a rate indicated in the instruction manual, and incubated at 37°C for 6 h. Eight independent BD-2 protein samples were synthesized using the PUREfrex® system. The total amount of synthesized protein was determined using the TaKaRa BCA Protein Assay Kit (TaKaRa Bio, Shiga, Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Protein samples were prepared by the syntheses with or without a template DNA for BD-2 using PUREfrex®2.1 kit. In SDS-PAGE, the synthesized BD-2 (total protein 100 μg), template DNA-free protein (total protein 100 μg) and recombinant BD-2 protein (3 μg, Abcam, ab243124, Cambridge, MA, USA) were electrophoretically separated on an SDS-polyacrylamide gel (Mini-PROTEIN TGX, 4-20%, Bio-Rad, CA, USA), stained with Quick-CBB PLUS (FUJIFILM Wako Pure Chemical) for 1 h, and de-stained with distilled water for 1 h. For western blotting, the synthesized BD2 protein (total protein 7.5 μg), template DNA-free protein (total protein 7.5 μg) and recombinant BD-2 protein (120 ng) were electrophoretically separated on an SDS-polyacrylamide gel (4-20%) and transferred to a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot Turbo

160 Transfer System® (Bio-Rad). The PVDF membrane with BD-2 protein was blocked with
161 PVDF Blocking Reagent for CanGet Signal® (TOYOBO, Tokyo, Japan) for 1 h at room
162 temperature and then washed in Tris-buffered saline supplemented with Tween 20. The
163 membrane was reacted with anti-BD-2 antibody (1/1000 dilution, GeneTex International
164 Co.: GTX56023, Hsinchu, Taiwan) in CanGet Signal® Solution 1 (TOYOBO) overnight
165 at 4°C. After washing, the membrane was reacted with horseradish peroxidase (HRP)-
166 conjugated anti-rabbit IgG (1/15000 dilution, GeneTex) in CanGet Signal® Solution 2
167 (TOYOBO) for 1 h at room temperature, and the BD-2 immuno-signal on the membrane
168 was developed using ECL™ Western Blotting Detection Reagents (GE Healthcare Japan,
169 Tokyo, Japan) and visualized using Image Quant LAS 500 (GE Healthcare). Over three
170 independent, synthesized BD-2 protein were analyzed by SDS-PAGE and western
171 blotting.
172
173 **Enzyme-linked immunosorbent assay (ELISA)**
174 The amount of synthesized BD-2 protein was determined using Human BD-2/beta-
175 Defensin-2 ELISA kit (Arigo biolaboratories, Hsinchu, Taiwan) in accordance with
176 manufacturer's instruction. Briefly, the synthesized BD-2 and template DNA-free
177 protein were appropriately diluted in a diluent buffer and reacted with anti-BD-2 antibody

178 in the microtiter plate of a kit at 37°C for 1.5 h. After washing, the immune reactant was
179 incubated with a second horseradish peroxidase (HRP)-conjugated antibody for 1h and
180 HRP-streptavidin solution for 30 min at 37°C, and then reacted with 3,3',5,5'-
181 tetramethylbenzidine substrate for 15 min in the dark. After termination of the reaction,
182 the absorbance of the immuno-complex was determined at 450 nm using a microplate
183 reader. Data are expressed as mean ± SD of each 5 samples of the synthesized sBD-2 and
184 template DNA (tDNA) free protein (Fig. 1C)
185
186 **Liposome preparation**
187 Bilayer liposomes were prepared by a spontaneous transfer method with DOPC-DOPE
188 and Egg-PC according to the modification method of Hamada *et al.* [28]. Briefly, the
189 mixture of 0.9 mM DOPC and 0.1 mM DOPE, and 0.454 mM Egg-PC were dissolved in
190 chloroform/methanol (2:1 v/v), dried with nitrogen gas, re-dissolved in mineral oil (MP
191 Biomedicals, Illkirch, France) and sonicated at 50°C for 1 h. The DOPC-DOPE solution
192 was overlaid on a 0.5 M glucose solution at a volume ratio of 0.71:1 in a tube and
193 incubated for 2 h at room temperature. Re-dissolved Egg-PC and 0.5 M sucrose solution
194 were mixed at a volume ratio of 16:1 and emulsified. A water-in-oil droplet emulsion
195 was prepared from Egg-PC and the sucrose solution, overlaid on the DOPC-DOPE and

196 glucose solution, and incubated at 4°C for 1 h (Fig. 2A). The formed bi-layer liposomes
197 were observed using an inverted microscope (ECLIPSE Ti-U, Nikon, Tokyo, Japan). The
198 bi-layer liposomes were prepared by a spontaneous transfer method more than ten times.
199
200 **Encapsulation of synthesized BD-2 in liposomes**
201 The synthesized BD-2 protein was concentrated by lyophilization, dissolved in 0.5 M
202 sucrose solution and thoroughly mixed with Egg-PC for 30 s to prepare an emulsion. This
203 water-in-oil droplet emulsions including BD-2 was overlaid on the layer of DOPC-DOPE
204 and glucose solution, and incubated at 4°C for 1 h according to the modified method of
205 Hamada *et al.* [28]. The liposome-encapsulated BD-2 in the intermediate phase was
206 collected by ultra-filtration (Amicon Ultra-0.5 Centrifuge Filter Devices, 3K, Merck
207 Millipore, Co. Cork, Ireland) and detected by western blotting (Fig. 3A). The protein
208 encapsulation was confirmed by three independent BD-2 protein samples.
209
210 **Delivery of synthesized BD-2 in liposomes to oral epithelial cells**
211 Human oral epithelial cells (TR146 cells) were seeded at 0.9 x 10^4 cells/cm^2 and cultured
212 in Ham's F-12 medium (FUJIFILM Wako Chemical Co.) supplemented with 10% fetal
213 bovine serum, penicillin and streptomycin for 5 days. Cells were cultured with or without

214 the synthesized BD-2 protein and template DNA-free protein for 16 h and the conditioned
215 medium (medium fraction) was collected and mixed with phenylmethylsulfonyl fluoride
216 (Merck Sigma-Aldrich Japan, Tokyo, Japan). Cell lysates (cellular fraction) were
217 dissolved in RIPA Lysis Buffer containing a protease inhibitor cocktail and sodium
218 orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cell and medium
219 fractions were separated by SDS-PAGE, transferred to PVDF membranes, and analyzed
220 by western blotting for traces of BD-2. The delivery of synthesized BD-2 protein samples
221 to oral epithelial cells was confirmed at three independent experiments. The concentration
222 of BD-2 in the cell fraction was determined by ELISA (Cont. n=6 samples, tDNA free
223 n=6 samples, sBD-2 n=12 samples) (Fig. 4A).
224
225 **Adhesion assay of *P. gingivalis* to oral epithelial cells**
226 The adhesion assay of *P. gingivalis* to oral epithelial cells was performed according to the
227 modified method described by Zanaboni *et al.* [29]. Briefly, epithelial cells were seeded
228 at 2 x 10^4 cells/cm^2 in 96-well black plate (Sumitomo Bakelite, Tokyo, Japan) and
229 cultured for 5 days, and then cultured with the synthesized BD-2 protein (100 ng/mL), a
230 template DNA-free protein and recombinant BD-2 (25 ng/mL) for 16 h. *P. gingivalis*
231 ATCC33277 was anaerobically cultured in a brain heart infusion (BD Bioscience,

232 Franklin, Lakes, NJ) broth containing hemin (5 μg/mL) and menadione (1 μg/mL) for 24
233 h, washed in phosphate-buffered saline solution (PBS), and labelled with Cyto Tell
234 UltraGreen (AAT Bioquest Inc., Sunnyvale, CA, USA) for 15 min at 37°C in the dark.
235 The labeled *P. gingivalis* was washed and prepared at 2.5 x 10^6/well at 100 multiplicity
236 of infection, incubated with oral epithelial cells that were pre-treated with the synthesized
237 BD-2 protein, a template DNA-free protein and recombinant BD-2 for 2 h at 37°C. Cells
238 and adhered bacteria were washed in PBS, and the fluorescence intensity of the bottom
239 surface of the culture well was determined using a fluorescence microplate reader
240 (TECAN Infinite® M200Pro, Switzerland) at 485/535 nm (excitation/emission) to
241 evaluate the adhesion of bacteria to oral epithelial cells. In another experiment using
242 liposome-encapsulated BD-2, oral epithelial cells and *P. gingivalis* were individually pre-
243 treated with the encapsulated BD-2 protein (300 ng/mL), a template DNA-free protein
244 and liposomes (Control) for 16 h, and the pre-treated cells and bacteria were co-incubated
245 at 37°C for 2 h. After cells and bacteria were washed in PBS, the fluorescence intensity
246 of the cultured wells was determined using a fluorescence microplate reader (TECAN).
247 The concentrations of the synthesized BD-2 and its liposome-encapsulated protein were
248 determined from the result of preliminary experiments (100 ng/mL and 300 ng/mL,
249 Supplemental data). Data are expressed as the mean ± SD of all samples from three

250 independent experiments, (A) n=24 samples (n=8/experiment) and (B) n=30 samples
251 (n=10/experiment).
252
253 **Statistical analysis**
254 Statistical analyses were performed using Excel Analysis 2012 for Windows (SSRI,
255 Tokyo, Japan). The comparisons among multiple groups were performed by a one-way
256 analysis of variance (ANOVA) followed by Tukey-Kramer analysis, and the significance
257 of differences between two groups was estimated using Student's t-test. *P* values less than
258 0.05 were considered to be significant.
259
260 ## Results
261 **Synthesized BD-2 protein**
262 The proteins were synthesized by the reaction mixture with or without a template DNA
263 of BD-2 using a CFPS system, and the total protein concentration of the two synthesized
264 protein samples were 6.36 mg/mL and 6.26 mg/mL, respectively. When 100 μg of the
265 synthesized proteins was analyzed by SDS-PAGE, some bands of proteins were detected
266 in both samples with or without a template DNA of BD-2, however, a clear specific band
267 with approximately 4 kDa molecular weight was not observed (Fig. 1A). In the western

blot analysis of BD-2, a specific immune signal was identified in the synthesized BD-2 sample with a molecular weight of 4 kDa, which was similar to that of recombinant BD-2, however, there was no band in the synthesized protein from a template DNA-free (Fig. 1B). Further, the BD-2 concentration in the synthesized BD-2 protein sample was 206.2 μg/mL and its concentration in the protein sample derived from template DNA-free was below the measurement limit by ELISA (Fig. 1C).

Encapsulation of synthesized BD-2 into prepared liposomes

Phospholipid bilayer liposomes were prepared from DOPC-DOPE and Egg-PC by a spontaneous transfer method (Fig. 2A). The prepared liposomes had various sizes, with diameters of 1-140 μm (Fig. 2B). The synthesized BD-2 was encapsulated in liposomes and collected by ultra-filtration (Fig. 3A). The BD-2 band with a molecular weight of approximately 4 kDa was detected in two fractions of the liposome-encapsulated BD-2 sample and its ultra-filtrated sample (Fig. 3B). This band was similar to that of the recombinant BD-2. However, BD-2 was not detected in the flow-through fraction obtained by ultra-filtration. These results suggest that the synthesized BD-2 protein was encapsulated in liposomes and collected by ultra-filtration with a nominal molecular weight limit of 3000.

286

287 **Delivery of liposome-encapsulated BD-2 to oral epithelial cells**

288 Oral epithelial cells were cultured with liposome-encapsulated BD-2 protein and a
289 template DNA-free protein and their cellular and medium fractions were assayed by
290 western blotting and ELISA (Fig. 4A). The immunological signal of BD-2 was clearly
291 identified in the cellular and medium fractions from the culture with the synthesized BD-
292 2 protein (Fig. 4B, Sample 3 and 6), and was similar to that of the liposome-encapsulated
293 BD-2 sample before culture (Sample 7) and a recombinant BD-2 (Sample 9). In contrast,
294 a very weak band of BD-2 was detected in the cellular fraction of the culture with only
295 liposome and liposome-encapsulated protein from a template DNA-free (Sample 1 and
296 2) and in the medium fraction of oral epithelial cells at the growth stage (Sample 7).
297 However, there was no BD-2 signal in the medium fraction of the culture with only
298 liposome and liposome-encapsulated protein from a template DNA-free (Sample 4 and
299 5).

300 To investigate the amount of BD-2 protein delivered to oral epithelial cells, the BD-
301 2 protein in the cellular fraction was determined by ELISA (Fig. 4C). BD-2 concentration
302 in the cellular fraction of culture with liposome-encapsulated BD-2 protein was 1.52
303 ng/mL, which was significantly higher than that of liposomes without BD-2 (0.29 ng/mL)

304 and with a template DNA-free protein (0.27 ng/mL) (P<0.01). These results showed that
305 the synthesized BD-2 protein encapsulated in liposomes was delivered to oral epithelial
306 cells.
307
308 **Inhibitory effect of BD-2 on adhesion of *P. gingivalis* to oral epithelial cells**
309 When oral epithelial cells and *P. gingivalis* were treated with the synthesized BD-2 (100
310 ng/mL), the adhesion of bacteria to cells was inhibited by approximately 7% in
311 comparison to a template DNA-free protein (P<0.01), and this inhibitory activity was
312 similar to that of recombinant BD-2 (Fig. 5A). The raw values of fluorescence intensity
313 were 29991 ± 1079 (protein-free; Control), 28243± 852 (synthesized BD-2), 30126 ±
314 1988 (template DNA-free protein) and 28117 ± 1490 (recombinant BD-2). When the
315 effect of liposome-encapsulated BD-2 (300 ng/mL) was investigated, the synthesized BD-
316 2 protein (300 ng/mL) in liposomes decreased adhesion of *P. gingivalis* to oral epithelial
317 cells by 5%, compared to a template DNA-free protein (Fig. 5B). The raw values of
318 fluorescence intensity were 31854 ± 1481 (protein-free; Control), 30516 ± 1127
319 (synthesized BD-2) and 31491 ± 1567 (template DNA-free protein). These results
320 suggested that the artificially synthesized, liposome-encapsulated BD-2 significantly
321 inhibited the adhesion of *P. gingivalis* to oral epithelial cells.

322

323 **Discussion**

324 This study investigated the possibility that the artificially synthesized BD-2 protein
325 contributes to improve oral healthcare. Human gingival epithelial cells (HGECs) were
326 stimulated with *P. gingivalis* ATCC33277 and the expression of BD-2 mRNA in HGECs
327 was increased [30]. BD-2 level in gingival crevicular fluid (GCF) in the chronic
328 periodontitis group was significantly higher than that in healthy control group, and GCF
329 BD-2 concentration was positively correlated with copy numbers of *P. gingivalis* and
330 *Tannerella forsythia* in GCF [31, 32]. These reports suggested that cellular production of
331 BD-2 protein was elevated in periodontal diseases with periodontopathic bacteria in the
332 periodontal pockets.

333 On the other hand, the synthesis of BD-2 using CFPS has not been extensively
334 explored. Xu and Chen *et al.* [33, 34] formed an *E. coli*-cell-free system for production
335 of a fusion protein containing BD-2, in which a plasmid was used for BD-2 and Trx-Tag;
336 they showed that the concentrations of BD-2 fused with green fluorescent protein and
337 trxA-BD-2 fusion protein were 0.25 mg/mL and 0.35 mg/mL, respectively. In the present
338 study, the mean concentration of BD-2 protein synthesized using a PURE system was
339 206.2 μg/mL when its concentration was determined using a BD-2 ELISA kit. Our BD-2

concentration was similar to that reported by Xu and Chen [18, 33, 34], and the procedure of CFPS system in the present study was simple and did not require a plasmid with the BD-2 gene. BD-2 demonstrated antibacterial activity against *P. gingivalis* and susceptibility against *P. gingivalis* ATCC33277 at a minimum inhibitory concentration (MIC) of 34.6 μg/mL compared to the MIC (<250 μg/mL) of *A. actinomycetemcomitans* Y4 [11,12]. The concentration of BD-2 synthesized in the present study (206.2 μg/mL) exceeded MIC (34.6 μg/mL) of BD-2 for *P. gingivalis* ATCC33277 [12]. The artificially synthesized BD-2 significantly inhibited the adhesion of *P. gingivalis* to oral epithelial cells at 100 ng/mL, and liposome-encapsulated BD-2 significantly inhibited bacterial adhesion at a concentration of 300 ng/mL. These results suggest that BD-2, which was artificially synthesized using the CFPS system, has effective antimicrobial activity against *P. gingivalis*.

BD-2 inhibited the growth of several bacteria including *P. gingivalis*, *F. nucleatum, E. coli* and *P. aeruginosa* [8-12]. The mechanism of antimicrobial activity of BD-2 has not yet been defined. BD-2 molecules aggregates and are inserted into the bacterial membrane, and forms multimeric pores in bacterial membranes, thereby change the permeability of bacterial membrane and causes membrane depolarization, and consequently disrupts bacterial membrane [8, 10]. Cationic and amphiphilic BD-2

358 interacts with the lipid bilayer of bacterial membrane and leads to a leak of internal
359 contents from the bacterial cell [8]. The mechanism of inhibition of *P. gingivalis*-adhesion
360 by synthesized BD-2 in the present study has not yet been accurately elucidated. However,
361 the characteristics of BD-2 influence the cell membrane of *P. gingivalis* and appear to
362 disturb bacterial adhesion to oral epithelial cells.

363 Several antimicrobial medications and disinfectants such as doxycycline,
364 minocycline, metronidazole and chlorhexidine are contained in some DDS devices that
365 are capsules, fibers, gels/pastes, particles and strips, and theses complex are delivered to
366 inflammatory periodontal tissues and work as the chemical treatment of periodontal
367 diseases [35-39]. Mou *et al.* [36] reported that minocycline- and zinc oxide-loaded serum
368 albumin encapsulated in hydrogels showed antimicrobial activity against *P. gingivalis* and
369 *Prevotella intermedia*, and Chaturvedi *et al.* [38] showed that nanofibers containing
370 doxycycline improved clinical indicators such as probing depth (PD) and gingival index
371 (GI) in patients with chronic periodontitis. Some preparations with carrier device are
372 marketed for oral care and management of periodontal diseases and administered into
373 periodontal pockets [3]. On the other hand, liposomes are also used as a carrier of
374 medications in the studies and treatments of periodontal diseases. When superoxide
375 dismutase encapsulated into liposome that was composed of soya lecithin and cholesterol

376 and a diameter of 600 nm was applied to around teeth after scaling and root planing in
377 dogs with experimental periodontitis, the levels of PD and GI were significantly
378 decreased [40]. Lactoferrin is known as an antimicrobial peptide that has an iron-binding
379 characteristic, and when bovine lactoferrin with multi-lamellar liposome composed of soy
380 phosphatidylcholine was orally administered to the individuals with multiple sites of
381 more than 3 mm PD for 4 weeks, PD was significantly reduced [41]. Furthermore, Shi *et*
382 *al.* [42] prepared the liposomes composed of Egg-PC, cholesterol and 1,2-distearoyl-sn-
383 glycereo-3-phosphorylethanolamine-polyethylene glycol 2000, with a size within 200 nm,
384 and showed that resveratrol encapsulated into this liposome decreased inflammatory
385 cytokine level in macrophages and alveolar bone resorption, and ameliorated
386 experimental periodontitis in mice. These reports suggested a possibility that AMP,
387 antioxidant and enzyme encapsulated into small-sized liposomes demonstrated the useful
388 effects on periodontal treatments. In contrast, the liposomes prepared in the present study
389 had diameters ranging from 1 to 140 μm and were classified as giant liposome [24, 43].
390 Giant liposomes encapsulate some biological molecules and chemical materials including
391 DNA, RNA, proteins and drugs [44-46], and act as a container of protein synthesis
392 reaction by the PURE system [47] and as vaccine carriers and immunological adjuvants
393 [48], suggesting that giant liposomes are also useful carriers in DDS. We suppose that

394 artificially synthesized BD-2 encapsulated in liposomes may be useful for the prevention
395 of periodontal diseases. Although further studies of the *in vitro* and *in vivo* effects of
396 liposomes encapsulating AMPs are needed to proceed our project in the future, but we
397 believe that the results of this study provide the foundation for the next generation of oral
398 healthcare systems using AMPs.
399
400 **Acknowledgements**
401 This study was supported by Grants-in-Aid for Scientific Research from the Japan Society
402 for the Promotion of Science (17H04418, 20K09941, 20K23083 and 22K17040). We
403 thank GeneFrontier Co., Ltd. (Chiba, Japan) for supporting the design of the template
404 DNA sequences with AT-rich codons for BD-2 synthesis.
405
406 **Author contributions**
407 YH: Planning study, performing experiments (template DNA preparation, synthesized
408 BD-2 analysis, bacterial adhesion assay etc.), writing and reviewing a manuscript and
409 summarizing study. JK: Co-planning study, performing experiments (liposome
410 preparation, western blotting and ELISA), and supporting the preparation of figures in
411 the manuscript. RK: Performing experiments (cell culture, ELISA and western blotting).

KY: Observing liposomes. MB: Supporting SDS-PAGE and western blotting procedures, KK: Instructing liposome preparation and delivery experiments. HY: Advising study performance. YS: Instructing cell-free protein synthesis and evaluating the result data,

Conflict of interest

The authors declare no conflicts of interest related to this study.

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591

592

593 **Figure legends**

594 **Fig. 1** SDS-PAGE, western blotting and ELISA of synthesized BD-2 protein. The
595 synthesized proteins and a recombinant BD-2 protein were analyzed by (A) SDS-PAGE
596 and Coomassie Brilliant Blue staining, and (B) western blotting. These results were
597 confirmed by over three independent protein samples. (C) BD-2 concentration in the
598 synthesized proteins was determined by ELISA. Data are expressed as mean ± SD of 5
599 samples. $^{**}P<0.01$. SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel
600 electrophoresis, ELISA: enzyme-linked immunosorbent assay, sBD-2: synthesized BD-2,
601 tDNA free: template DNA-free protein, rBD-2: recombinant BD-2

602

603 **Fig. 2** Liposome preparation and observation. (A) Liposomes were prepared according to
604 a spontaneous transfer method with DOPC, DOPE and Egg-PC. (B) The prepared
605 liposomes were observed using an inverted microscope at magnification x 20. Scale bars:

606 100 μm. DOPC: 1,2-Dioleoyl-sn-glycero-3-phosphocholine, DOPE: 1,2-dioleoyl-sn-
607 glycero-3 phosphoethanolamine, Egg-PC: 3-sn-phosphatidylcholine from egg yolk
608
609 **Fig. 3** Encapsulation of synthesized BD-2 protein into liposomes. (A) Procedure of
610 encapsulation of synthesized BD-2 in liposomes and collection. (B) Synthesized BD-2
611 samples were analyzed by western blotting. Sample 1: synthesized BD-2 sample, Sample
612 2: liposome-encapsulated Sample 1, Sample 3: ultra-filtrated Sample 2, Sample 4: flow-
613 through of ultra-filtrated Sample 2, Sample 5: recombinant BD-2. This result was
614 confirmed by three independent protein samples.
615
616 **Fig. 4** Delivery of liposome-encapsulated BD-2 to oral epithelial cells. (A) Protocol of
617 delivery of the liposome-encapsulated BD-2 to cells, collection and assays. (B) Oral
618 epithelial cells were cultured with liposome-encapsulated samples of protein-free, a
619 template DNA-free protein and the synthesized BD-2 protein for 16 h, and the cellular
620 and medium fractions were collected and analyzed by western blotting for BD-2.
621 Sample 1: cellular fraction of protein-free, Sample 2: cellular fraction of a template DNA-
622 free protein, Sample 3: cellular fraction of synthesized BD-2 protein, Sample 4: medium
623 fraction of protein-free, Sample 5: medium fraction of a template DNA-free protein,

624 Sample 6: medium fraction of synthesized BD-2 protein, Sample 7:liposome-
625 encapsulated BD-2 protein before addition to cell culture, Sample 8: conditioned medium
626 of TR146 cells, Sample 9: recombinant BD-2. This result was confirmed by three
627 independent protein samples. (C) BD-2 concentration in cellular fractions derived from
628 protein-free, a template DNA-free protein and synthesized BD-2 protein were determined
629 by ELISA. Data are expressed as mean ± SD of 6-12 samples. **P<0.01. Cont.: protein-
630 free, tDNA free: template DNA-free protein, sBD-2: synthesized BD-2
631
632 **Fig. 5** Inhibitory effect of BD-2 on adhesion of *P. gingivalis* to oral epithelial cells. (A)
633 Effect of the synthesized BD-2. (B) Effect of the liposome-encapsulated synthesized BD-
634 2. The mean fluorescence intensity of Control in (A) and (B) were 29991 and 31854,
635 respectively, and values are shown as percentage of Control. Data are expressed as the
636 mean ± SD of all samples from three independent experiments, (A) n=24 samples
637 (n=8/experiment) and (B) n=30 samples (n=10/experiment). **P<0.01. Cont.: protein-
638 free, sBD-2: synthesized BD-2, tDNA free: template DNA-free protein, rBD-2:
639 recombinant BD-2

Figure 1

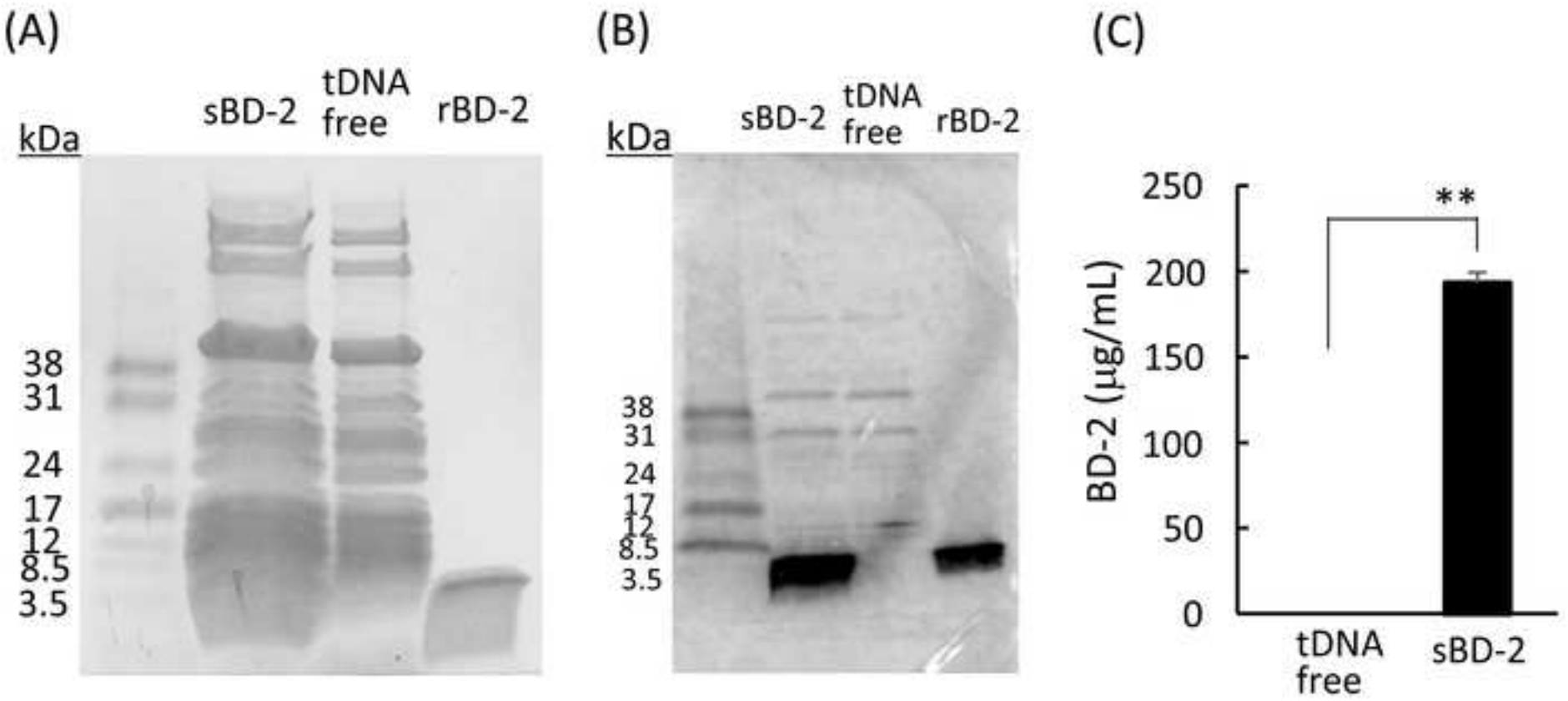

(A)
kDa
sBD-2
tDNA free
rBD-2
38
31
24
17
12
8.5
3.5
(B)
kDa
sBD-2
tDNA free
rBD-2
38
31
24
17
12
8.5
3.5
(C)
BD-2 (μg/mL)
250
200
150
100
50
0
**
tDNA free
sBD-2

Figure 2

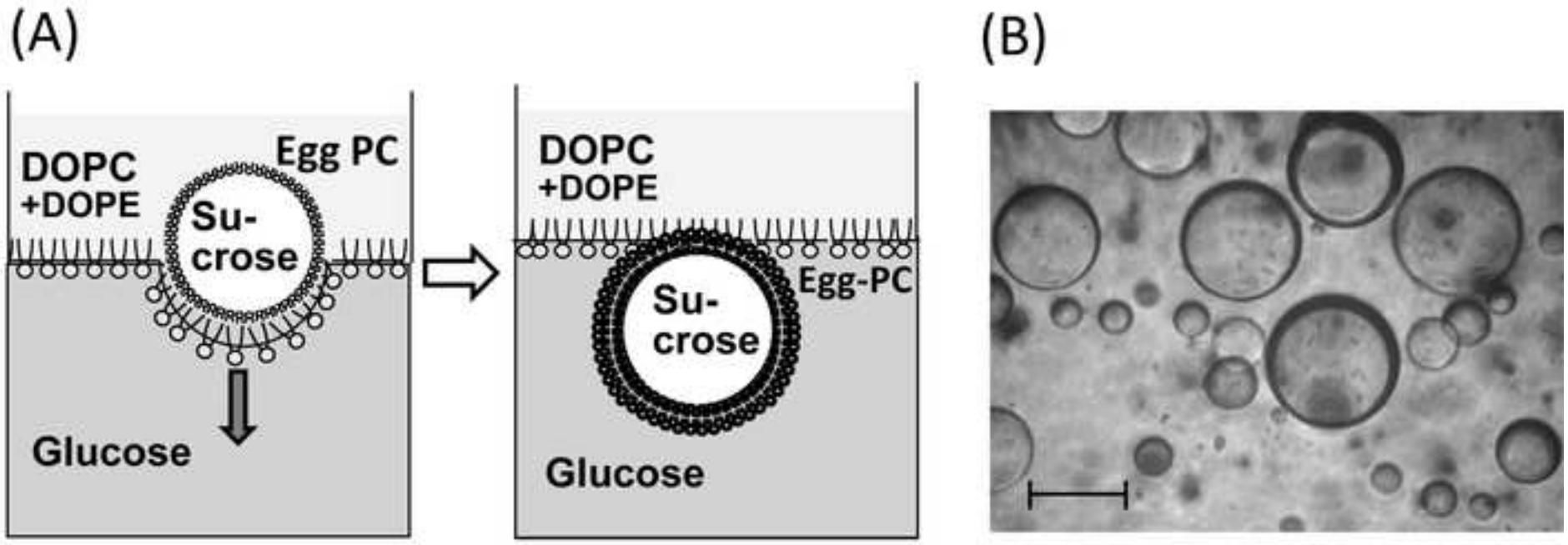

(A)
DOPC
+DOPE
Egg PC
Su-
crose
Glucose
DOPC
+DOPE
Egg-PC
Su-
crose
Glucose
(B)

Figure 3

(A)

< Procedure of sample preparation >

Synthesis of BD-2 (sBD-2)

⇩

Encapsulation of sBD-2 into Liposomes

⇩

Ultra-filtration

⇩

Collection of liposome-encapsulated sBD-2

(B)

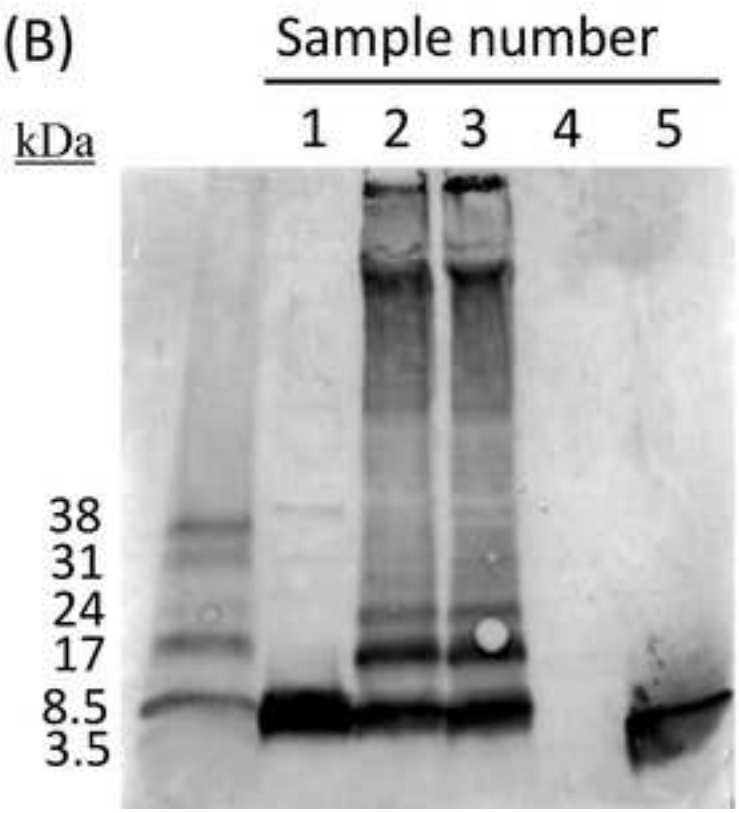


Figure 4

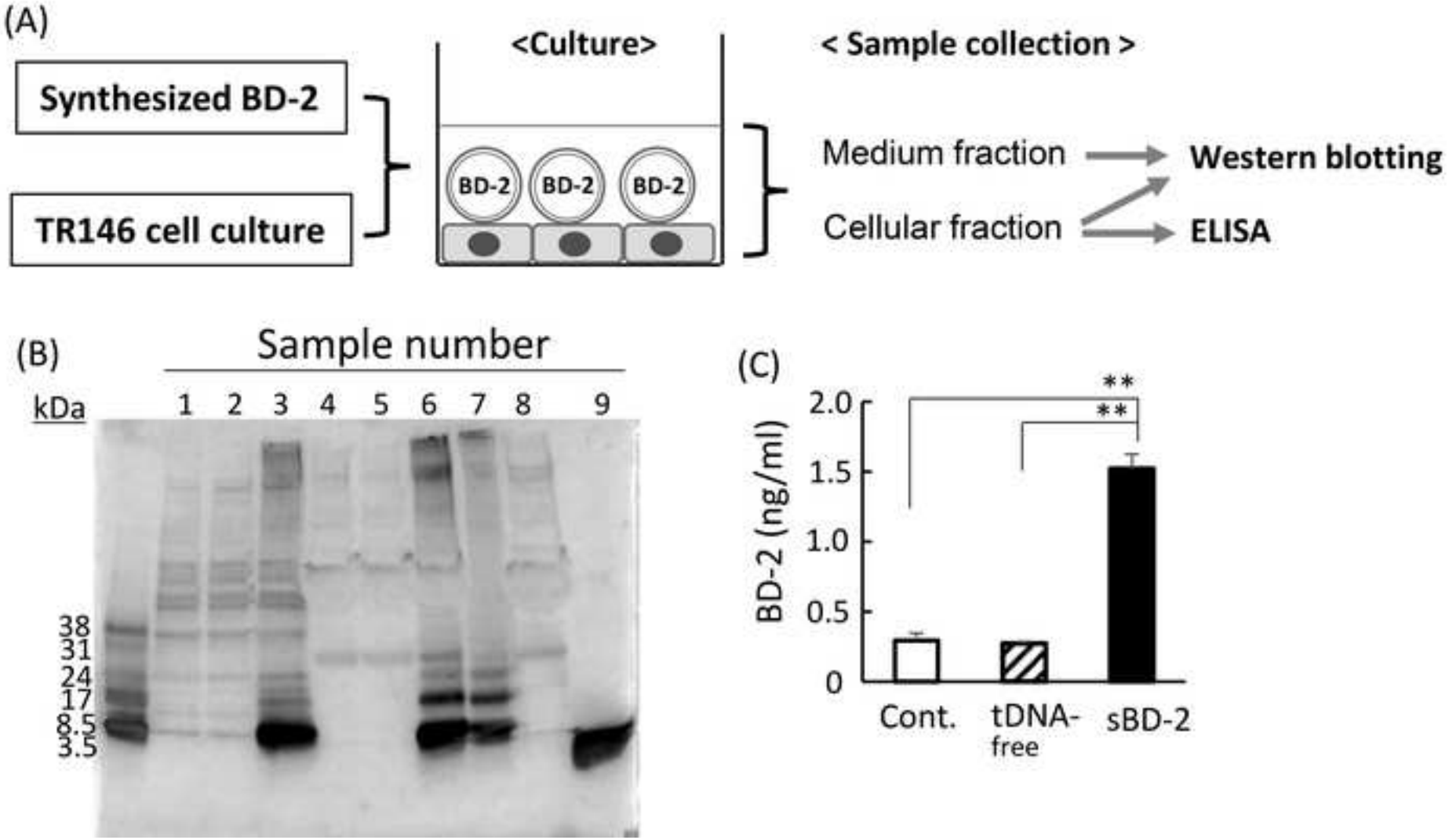
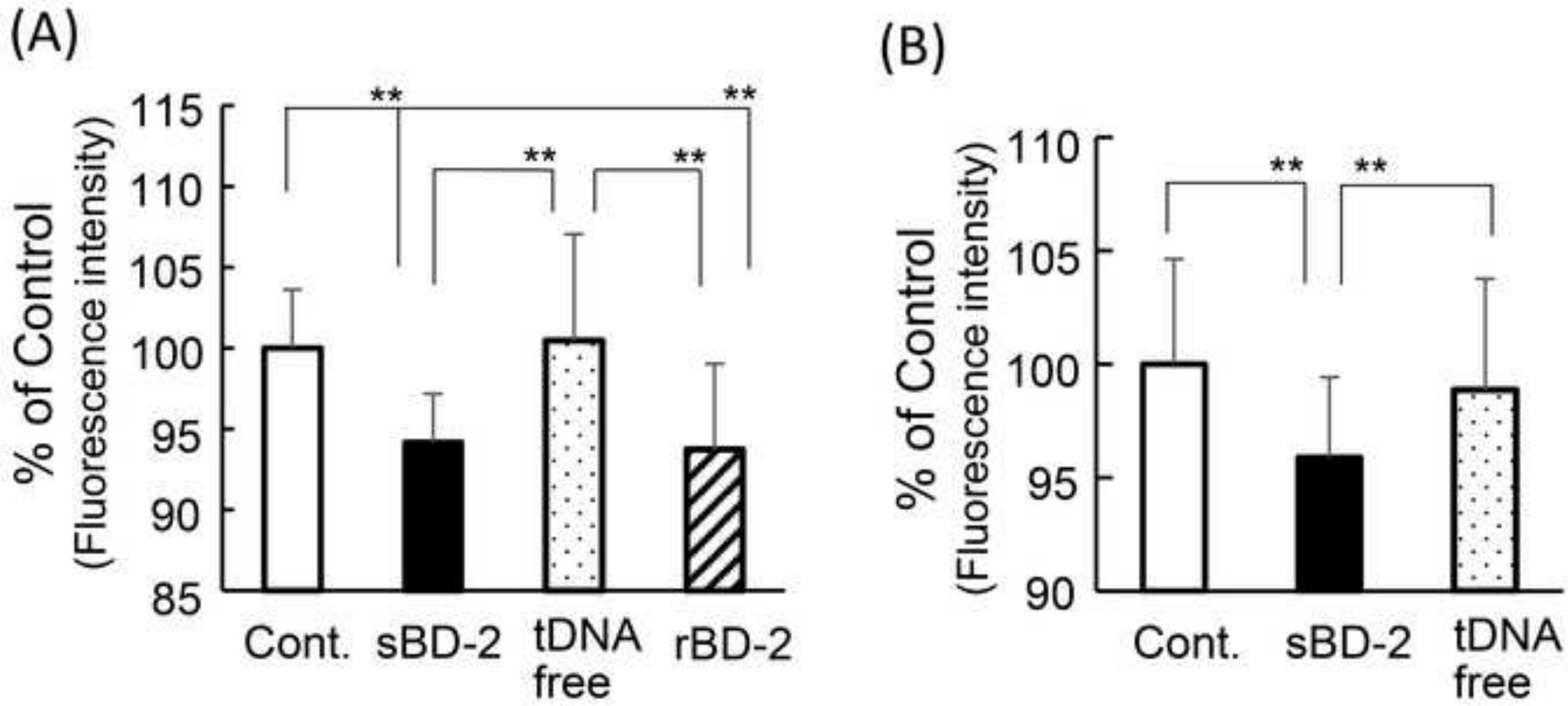
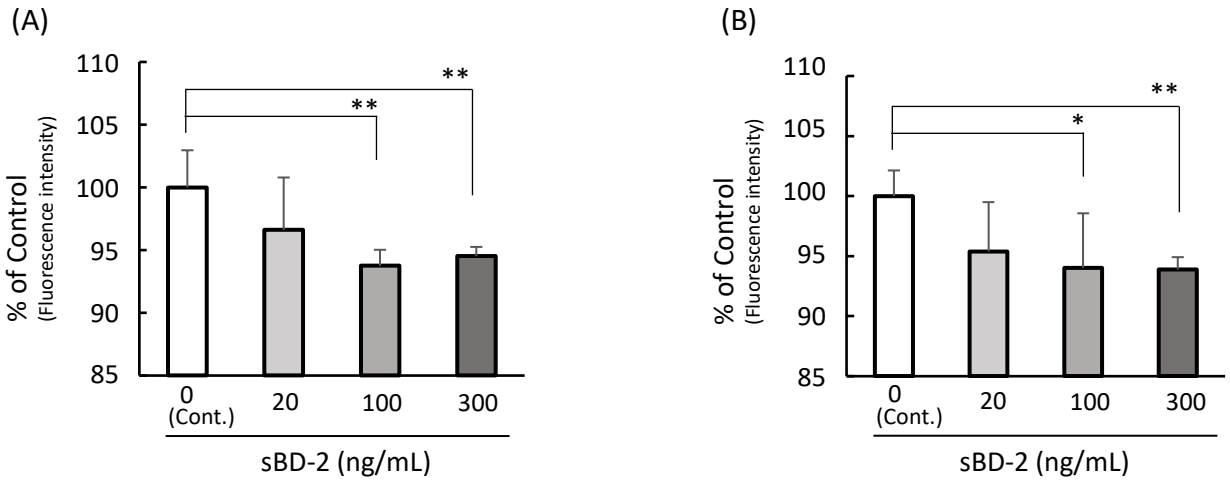

(A)
Synthesized BD-2
TR146 cell culture
<Culture>
BD-2
BD-2
BD-2
< Sample collection >
Medium fraction
Western blotting
Cellular fraction
ELISA
(B)
Sample number
kDa
1 2 3 4 5 6 7 8 9
38
31
24
17
8.5
3.5
(C)
BD-2 (ng/ml)
2.0
1.5
1.0
0.5
0
**
**
Cont.
tDNA-free
sBD-2

Figure 5


(A)
% of Control
(Fluorescence intensity)
115
110
105
100
95
90
85
**
**
**
**
Cont.
sBD-2
tDNA free
rBD-2
(B)
% of Control
(Fluorescence intensity)
110
105
100
95
90
**
**
Cont.
sBD-2
tDNA free



Supplemental data. Effect of the synthesized BD-2 and its liposome-encapsulated protein on the adhesion of *P. gingivalis* to oral epithelial cells. Oral epithelial cells (TR146 cells) were cultured with the synthesized BD-2 (A, sBD-2, 0-300 ng/mL) and liposome-encapsulated sBD-2 (B, 0-300 ng/mL) for 16 h. *P. gingivalis* labeled with a fluorescent reagent were co-cultured with the cells for 2 h in the medium containing sBD-2 and liposome-sBD-2. After washing the culture, a fluorescent intensity was determined using a fluorescence plate reader at 485/535 nm (excitation/emission). The values were expressed as percentage of each Control. Data are shown as mean ± SD of 4-5 samples (A) and 6 samples (B). **P*<0.05, ***P*<0.01.