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A Novel Screening System for Claudin Binder Using Baculoviral Display

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Abstract

Recent progress in cell biology has provided new insight into the claudin (CL) family of integral membrane proteins, which contains more than 20 members, as a target for pharmaceutical therapy. Few ligands for CL have been identified because it is difficult to prepare CL in an intact form. In the present study, we developed a method to screen for CL binders by using the budded baculovirus (BV) display system. CL4-displaying BV interacted with a CL4 binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), but it did not interact with C-CPE that was mutated in its CL4-binding region. C-CPE did not interact with BV and CL1-displaying BV. We used CL4-displaying BV to select CL4-binding phage in a mixture of a scFv-phage and C-CPE-phage. The percentage of C-CPE-phage in the phage mixture increased from 16.7% before selection to 92% after selection, indicating that CL-displaying BV may be useful for the selection of CL binders. We prepared a C-CPE phage library by mutating the functional amino acids. We screened the library for CL4 binders by affinity to CL4-displaying BV, and we found that the novel CL4 binders modulated the tight-junction barrier. These findings indicate that the CL-displaying BV system may be a promising method to produce a novel CL binder and modulator.

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Introduction

Tight junctions (TJ) are intercellular adhesion complexes in epithelial and endothelial cells; TJs are located in the most apical part of the complexes [1]. TJs have a barrier function and a fence function [2–4]. TJs contribute to epithelial and endothelial barrier functions by restricting the diffusion of solutes through the paracellular pathway. TJs maintain cellular polarity by preventing the free movement of membrane proteins between the apical and basal membranes [5]. Loss of cell-cell adhesion and cellular polarity commonly occurs in the early stages of cancer [6]. Modulation of the TJ barrier function can be a method to enhance drug absorption, and TJ components exposed on the surface of cancer cells can be a target for cancer therapy.

Biochemical analyses of TJs have identified TJ components, such as occludin, claudins (CLs) and junction adhesion molecule [7]. The CL family contains more than 20 integral tetra-transmembrane proteins that play pivotal roles in the TJ barrier and fence functions. CL1-deficient mice lack the epidermal barrier, while CL5-deficient mice lack the blood-brain barrier [8,9], indicating that the regulation of the TJ barrier by modulation of CLs may be a promising method for drug delivery. *Clostridium perfringens* enterotoxin (CPE) causes food poisoning in humans [10]. An interaction between the C-terminal domain of CPE (C-CPE) with CL4 deregulates the TJ barrier [11,12]. We previously found that C-CPE enhances jejunal absorption through its interaction with CL4, indicating that a CL binder is a potent drug-delivery system [13].

The majority of lethal cancers are derived from epithelial tissues [14]. Malignant tumor cells frequently exhibit abnormal TJ function, followed by the deregulation of cellular polarity and intercellular contact, which is commonly observed in both advanced tumors and the early stages of carcinogenesis [6]. Some CLs are overexpressed in various types of cancers. For example, CL3 and CL4 are overexpressed in breast, prostate, ovarian, pancreatic and gastric cancers. CL1, CL7, CL10 and CL16 are overexpressed in colon, gastric, thyroid and ovarian cancers, respectively [15,16]. These findings indicate that the CLs may be a target molecule for cancer therapy. A receptor for CPE is CL4 [11,12]. CPE has anti-tumor activity against human pancreatic and ovarian cancers without side effects [17,18]. The CLs binders will be useful for cancer-targeting therapy.

As above, recent investigations of CLs provide new insight into their use as pharmaceutical agents; for example, a CL binder may be used in drug delivery and anti-tumor therapy. Selection of a CL binder by using a recombinant CL protein is a putative method to

prepare a CL binder. However, CLs are four-transmembrane proteins with high hydrophobicity; there has been little success in the preparation of intact CL protein. Recently, a novel type of protein expression system that uses baculovirus has been developed. Membrane proteins are displayed on the budded baculovirus (BV) in their active form [19–21], indicating that the BV system may be useful for the preparation of a CL binder. In the present study, we investigated whether a CL binder was screened by using a CL-displaying BV.

Results

Preparation of CL4-displaying BV

C-CPE is the only known CL binder and modulator [12,13,22]. C-CPE has affinity to CL4 in a nanomolar range [23]. We chose C-CPE and CL4 as models of the CL binder and CL, respectively. Several reports indicate that membrane proteins expressed on the surface of BV are in an intact form [19–21]. To check the expression of CL4 on the BV, we performed immunoblot analysis of the lysate of CL4-BV against CL4. As shown in Fig. 1A, CL4 was detected in the virus lysates. To determine if the CL4 expressed on the virus has an intact form, we performed enzyme-linked immunosorbent assay (ELISA) with CL4-BV-coated immunoplates. C-CPE binds to the extracellular loop domain of CL4 [23]. After the addition of C-CPE to the CL4-BV-coated plate, the C-CPE bound to the CL4-BV-coated plate was detected by anti-his-tag antibody, followed by incubation with horseradish peroxidase-labeled antibody. C-CPE was dose-dependently bound to CL4-BV, whereas C-CPE did not interact with wild-BV (Fig. 1B). Deletion of the CL4-binding region (C-CPE303) attenuated the interaction of C-CPE with CL4-BV (Fig. 1C). Together, these results indicate that the CL4 displayed on BV may have an intact extracellular loop region.

Selection of C-CPE-phage by using CL4-BV

We next examined the interaction between C-CPE-phage and CL4-BV. As shown in Fig. 2A, C-CPE-phage bound to CL4-BV but not to wild-BV, and a scFv-phage did not bind to CL4-BV. To determine if CL-BV can be used to select CL binders, we prepared a mixture of C-CPE-phage and scFv-phage at a ratio of 2:10 and used CL4-BV to select CL4-binding phage in the mixtures. The amount of C-CPE-phage was increased to 11 of 12 clones in the mixture (Fig. 2B), indicating that CL-BV may be useful in the preparation of CL binders.

We previously found that each substitution of S304, S305, S307, N309, S313 and K318 with alanine increased the binding of C-CPE to CL4 [24]. Here, we prepared a phage library for C-CPE by randomly changing the functional 6 amino acids to any of the 20 amino acids. To confirm the diversity of the library, we checked the sequences of 17 randomly isolated clones. Each of the 17 clones had a different sequence, indicating that the library has a diverse population of C-CPE mutants (Table 1).

Then, we screened the CL4-binding phage by their affinity to CL4-BV. After addition of the C-CPE library to CL4-BV-adsorbed tubes, the CL4-BV-bound phages were recovered (1st screening). We repeated this screening process two more times (2nd screening and 3rd screening). If the number of CL4-bound phage is increased during the screening, the ratio of the incubated phage titers to the recovered phage titers will increase. As shown in Fig. 3A, the ratio was increased during screening from 4.5×10^{-7} to 5.5×10^{-5}, indicating that the screening system for CL4 binders may work. Indeed, the number of monoclonal phage clones with high affinity to CL4-BV was increased after the 3rd screening compared with that after the 2nd screening (Fig. 3B).

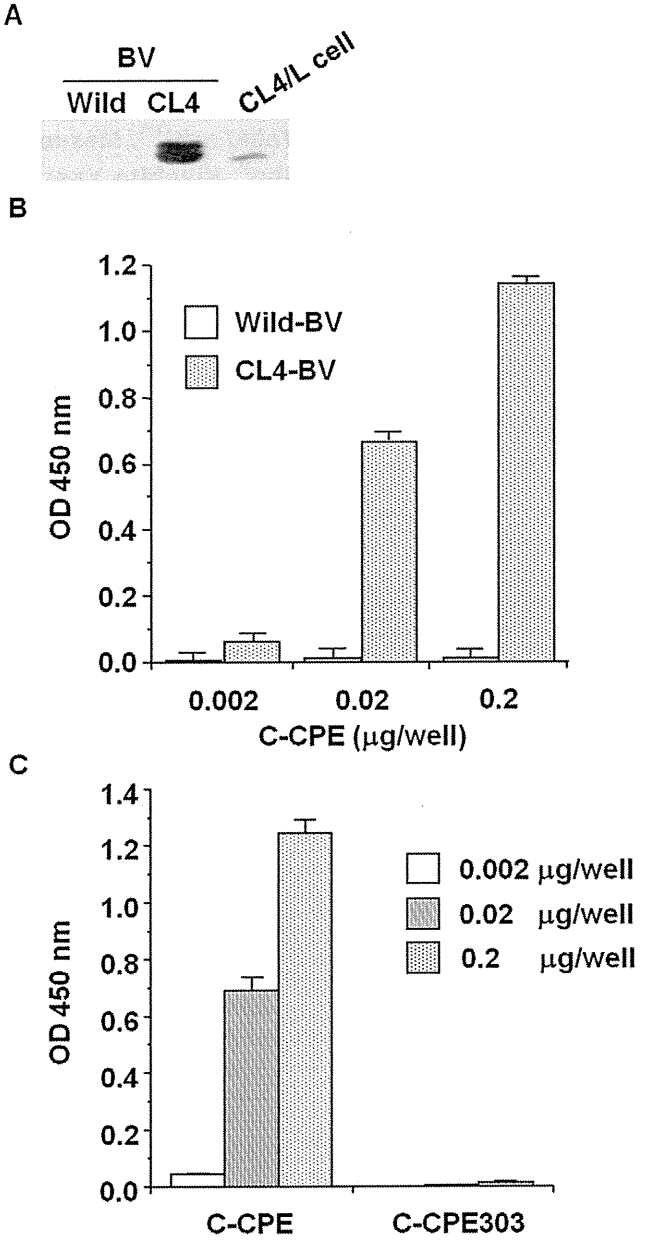

Figure 1. Preparation of CL4-displaying BV. A) Immunoblot analysis. Wild-BV and CL4-BV (0.1 μg/lane) were subjected to SDS-PAGE, followed by immunoblot analysis with anti-CL4 antibody. The lysate of CL4-expressing L (CL4/L) cells was used as a positive control. B, C) Interaction of a CL4 binder with CL4-BV. Immunotubes were coated with the wild-BV or CL4-BV, and C-CPE (B) or mutated C-CPE (C) was added to the BV-coated immunotubes at the indicated concentration. C-CPE bound to the BV-coated tubes was detected by ELISA with an anti-his-tag antibody.
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We analyzed the sequences of the CL4-BV-bound phages and got novel CL4-binder candidates with amino acid sequences that differed from the wild-type sequence (Table 2). To investigate their CL4-binding, we prepared the recombinant proteins of the binders and investigated their interaction with CL4 by ELISA with CL-BVs. As shown in Fig. 4A, the novel C-CPE derivatives had affinity to CL4 but not CL1. Next, we investigated whether the novel CL4 binders modulate TJ barrier in Caco-2 monolayer cell sheets, a popular model for the evaluation of TJ barriers [25].

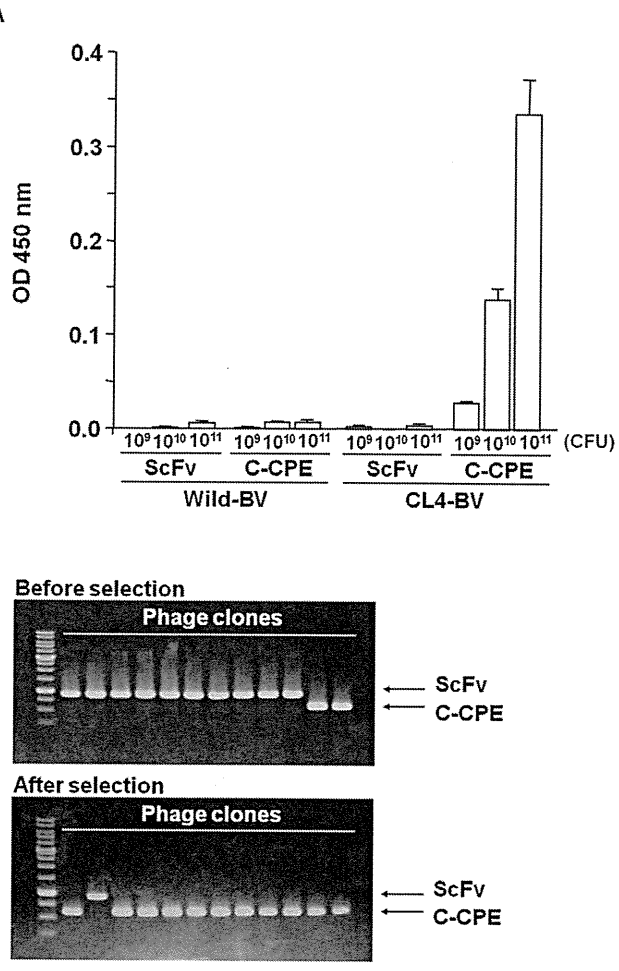

Figure 2. Selection of C-CPE-displaying phage by using the CL4-BV system. A) Interaction of C-CPE-displaying phage with CL4-BV. Wild-BV or CL4-BV was coated on an immunoplate, and then scFv-displaying phage or C-CPE-displaying phage was added to the BV-coated immunoplate at the indicated concentrations. The BV-bound phages were detected by ELISA with anti-M13 antibody as described in Materials and methods. Data are representative of two independent experiments. Data are means ± SD (n = 3). B) Enrichment of C-CPE-displaying phage by the BV system. A mixture of scFv-phage and C-CPE-phage (mixing ratio of scFv-phage to C-CPE-phage = 2:10) was incubated with a CL4-BV-coated immunotube, and the bound phages were recovered. Each phage clone was identified by PCR amplification, followed by agarose gel electrophoresis. Upper and lower pictures are before and after the selection, respectively. The putative sizes of the PCR products are 856 and 523 bp in scFv and C-CPE, respectively. The data are representative of two independent experiments.
doi:10.1371/journal.pone.0016611.g002

Treatment of the cells with C-CPE resulted in decreased transepithelial electrical resistance (TEER) values, a marker of TJ integrity, and the TEER values increased after removal of C-CPE. The C-CPE derivatives (clones 1–5) had TJ-modulating activity similar to that of C-CPE (Fig. 4B).

Discussion

CL is a promising target for pharmaceutical therapy. However, CL has low antigenicity, and there has been little success in the preparation of monoclonal antibody against the extracellular loop region of CL. The three-dimensional structure of CL has never been determined, so it is impossible to perform a theoretical design of a CL binder based on the structural information. In the present study, we developed a novel screening system for CL binders by using a BV system and a C-CPE phage display library, and we used this system to identify novel CL4 binders.

Table 1. C-CPE phage library.

	304	305	307	309	313	318
C-CPE	**S**	**S**	**S**	**N**	**S**	**K**
Clone 1	V	T	C	V	N	K
2	C	P	A	H	L	T
3	A	G	G	V	P	P
4	R	G	H	L	E	H
5	A	A	P	S	R	Q
6	P	A	P	D	P	A
7	C	T	T	T	N	K
8	H	P	S	P	G	H
9	R	G	G	R	N	R
10	A	P	S	T	Q	P
11	V	L	G	N	M	R
12	P	P	A	T	F	R
13	G	D	C	S	N	L
14	F	R	V	F	R	N
15	S	Q	Q	W	T	T
16	S	R	L	E	W	Q
17	K	R	E	R	Q	S

Phage clones were randomly picked up from the C-CPE phage library, and the amino acids sequences of C-CPE mutant were analyzed.
doi:10.1371/journal.pone.0016611.t001

In ligand screening, the preparation of a receptor for the ligand is very critical. Membrane proteins are especially difficult to prepare as recombinant protein with an intact structure. Functional membrane proteins such as cell-surface proteins are heterologously expressed on BV in their native forms [19–21]. Interactions between membrane proteins can be detected by using receptor-displaying and ligand-displaying BV [21]. In the present report, we found that CL4-BV interacts with a CL4 binder, C-CPE, but it does not interact with C-CPE303 that lacks the CL4-binding residues of C-CPE. The CL4-binding site of C-CPE corresponds to that of CPE; so, the second extracellular loop of CL appears to be the C-CPE-binding site [23,26]. These findings indicate that CL4 displayed on BV may have native form. We anticipate that CL-BV will be useful for the preparation of CL binders, such as peptides and antibodies.

To the best of our knowledge, the preparation of CL binder has been performed by only four groups. Offner et al. prepared polyclonal antibodies against extracellular domains of CL3 and CL4 [27], Ling et al. screened peptide types of CL4 binder by using a 12-mer peptide phage display library and CL4-expressing cells [28], Suzuki et al. generated a monoclonal antibody against the second extracellular loop of CL4 from mice immunized with a human pancreatic cancer cell line [29] and Romani et al. screened scFv against CL3 by using a human antibody phage display library [30]. However, the CL modulators have never been developed; thus, C-CPE is the only known CL4 modulator [12]. In the present study, we prepared a C-CPE phage library containing C-CPE mutants in which each of the 6 functional amino acids was randomly replaced with an amino acid, and we isolated CL4 binders by using CL4-BV as a screening ligand. Interestingly, all of

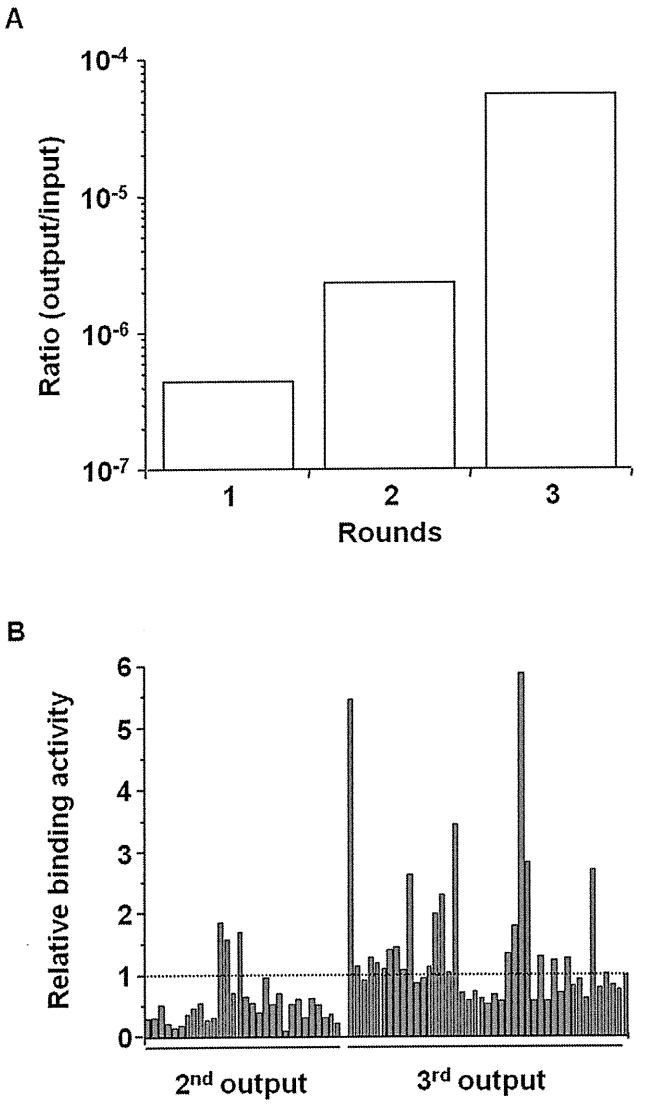

Figure 3. Screening of a novel CL4 binder. A) Enrichment of phages with affinity to CL4-BV. CL4-BVs coated on immunotubes were incubated with the C-CPE-derivative phage library at 1.6×10^{12} CFU titer (1^{st} input phage). The phages bound to CL4-BV were recovered (1^{st} output phage). The CL4-BV-binding phages were subjected to two additional cycles of the incubation and wash step, resulting in 2^{nd}, 3^{rd} output phage. The ratio of output phage to input phage titers was calculated. B) Monoclonal analysis of C-CPE-derivative phage. CL4-BV-bound phage clones were isolated from the 2^{nd} and 3^{rd} output phages, and the interaction of the monoclonal phage with CL4-BV was examined by ELISA with anti-M13 antibody as described in Materials and methods. Data are expressed as relative binding to that of C-CPE-phage indicated by the most right column.
doi:10.1371/journal.pone.0016611.g003

the CL4 binders modulated TJ barriers. We are investigating why the substitution of the amino acids with the other amino acids modulated CL4. These findings indicate that a BV screening system with a C-CPE library may be a powerful method to develop CL modulators.

The CL family forms various types of TJ barriers through combinations of its more than 20 members in homophilic/heterophilic CL strands [31,32]. Intercellular proteins ZO-1 and ZO-2 determine the localization of CL strands [33]. If a screening system to reconstitute heterogeneous CL strands with ZO-1 and/or ZO-2 is developed, then useful and effective CL modulators can be identified. In this point, the BV system has extremely superior features. G protein and G protein-coupled receptors have been

Table 2. CL4-binding phages.

	304	305	307	309	313	318
C-CPE	S	S	S	N	S	K
Clone 1	R	V	S	A	R	R
2	R	S	V	A	R	K
3	G	D	G	R	T	R
4	S	A	P	R	S	A
5	R	S	L	K	S	K

The sequences of C-CPE mutant in the CL4-binding phages were analyzed.
doi:10.1371/journal.pone.0016611.t002

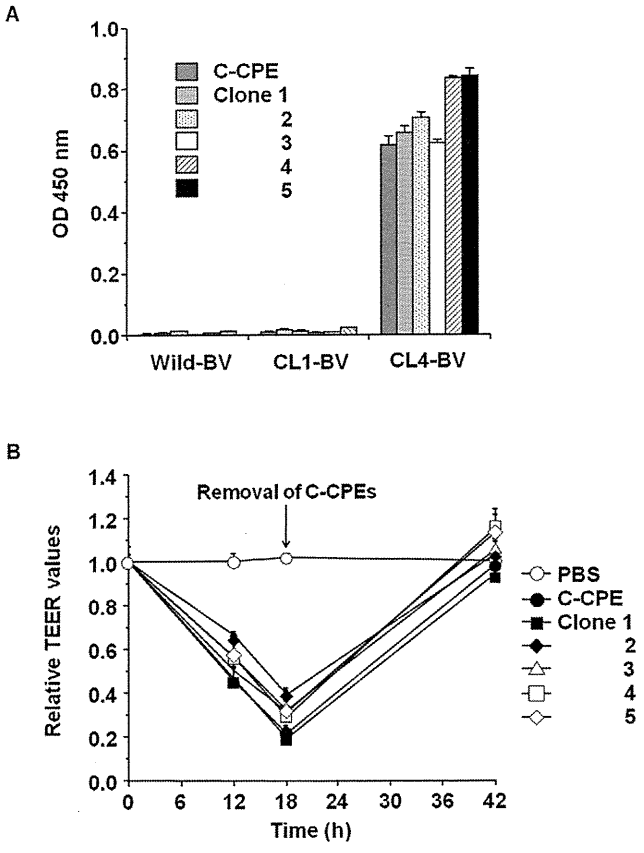

Figure 4. Isolation of a novel CL4 modulator. A) Interaction of the C-CPE derivatives with CL4. C-CPE derivatives were prepared as his-tagged recombinant proteins. The C-CPE derivatives (0.02 μg) were added to CL-BV-coated immunoplates, followed by detection of the C-CPE derivatives bound to CL-BV. Data are means ± SD (n = 4). B) Modulation of tight junction-barriers. Caco-2 cells were cultured on Transwell™ chambers. When TEER values reach a plateau, the cells were treated with C-CPE or C-CPE derivatives at the indicated concentrations. After 18 h of exposure to the C-CPEs, the cells were washed with medium to remove C-CPEs, and then the cells were cultured for an additional 24 h. Changes in TEER values were monitored during the C-CPEs treatment. Relative TEER values were calculated as the ratio of TEER values at 0 h. Data are representative of two independent experiments. The data are means ± SD (n = 4).
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functionally reconstituted in BV [20,34], and functional γ-secretase complexes have also been reconstituted on BV [35]. In the near future, the reconstituted CL system on BV will be developed and used for the screening of CL binders and modulators, hopefully leading to breakthroughs in pharmaceutical therapies that target CLs.

Materials and Methods

Recombinant BV construction and Sf9 cell culture

Recombinant BV was prepared by using the Bac-to-Bac expression system, according to the manufacturer's instructions (Invitrogen, Gaithersburg, MD). Briefly, mouse CL1 and CL4 cDNA (kind gifts from Dr. M Furuse, Kobe University, Japan) were inserted into pFastBac1, and the resulting plasmids were transduced into DH10Bac *E. Coli* cells. Recombinant bacmid DNA was extracted from the cells. Sf9 cells were transduced with the bacmid coding CL, and the recombinant BV was recovered by centrifugation of the conditioned medium [36].

Preparation of the BV fractions

Sf9 cells (2×10^6 cells) were infected with recombinant BV at a multiplicity of infection of 5. Seventy-two hours after infection, the BV fraction was recovered from the culture supernatant of infected Sf9 cells by centrifugation. The pellets of the BV fraction were resuspended in Tris-buffered saline (TBS) containing 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and then stored at 4°C until use. The expression of CL1 and CL4 in the BV was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with anti-CL antibodies (Zymed Laboratory, South San Francisco, CA).

Preparation of mutant C-CPE library

C-CPE fragments in which the functional amino acids (S304, S305, S307, N309, S313 and K318) [24] were randomly mutated were prepared by polymerase chain reaction (PCR) with pET-H_{10}PER as a template, a forward primer (5′-catgccatggccgatatagaaaaagaaatccttgatttagctgctg-3′, Nco I site is underlined) and a reverse primer (5′-ttttccttttgcggccgcaaa*nnn*ttgaaataatat*nnn*ataagggta*nnn*tcc*nnn*ata*nnn**nnn*attagcttt-3′, Not I site is underlined, and the randomly mutated amino acids are in italics). The PCR fragments were inserted into a pY03 phagemid at the NcoI/NotI sites [22]. The resultant phagemid containing the C-CPE mutant library was transduced into *E. coli* TG1 cells, and then the cells were stored at −80°C.

Preparation of phage

TG1 cells containing phagemid coding a scFv, C-CPE, C-CPE mutant or C-CPE mutant library were culture in 2YT medium containing 2% glucose and ampicillin. When the cells grew to a growing phage, M13K07 helper phages (Invitrogen) were added, and the medium was changed into 2YT medium containing ampicillin and kanamycin. After an additional 6 h of culture, the phages in the conditioned medium were precipitated with polyethylene glycol. The phages were suspended in phosphate-buffered saline (PBS) and stored at 4°C until use.

ELISA

Wild-BVs or CL-BVs (0.5 μg/well) were adsorbed onto an immunoplate (Greiner Bio-One, Frickenhausen, Germany). The wells were washed with PBS and blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan). C-CPEs or phages were incubated in the immunoplate, and the BV-bound C-CPEs or phages were detected by using anti-his-tag antibody (Novagen, Darmstadt, Germany) or anti-M13 antibody (Amersham-Pharmacia Biotech, Uppsala, Sweden), respectively, horseradish peroxidase-labelled secondary antibody and TMB peroxidase substrate (Nacalai Tesque, Kyoto, Japan). The immunoreactive C-CPEs or phages were quantified by the measurement of absorbance at 450 nm. In the screening of phages, the data were normalized by the amounts of phages, which were quantified by ELISA for the FLAG-tag contained in the coat protein.

Selection of phage by using BV

A total of 0.5 μg of BV was adsorbed onto an immunotube (Nunc, Roskilde, Denmark). The tube was washed with PBS and blocked with TBS containing 4.0% BlockAce. The BV-coated tubes were incubated with mixture of phages, and then the tubes were washed 15 times with PBS and 15 times with PBS containing 0.05% Tween 20. The phages bound to the tube were eluted with 100 mM HCl. TG1 cells were infected with the eluted phages, and phages were prepared as described above. The resulting phages were subjected to repeated selection by using the BV-coated immunotubes.

Identification of a phage clone

To identify an isolated phage clone, we performed PCR or sequencing analysis. We amplified the inserted fragment into the phagemid by PCR using forward primer 5′-caggaaacagctatgac-3′ and reverse primer 5′-gtaaatgaattttctgtatgagg-3′. The resultant PCR products were subjected to agarose gel electrophoresis followed by staining with ethidium bromide. We performed a sequence analysis with primer 5′-gtaaatgaattttctgtatgagg-3′.

Measurement of phage titer

To quantify the concentration of phages, we measured the titer (colony formation unit (CFU)/ml) of the phage solution. Briefly, the phage solution was diluted to 10^{-5}–10^{-10} with PBS. The diluted solution was seeded onto Petrifilm™ (Tech-Jam, Osaka, Japan). After 24 h of incubation, the colonies were counted, and the titer was calculated.

Purification of C-CPE mutants

C-CPE and C-CPE303, in which the CL-4 binding region of C-CPE was deleted, were prepared as described previously [13]. To prepare plasmid containing C-CPE mutants, the C-CPE mutant fragment was PCR-amplified by using phagemids coding C-CPE mutants as a template. The resulting PCR fragment was inserted into pET16b, and the sequence was confirmed. The plasmids were transduced into *E. coli* strain BL21 (DE3), and production of mutant C-CPEs was induced by the addition of isopropyl-D-thiogalactopyranoside. The harvested cells were lysed in buffer A (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol) that was supplemented with 8 M urea when necessary. The lysates were applied to HiTrap™ Chelating HP (GE Healthcare, Buckinghamshire, UK), and mutant C-CPEs were eluted with buffer A containing 100–400 mM imidazole. The buffer was exchanged with PBS by using a PD-10 column (GE Healthcare), and the purified protein was stored at −80°C until use. Purification of the mutant C-CPEs was confirmed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-his-tag antibody (Novagen). Protein was quantified by using a BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical, Rockford, IL).

TEER assay

Caco-2 cells were seeded in Transwell^{TM} chambers (Corning, NY) at a subconfluent density. The TEER of the Caco-2 monolayer cell sheets on the chamber was monitored by using a Millicell-ERS epithelial volt-ohmmeter (Millipore, Billerica, MA). When TEER values reached a plateau, indicating that TJs were well-developed in the cell sheets, the Caco-2 monolayers were treated with C-CPE or C-CPE mutants on the basal side of the chamber. Changes in TEER values were monitored. The TEER values were normalized by the area of the Caco-2 monolayer, and the TEER value of a blank Transwell^{TM} chamber (background) was subtracted.

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Author Contributions

Conceived and designed the experiments: MK TS TH KY. Performed the experiments: HK AT MK YS TY TS. Analyzed the data: HK AT MK KY. Contributed reagents/materials/analysis tools: HK AK TS TH. Wrote the manuscript: HK MK TY.

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A toxicological evaluation of a claudin modulator, the C-terminal fragment of *Clostridium perfringens* enterotoxin, in mice

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Tight junctions (TJs) maintain cellular polarity between the apical and basolateral region of epithelial cells. Claudin, a tetra-transmembrane protein, plays a pivotal role in the barrier function of TJs. We previously found that a claudin modulator, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), may be a promising candidate for improving the mucosal absorption of drugs. C-CPE is a fragment of enterotoxin, and putative CPE claudin receptors are highly expressed in liver and kidney. The safety and antigenicity of C-CPE must be evaluated for future clinical application. Therefore, we evaluated whether C-CPE administration in mice leads to tissue injury or production of antibodies. Intravenous administration of C-CPE at 5 mg/kg, which is a more than 25-fold higher dose than that used in a murine mucosal absorption model, did not increase biochemical markers of liver and kidney injury even after 11 injections once a week. Nasal C-CPE administration (2 mg/kg) once a week for 11 administrations also did not increase these biochemical markers, but 6 administrations of C-CPE resulted in elevation of C-CPE-specific serum IgG. These results indicate that development of a less antigenic claudin modulator will be essential for future clinical application of a C-CPE-based mucosal absorption enhancer.

1. Introduction

The use of biologics, such as antibodies, peptides, and nucleic acids, in new drugs is becoming increasingly prominent. Biologics are biodegradable and poorly absorbed in the mucosa, and therefore they are often employed as injectable drugs. The development of a non-invasive system for delivery of drugs across the mucosal epithelium would improve quality of life and patient compliance. Since orally administered drugs can be degraded by digestive enzymes and first pass effects in the liver, developing ways to administer drugs through nasal and pulmonary transmucosal absorption has a high priority. However, passing biologics across the mucosal epithelium is extremely difficult because the mucosa's primary function is as a physical and biological barrier preventing the entry of pathogens and toxic substances into the body.

Epithelial cell sheets develop intercellular junctions to prevent the free movement of solutes between sheets. Adjacent epithelial cells adhere to one another via tight junctions (TJ), adherent junctions, and gap junctions. Among these, the TJ plays a key role in sealing the intercellular space and preventing leakage of solutes. Modulation of the TJ barrier has proven to be a promising strategy for enhancement of mucosal drug absorption. Tight junction modulators, such as surfactants, chelators, and nitric oxide donors, have been investigated as potential absorption enhancers since the 1960 s (Aungst 2000; Citi 1992; Engel and Riggi 1969; Tomita et al. 1996).

The detection and development of absorption-enhancers focuses on modulating activity of the TJ barrier. Such enhancers are called "the first generation TJ modulators" (Kondoh et al. 2008). The identification of claudin, a structural and functional TJ component, provided new insight into absorption-enhancers, and led to a TJ-components-based strategy for enhancer development, the second generation TJ modulators. Claudins are ~23 kDa proteins bearing tetra-transmembrane domains and comprise a family of more than 20 members (Furuse and Tsukita 2006). The expression profiles and barrier function of the various claudin family members differ among tissues. For instance, claudins-1 and -5 are critical for epidermal barrier and blood-brain-barrier functions, respectively (Furuse et al. 2002; Nitta et al. 2003). Modulation of the claudin barrier has been proposed as a novel strategy for absorption enhancement (Furuse et al. 1998; Tsukita and Furuse 1998).

Clostridium perfringens enterotoxin (CPE) is a cause of food poisoning in humans (McClane and Chakrabarti 2004). A receptor of CPE is identical to claudin-3/4, and the C-terminal fragment of CPE (corresponding to amino acids 184–319) modulates the TJ barrier by its interaction with claudin-3/4 (Sonoda et al. 1999). We found that the claudin modulator the C-terminal fragment of CPE was 400-fold more potent at enhancing intestinal absorption than a clinically used absorption-enhancer, sodium caprate (Kondoh et al. 2005). However, the C-terminal fragment of CPE did not enhance intestinal absorption of a peptide drug when co-administered (Uchida et al. 2010). The N-terminal truncated fragment (C-CPE), comprising amino acids 194–319, did enhance intestinal, nasal, and pulmonary absorption of a biologically active peptide (Uchida et al. 2010). Thus, C-CPE may be a promising enhancer of mucosal drug absorp-

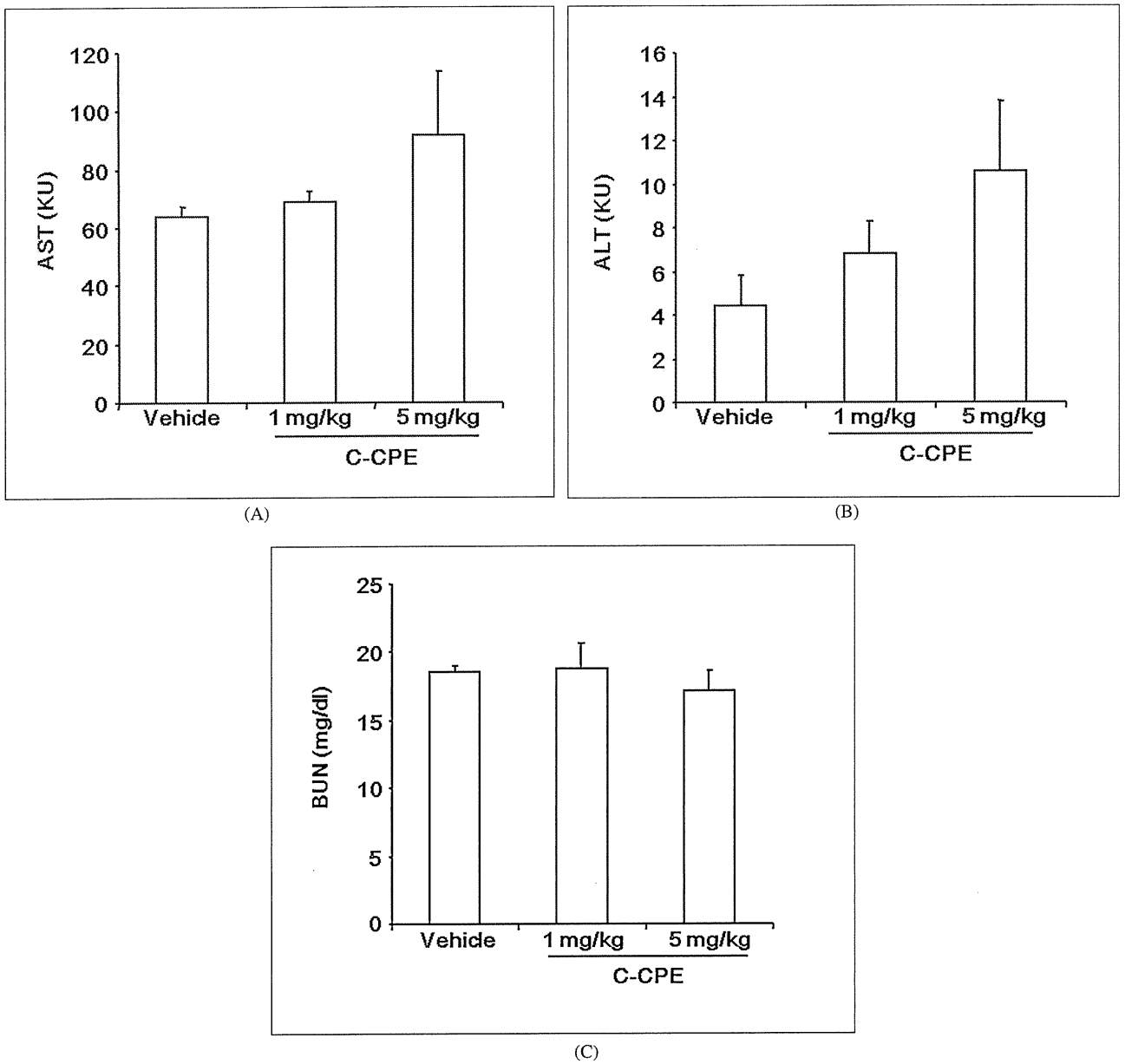


Fig. 1: Effect of systemic injection of C-CPE on biochemical markers for liver and kidney injury. Mice received intravenous injections of C-CPE at 0, 1, or 5 mg/kg once a week for 11 weeks. Blood was recovered 24 h after the last administration of C-CPE. Serum AST (A), ALT (B) and BUN (C) were measured using a commercially available kit as described in the Expreimental section. Data are presented as mean ± SEM (n = 3 or 5)

tion. Although data on safety and antigenicity is critical for any future clinical application of C-CPE, its potential side effects have not been investigated.

In the present study, we investigated the effect of C-CPE administration on liver and kidney tissues in which claudin-3/4 is expressed, as well as studied induction of anti-C-CPE antibodies.

2. Investigations, results and discussion

Administration of C-CPE enhanced the mucosal absorption of dextran with a molecular weight of 20 kDa, indicating that C-CPE (13 kDa) might enter into the systemic flow from the mucosal membrane with drugs (Kondoh et al. 2005). C-CPE constitutes the receptor-binding domain of CPE, and binds to claudin-3 and claudin-4 (Fujita et al. 2000; Katahira et al. 1997). Since claudins-3 and -4 are highly expressed in the liver and kidney (Morita et al. 1999), we evaluated the effect of C-CPE on these tissues. To investigate the potential effects of C-CPE on liver and kidney, we systemically injected C-CPE into mice once a week for 11 weeks and measured biochemical markers of liver (AST and ALT) and kidney (BUN) injury 24 h after the last injection. As shown in Figs. 1A, 1B and 1C, intravenous administration of C-CPE did not affect serum AST, ALT and BUN levels, even at a dose as high as 5 mg/kg. C-CPE was mucosally administered at 0.02–0.4 mg/kg (Uchida et al. 2010). Therefore, even if all C-CPE was absorbed, no side effects in liver or kidney are likely to occur.

Since C-CPE is a polypeptide, its antigenicity could interfere with its clinical use. We therefore investigated whether repeated mucosal administration of C-CPE activates serum C-CPE-specific IgG responses. Mice were surgically operated upon in jejunal and pulmonary absorption studies, and consequently could not be repeatedly treated with C-CPE. Therefore, to investigate the antigenicity of C-CPE following mucosal administration, C-CPE was intranasally administered to mice once a week for 10 weeks. Serum IgG production was monitored every week. C-CPE treatment did not increase C-CPE-specific serum IgG after 4 administrations of C-CPE at 2 mg/kg. However, 6 administrations of C-CPE did cause production of C-CPE-

specific serum IgG (Fig. 2). A dose of 1 mg/kg is equal to that used in a previously published study on mucosal absorption (Uchida et al. 2010). Repeated mucosal administration of C-CPE in our study at twice this dose (2.0 mg/kg) did not increase serum AST, ALT and BUN levels (Figs. 3A 3B and 3C). These findings indicate that while C-CPE does not cause tissue damage at clinically relevant doses, it may be limited in its clinical applications as a mucosal absorption enhancer only by its antigenicity.

There are two potential directions for clinical applications of claudin modulators. The first is preparation of a claudin modulator based on C-CPE. An antigenic determinant assay of CPE revealed that the C-terminal fragment corresponding to amino acids 286–305 was immunogenic (Sugii 1994). Mutating the antigenic domain while maintaining its claudin-binding activity would contribute to development of a low antigenic claudin modulator. In general, smaller peptides are less antigenic. The C-terminal fragment corresponding to amino acids 290–319 constituted the receptor-binding domain of CPE (Hanna et al. 1991). Preparation of a claudin-modulating peptide with low antigenicity and high claudin-modulating activity using this 30 amino acid fragment may lead to a claudin modulator useful as an enhancer of drug absorption.

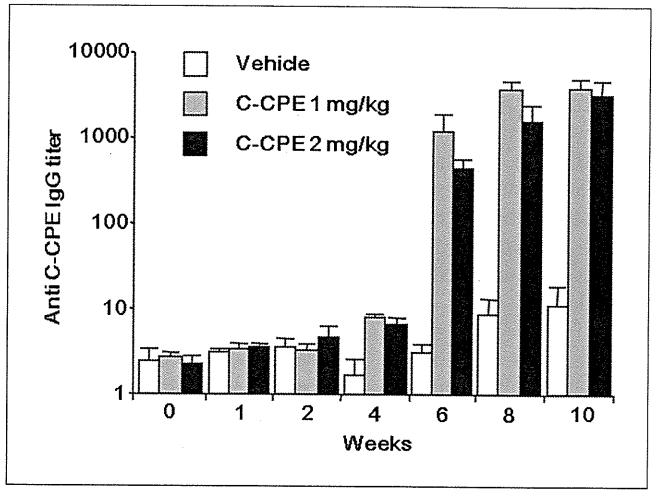
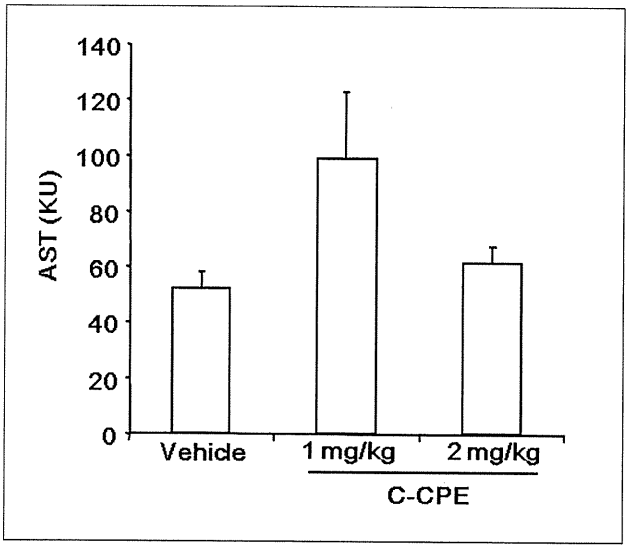
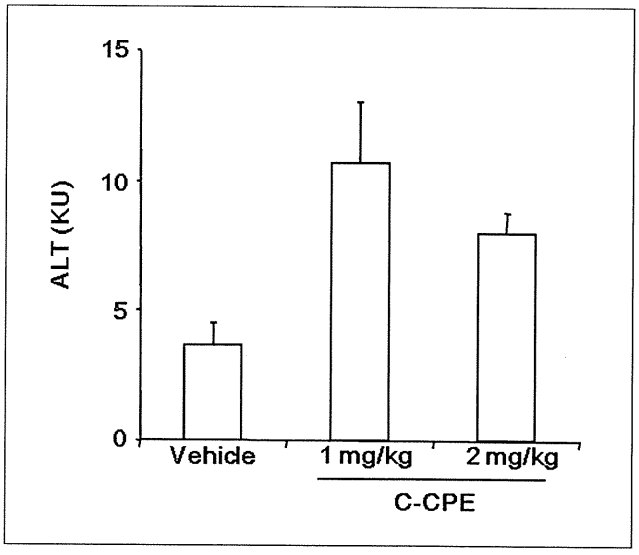


Fig. 2: Effect of mucosal administration of C-CPE on production of anti-C-CPE IgG. Mice received nasal injections of C-CPE at 0, 1, or 2 mg/kg once a week for 10 weeks. Blood was collected each week and serum C-CPE specific IgG levels were measured as described in Materials and methods. Data are presented as means ± SEM (n = 4 or 5)



(A)



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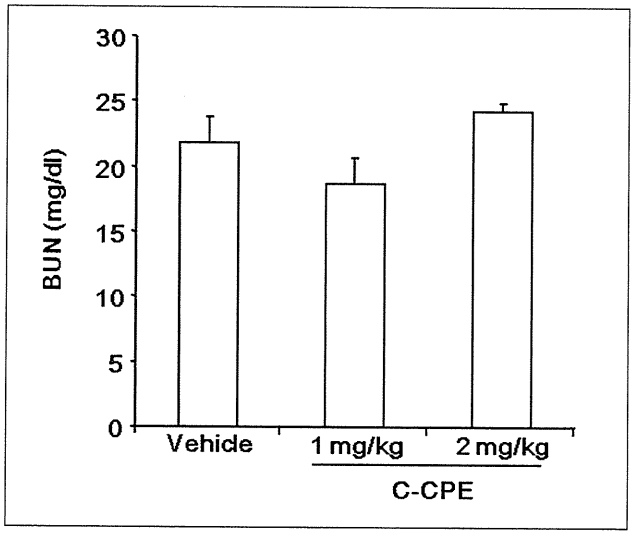

(C)

Fig. 3: Effect of repeated mucosal administration of C-CPE on biochemical markers of liver and kidney injury. Mice were nasally administered with C-CPE at 0, 1, or 2 mg/kg once a week for 11 weeks. Seven days after the last administration, blood was recovered, and serum AST (A), ALT (B) and BUN (C) were measured using a commercially available kit. Data are presented as mean ± SEM (n = 4 or 5)

The second potential direction for clinical applications of claudin modulators lies in the preparation of a human antibody to modulate the claudin-barrier. Claudin is characterized by low antigenicity, and it is therefore difficult to prepare an antibody to bind its extracellular domain (Evans et al. 2007). Recently, Romani et al. successfully prepared a human single-chain antibody to claudin-3, and Suzuki et al. also prepared a monoclonal antibody against claudin-4 (Romani et al. 2009; Suzuki et al. 2009). Development of a humanized antibody against claudin will contribute greatly to the clinical applications of claudin modulators.

In summary, we found that C-CPE administration does not result in significant tissue injury in mice. However, our findings also suggest that discovering a means of reducing C-CPE's antigenicity is critical for continued development of a C-CPE-based claudin modulator useful as an enhancer of drug absorption.

3. Experimentals

3.1. Animals

BALB/c female mice (6 wk) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), and were housed in an environmentally controlled room at 23 ± 1.5 °C with a 12-h light/12-h dark cycle. The mice had free access to water and commercial chow (Type MF, Oriental Yeast, Tokyo, Japan). Experimental protocols involving mice were performed according to the ethics guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

3.2. Preparation of C-CPE

C-CPE was prepared as described previously (Uchida et al. 2010). Briefly, pET16b vector plasmids coding the C-terminal fragment of CPE (amino acids 194–319) were transduced into *E. coli* BL21 (DE3), and protein expression was stimulated by addition of isopropyl-1-thio-β-D-galactoside. Cell lysates were applied to HisTrap™ Chelating HP columns (GE Healthcare, Buckinghamshire, UK), and C-CPE was eluted with imidazole. The solvent was exchanged with phosphate-buffered saline by gel-filtration, and the purified proteins were stored at −80 °C until use. Purification of the proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue. C-CPE was quantified by using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL) using bovine serum albumin as a standard.

3.3. Biochemical assay

C-CPE was administered to mice intravenously or nasally once a week for 11 weeks, and blood was collected from the mice by cardiac puncture one day after the last administration. Serum aspartate aminotransferase (AST), alanin aminotransferase (ALT) levels and blood urea nitrogen (BUN) were measured using commercially available Transaminase-CII and Blood Urea Nitrogen-B Test (WAKO Pure Chemical, Osaka, Japan) kits, respectively.

3.4. C-CPE-specific antibody production

C-CPE was administered to mice intravenously or nasally once a week. Serum was collected 7 days after each administration of C-CPE. The titers of C-CPE-specific antibody in serum were determined using an enzyme-linked immunosorbent assay. Briefly, an immunoplate was coated with C-CPE (1 μg/well in a 96-well plate). Ten-fold serial dilutions of samples were added to the wells, followed by reaction with horseradish peroxidase-conjugated anti-mouse IgG. The presence of C-CPE-specific antibodies was determined using TMB peroxide substrate. End point titers were expressed as the dilution ratio, which gave 0.1 above control values obtained for serum of naïve mice at an absorbance of 450 nm.

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Mutated C-terminal fragments of *Clostridium perfringens* enterotoxin have increased affinity to claudin-4 and reversibly modulate tight junctions in vitro

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ABSTRACT

Passage across epithelial cell sheets is the first step in drug absorption. Tight junctions (TJs) are located between adjacent epithelial cells and seal the intercellular space preventing leakage of solutes. Claudin, a tetra-transmembrane protein family, is a pivotal functional and structural component of the TJ barrier. Modulation of the claudin-based TJ seal is a strategy for mucosal drug absorption. We previously found that a claudin-4 binder, a C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE194), was a modulator of the TJ seal and a potent mucosal absorption enhancer. In the present study, we attempted to improve claudin-4 binders by modification of C-CPE194. Substitution of Asn at position 309 and Ser at position 313 with Ala increased the affinity to claudin-4 by 9.9-fold as compared to C-CPE194. Deletion of 10 amino acids in the N-terminal domain of the double-alanine-substituted mutant increased affinity to claudin-4 by 23.9-fold as compared to C-CPE194. These C-CPE194 mutants reversibly modulated the TJ seal in human intestinal epithelial cell sheets. The N-terminal-truncated mutant was the most potent modulator of the TJ seal. These findings indicate that the C-CPE mutant may be a promising lead for the development of a clinical TJ modulator.

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1. Introduction

Epithelium surrounds organisms and separates the inside of the body from the outside environment. Passage across the epithelium is the first step in drug absorption. Routes for solute movement across the epithelium are classified into transcellular and paracellular routes. Methods to deliver drugs through the transcellular route by simple diffusion or transporter- or receptor-mediated active transport have been developed since the 1990s, and some of these methods have been used clinically [1,2]. Absorption enhancers that deliver drugs through the paracellular route have been investigated since the 1960s, but most paracellular delivery systems have been investigated from the point of epithelial barrier-modulating activity [3,4]. Theoretical approaches for paracellular drug transport based on components of the epithelial barrier have never been fully developed because biochemical studies of the epithelial barrier have been sparse.

Tight junctions (TJs) are localized between adjacent epithelial cells and seal the intercellular space to prevent the leakage of solutes across the epithelial cell sheets. Modulation of the TJ barrier has been a strategy to enhance the epithelial absorption of drugs. However, biochemical and functional structures of TJs were not identified until 1998. Freeze-fracture replica electron microscopy analysis showed that TJs form a series of continuous, anastomotic and intramembranous particle strands, and the first structural and functional component of TJs, claudin, was identified in 1998 [5,6]. Claudin is a tetra-transmembrane protein with a molecular mass of ~23 kDa and comprises a family of 27 members [7,8]. Interestingly, the expression profiles and barrier functions of claudin family members differ among tissues. For instance, the epidermal barrier is disrupted in claudin-1-deficient mice, while the blood–brain barrier is deregulated in claudin-5-deficient mice [9,10]. These findings support the use of claudin-targeting strategies to enhance paracellular drug absorption.

Clostridium perfringens enterotoxin (CPE), a 35-kDa polypeptide, causes food poisoning in humans [11]. The CPE receptor is identical

Abbreviations: TJ, tight junction; C-CPE, the C-terminal fragment of *Clostridium perfringens* enterotoxin corresponding to 184-319 amino acids; CPE, *Clostridium perfringens* enterotoxin; DDM, *n*-dodecyl-β-D-maltoside; C-CPE194, C-terminal fragment of *Clostridium perfringens* enterotoxin corresponding to 194–319 amino acids; C-CPE205, C-terminal fragment of *Clostridium perfringens* enterotoxin corresponding to 205–319 amino acids; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BV, budded baculovirus; TBS, tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; TEER, transepithelial electric resistance.

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to claudin-3 and -4, and the C-terminal fragment of CPE (C-CPE; amino acids 184–319) binds to claudin-3/-4 [12–15]. Interestingly, C-CPE reversibly modulates TJ seals in epithelial cell sheets [15]. We previously found that the jejunal absorption-enhancing activity of a claudin binder was 400-fold more potent than a clinically used absorption enhancer, sodium caprate, and that the claudin binder enhanced jejunal, pulmonary and nasal absorption of a biologically active peptide [16,17]. Thus, claudin binders are promising absorption enhancers, but claudin binders have never been fully developed because of the difficulty in preparation of the claudin proteins needed for screening claudin binders and the low antigenicity of claudin. To develop claudin binders using C-CPE as a prototype, we previously prepared alanine-substituted mutants and N-terminus-truncated mutants, and we investigated their claudin-4-binding and TJ-modulating activities [17–19]. Based on these findings on the functional domain of C-CPE, we modified C-CPE and prepared claudin-4 binders with higher affinity and TJ-modulating activity in the present study.

2. Materials and methods

2.1. Reagents

n-Dodecyl-β-D-maltoside (DDM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Anti-claudin antibodies and anti-his-tag antibody were obtained from Invitrogen (Carlsbad, CA). CM5 sensor chips, amine-coupling reagents (*N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide, *N*-hydroxysuccinimide, and ethanolamine–HCl) and HBS-EP+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P20) were obtained from GE Healthcare (Buckinghamshire, UK). All reagents used were of research grade.

2.2. C-CPE mutants

C-CPE is the first claudin binder to be identified [15]. N-terminal region-truncated C-CPE mutants are also claudin-4 binders: C-CPE194 (amino acids 194–319) and C-CPE205 (amino acids 205–319) [17,20]. The DNA fragment encoding alanine-substituted C-CPE194 or C-CPE205 was amplified by polymerase chain reaction (PCR) using the alanine-substituted primers and a template plasmid encoding C-CPE194 or C-CPE205, respectively [17]. The resulting PCR products were cloned into a pET16 vector. The plasmids were transduced into *E. coli* BL21 (DE3), and protein expression was stimulated by the addition of isopropyl-1-thio-β-D-galactoside. C-CPE mutants were purified from the cell lysates by affinity chromatography with HisTrap™ HP (GE Healthcare). The solvent was exchanged with phosphate-buffered saline (PBS) by gel filtration, and the purified proteins were stored at −80 °C until used. Purification of the proteins was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by staining with Coomassie Brilliant Blue. C-CPE mutants were quantified using a BCA protein assay kit with bovine serum albumin as a standard (Thermo Fisher Scientific Inc., Rockford, IL).

2.3. Claudin-displaying budded baculovirus (BV)

Claudin-displaying BV was prepared as described previously [21]. Claudin-1 and -4 cDNA fragments were cloned into the baculoviral transfer vector pFastBac1 (Invitrogen). Recombinant baculoviruses were generated using the Bac-to-Bac system according to the manufacturer's instructions (Invitrogen). Sf9 cells were cultured in Grace's Insect medium (Invitrogen) containing 10% fetal bovine serum at 27 °C and infected with the recombinant baculovirus. Seventy-two hours after infection, the BV fraction was isolated from the culture supernatant of the infected Sf9 cells by centrifugation at 40,000*g* for 25 min. The pellets of the BV fraction were suspended in Tris-buffered saline (TBS) containing protease inhibitor cocktail (Sigma–Aldrich, St. Louis) and then stored at 4 °C. The expression of claudins in the BV fraction was confirmed by SDS–PAGE and immunoblot with antibodies against claudins.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The claudin-displaying BVs were adsorbed to the wells of 96-well immunoplates (Nunc, Roskilde, Denmark) overnight at 4 °C. The wells were blocked with 1.6% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) for 2 h at room temperature. C-CPE mutants were added to the wells and incubated for an additional 2 h at room temperature. The wells were incubated with anti-his-tag antibody for 2 h at room temperature. The immunoreactive proteins were detected by a horseradish peroxidase-labeled secondary antibody with 3,3′,5,5′-tetramethylbenzine as a substrate. The reaction was terminated by the addition of 0.5 M H_2SO_4, and the immunoreactive proteins were measured at 450 nm.

2.5. Recombinant claudin-4 protein

Recombinant claudin-4 protein was prepared by an expression system using Sf9 cells and recombinant baculovirus as previously reported [17]. Briefly, the C-terminal his-tagged claudin-4 cDNA fragment was cloned into pFastBac1, and recombinant baculovirus was generated using the Bac-to-Bac baculovirus expression system. Sf9 cells were infected with the recombinant baculovirus. After 52–56 h of infection, the cells were harvested by centrifugation. The cells were resuspended in a solution of 10 mM HEPES (pH 7.4), 120 mM NaCl with protease inhibitors (Complete Mini, EDTA-free, Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride and 20 U/ml DNase I. The cells were lysed with 2% DDM and then centrifuged. The resultant supernatant was applied to HisTrap™ HP, and claudin-4 was eluted with imidazole. The solvent for claudin-4 was changed to PBS containing 0.2% DDM by gel filtration with a HiTrap desalting column (GE Healthcare). Purification of claudin-4 was confirmed by SDS–PAGE followed by staining with Coomassie Brilliant Blue.

2.6. Surface plasmon resonance (SPR) analysis

SPR analysis was performed with a Biacore T100 instrument (GE Healthcare). Amine-coupling chemistry was used to immobilize claudin-4 at 25 °C on a CM5 sensor chip surface docked in a Biacore T100 and equilibrated with HBS-EP+. The carboxymethyl surface of the CM5 chip was activated for 2 min with a 1:1 ratio of 0.4 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide and 0.1 M *N*-hydroxysuccinimide at a flow rate of 10 μl/min. Claudin-4 was diluted to 2.5 μg/ml in 10 mM MES buffer (pH 6.5) and injected for 2 min over the surface at a flow rate of 10 μl/min. Excess activated groups were blocked by a 5-min injection of 1 M ethanolamine (pH 8.5) at a flow rate of 10 μl/min. Approximately 1000 RU of claudin-4 was immobilized using this protocol. Single-cycle kinetics experiments were performed at 25 °C with a flow rate of 30 μl/min [22]. C-CPE or its derivatives were serially diluted (1.25, 2.5, 5, 10 and 20 nM) in running buffer (HBS-EP+). Within a single binding cycle, samples of C-CPE or its derivatives were injected sequentially in order of increasing concentration over both the ligand and the reference surfaces. The reference surface, an unmodified flowcell, was used to correct for systematic noise and instrumental drift. Prior to the binding cycle for C-CPE or its derivatives, buffer was injected. These blank responses were used as a double-reference for the binding data [23]. The sensorgrams were globally fitted using a

1:1 binding model to determine k_a, k_d and K_D values with the Biacore T100 Evaluation Software version 2.0.1.

2.7. Transepithelial electric resistance (TEER) assay

Caco-2 cells were seeded onto Transwell™ chambers (Corning, NY) at a subconfluent density. The TEER of the Caco-2 monolayer cell sheets were monitored with a Millicell-ERS epithelial volt-ohmmeter (Millipore, Billerica, MA). When TJs were developed reaching a plateau in the TEER values, the cells were treated with C-CPEs on the basal side of the chambers. Changes in TEER values were monitored. The TEER values were normalized by the area of the Caco-2 monolayer cell sheets, and the TEER value of a blank chamber was subtracted. Relative TEER values were calculated by the ratio to TEER in the vehicle-treated chambers or before treatment.

3. Results

3.1. Preparation of C-CPE mutants

C-CPE is a 400-fold more potent mucosal absorption-enhancer than a clinically used absorption-enhancer, sodium caprate [16]. Partial N-terminal-deleted C-CPE derivatives, C-CPE194 and C-CPE205, had over 30- and 10-fold higher solubility in PBS than C-CPE without loss of binding to claudin-4, respectively [17]. An alanine-substitution analysis of C-CPE indicated that replacement of Asn at position 309 or Ser at position 313 by alanine may improve its binding to claudin-4 and modulation of the TJ barrier [18]. Based on these findings, we speculated that the combination of N-terminal deletion and alanine substitution may produce a potent claudin modulator. We first focused on C-CPE194 because it has the highest solubility. We prepared C-CPE194$_{N309A}$, C-CPE194$_{S313A}$ and C-CPE194$_{N309A/S313A}$ (Fig. 1). To determine if these C-CPE mutants could bind to claudin-4, we performed ELISA with claudin-4-displaying BV. As shown in Fig. 2, all of these C-CPE mutants dose-dependently bound to claudin-4-displaying BV but not wild-type BV. Next, we quantitatively investigated their affinity to claudin-4 by SPR analysis (Table 1). Substitution of Asn at position 309 did not affect affinity to claudin-4 (K_D values of C-CPE194, 455 pM; C-CPE194$_{N309A}$, 451 pM). Replacement of Ser at position 313 by alanine elevated affinity to claudin-4 (K_D value of C-CPE194$_{S313A}$, 117 pM). Interestingly, the double alanine-substituted C-CPE194$_{N309A/S313A}$ had synergistically higher affinity to claudin-4 (K_D value, 46 pM). Based on these results, we tested the affinity of C-CPE205 with replacements of both the Asn at position 309 and the Ser at position 313 by alanine (C-CPE205$_{N309A/S313A}$) (Fig. 1). The affinity of C-CPE205$_{N309A/S313A}$ to claudin-4 was the highest (K_D value, 19 pM) among the C-CPE mutants (Table 1).

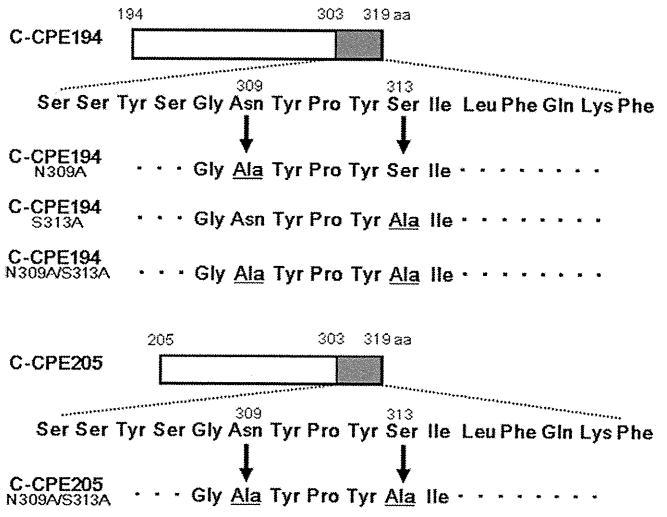

Fig. 1. Schematic illustration of C-CPE mutants. The C-terminal 16 amino acid fragment contains claudin-4-binding domains. A site-directed mutagenesis analysis of the C-terminal domain revealed that alanine substitution with Asn at position 309 or Ser at position 313 increased its binding to claudin-4 [18,19]. C-CPE194 and C-CPE205 are the C-terminal fragments of CPE corresponding to amino acids 194–319 and 205–319, respectively [17].

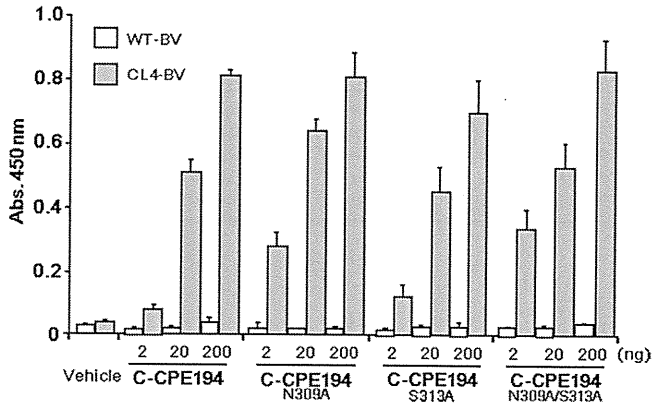

Fig. 2. Interaction of C-CPE mutants with claudin-4. Vehicle or C-CPE mutants were added to the wells of immunoplates coated with wild-type BV (WT-BV) or claudin-4-dysplaying BV (CL4-BV). After 2 h of incubation, anti-tag antibody, which recognized C-CPE mutants, was added to the wells, and then the C-CPE mutant-bound BV was detected by the addition of the horseradish peroxidase-labeled antibody, as described in Materials and methods. Data are representative of two independent experiments. The data are means ± SD (n = 4).

3.2. Effect of C-CPE mutants on the TJ seal

Next, we investigated the effects of C-CPE mutants on the modulation of the TJ seal. A monolayer culture of human colon Caco-2 cells is the most popular in vitro model for the assessment of TJ-seal modulation in the mucosal epithelium. As shown in Fig. 3A, treatment of monolayer cultures of Caco-2 cells with C-CPE mutants for 18 h dose-dependently decreased the integrity of TJ seals. The double alanine-substituted mutant C-CPE205$_{N309A/S313A}$ had the highest TJ-seal-modulating activity among the C-CPE mutants (CPE194: 62.7% and 31.7% of control at 5 and 20 μg/ml, respectively; C-CPE205$_{N309A/S313A}$: 14.6% and 10.3% of control at 5 and 20 μg/ml, respectively). The reversibility of TJ-seal modulation by a TJ modulator is important for safety. To investigate whether the C-CPE mutants reversibly modulated TJ integrity, C-CPE mutants were removed from the culture medium after 18 h, and then the cells were cultured for an additional 24 h. The TJ integrity was recovered 24 h after removal of the C-CPE mutants (Fig. 3B). These data indicate that the C-CPE mutants are reversible TJ modulators.

4. Discussion

Noninvasive drug administration is ideal for patient compliance and quality of life. Mucosal epithelium functions as a biological barrier preventing the free movement of solutes between the inside of the body and the outer environment. A strategy for noninvasive drug absorption is to modulate the TJ seal between adjacent mucosal epithelial cells. A C-terminal polypeptide fragment of CPE, referred to as C-CPE, modulates the TJ seal and binds to claudin-4 [15]. C-CPE enhanced the jejunal absorption of dextran by >400-fold as compared to a clinically used enhancer, and deletion of the claudin-4-binding domain attenuated its absorption-enhanc-

Table 1
Binding kinetics of C-CPE mutants to claudin-4.

Derivatives	ka(1/Ms)	kd (1/s)	K_D
C-CPE194	7.13×10^5	3.24×10^{-4}	455pM
C-CPE194$_{N309A}$	6.50×10^5	2.93×10^{-4}	451pM
C-CPE194$_{S313A}$	5.59×10^5	6.53×10^{-5}	117pM
C-CPE194$_{N309A/S313A}$	6.67×10^5	3.05×10^{-5}	46pM
C-CPE205$_{N309A/S313A}$	7.55×10^5	1.45×10^{-5}	19pM

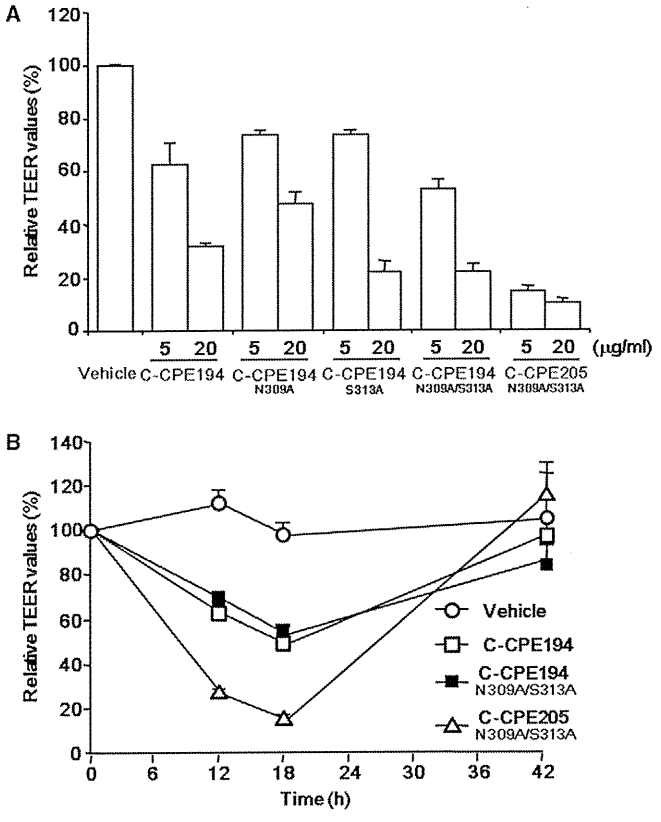

Fig. 3. Effect of C-CPE mutants on TJ-barrier in Caco-2 monolayer cell sheets. (A) Effect of C-CPE mutants on TJ-integrity. Caco-2 cells were cultured on Transwell™ inserts. When TEER values reached a plateau, the cells were treated with C-CPE or C-CPE mutants at 0, 5 or 20 μg/ml. After 18 h of C-CPE mutant treatment, the TEER values were monitored as described in the Materials and methods. The results were calculated as the percent of the TEER values to that of the vehicle-treated group. Data are representative of three independent experiments. The data are means ± SD ($n = 4$). (B) Reversibility of TJ modulation by C-CPE mutants. The cells were treated with C-CPE mutants (20 μg/ml) for 18 h, and then the cells were washed with the medium to remove the C-CPE mutants. The cells were cultured for an additional 24 h. Changes in TEER values were monitored during the C-CPE mutant treatment. The results were calculated as the percent of the TEER values to the values at 0 h. Data are representative of three independent experiments. The data are means ± SD ($n = 4$).

ing activity [16]. Claudin-4 binder may be useful for the development of a noninvasive drug delivery system. In the present study, we modified C-CPE based on our past functional domain mapping, and we found that the partial N-terminal-deleted and alanine-substituted mutants, C-CPE194$_{N309A/S313A}$ and C-CPE205$_{N309A/S313A}$, had higher affinity for claudin-4 and/or higher TJ-modulating activity than the parental C-CPE.

Why did substitution of Asn at position 309 and Ser at position 313 with Ala in C-CPE increase its affinity to claudin-4? Tyr residues at positions 306, 310 and 312 and Leu at position 315 in CPE are involved in the interaction between CPE and claudin-4 [18]. Winkler et al. proposed that C-CPE may interact with the hydrophobic turn in the second loop of claudin through its hydrophobic pit on the surface of C-CPE formed by those Tyr and Leu residues [24]. The substitution of Asn at position 309 and Ser at position 313 with Ala may reduce polarity and steric hindrance in the hydrophobic pit of C-CPE leading to increased binding of the Tyr and Leu residues to the hydrophobic region of claudin. Replacement of Ser at 305 or 307 by Ala also increased the binding of C-CPE to claudin-4 [18]. Tyr at position 306 is located between Ser residues at positions 305 and 307. Reduction of the polarity and steric hindrance surrounding Tyr at position 306, 310 and 312 and Leu at position 315 might increase the affinity of C-CPE to claudin-4.

C-CPE194$_{N309A/S313A}$ and C-CPE205$_{N309A/S313A}$ had 9.9- and 23.9-fold higher affinity to claudin-4 as compared to C-CPE194, respectively. However, their affinities to claudin-4 did not always affect their TJ-barrier-modulating activity. C-CPE205$_{N309A/S313A}$ modulated the TJ barrier more than C-CPE194, but C-CPE194$_{N309A/S313A}$ had TJ-barrier-modulating activity similar to C-CPE194. One possible explanation for this discrepancy may be differences in their cellular uptake activities. Claudin contains a clathrin-sorting signal in its C-terminal intracellular domain [25]. Treatment of cells with the C-terminal fragment of CPE caused a disappearance of claudin-4 in TJs and a decrease in claudin-4 protein [15]. Claudin-4 binders may first bind to claudin-4, after which the binders bound to claudin-4 may be taken up into the cytosol via endocytosis leading to the degradation of claudin-4. C-CPE205$_{N309A/S313A}$ might show higher endocytic activity by interacting with claudin-4 in a manner different than the other C-CPE mutants. These C-CPE mutants had similar association kinetics to claudin-4 but different dissociation kinetics to claudin-4 (Table 1). A longer interaction between C-CPE205$_{N309A/S313A}$ might increase the endocytosis of C-CPE mutant-bound claudin-4. Further experiments are needed to prove this hypothesis.

Preparation of antibodies against the extracellular domain of claudin-1, -3 and -4 has been recently reported [26–28]. However, to our knowledge, C-CPE is still the only modulator of the claudin barrier. C-CPE may be a promising lead for the development of TJ modulators. We have developed a screening system for claudin binders using a baculoviral display system [29]. We are attempting to develop novel claudin binders by the combination of a C-CPE mutant library and the baculoviral display system. Claudin-4-targeting is also a potent strategy for cancer-targeting and mucosal vaccination [21,27,30], for which C-CPE194$_{N309A/S313A}$ and C-CPE205$_{N309A/S313A}$ can be used. Taken together, our findings regarding C-CPE mutants will contribute to the development of not only drug-absorption enhancers but also claudin-targeted drug development, such as for cancer therapy and vaccines.

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A facile preparation method of a PFC-containing nano-sized emulsion for theranostics of solid tumors

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ABSTRACT

Theranostics means a therapy conducted in a diagnosis-guided manner. For theranostics of solid tumors by means of ultrasound, we designed a nano-sized emulsion containing perfluoropentane (PFC5). This emulsion can be delivered into tumor tissues through the tumor vasculatures owing to its nano-size, and the emulsion is transformed into a micron-sized bubble upon sonication through phase transition of PFC5. The micron-sized bubbles can more efficiently absorb ultrasonic energy for better diagnostic images and can exhibit more efficient ultrasound-driven therapeutic effects than nano-sized bubbles. For more efficient tumor delivery, smaller size is preferable, yet the preparation of a smaller emulsion is technically more difficult. In this paper, we used a bath-type sonicator to successfully obtain small PFC5-containing emulsions in a diameter of ca. 200 nm. Additionally, we prepared these small emulsions at 40 °C, which is above the boiling temperature of PFC5. Accordingly, we succeeded in obtaining very small nano-emulsions for theranostics through a very facile method.

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1. Introduction

'Theranostics', 'theranosis', or 'theragnosis' is a newly created term in the fields of imaging diagnosis and drug delivery systems. As a word, 'theranostics' (Chen, 2011; Lammers et al., 2010, 2011; MacKay and Li, 2010) is a combination of therapy and diagnosis, and is defined as therapy conducted in a diagnosis-guided manner. A typical example of theranostics is found in a carrier system containing both a contrast agent for diagnosis and a drug for therapy. Theranostics has been studied with various types of drug carriers including liposomes (Kamaly and Miller, 2010), small molecules (Kalber et al., 2011), nano-particles (Jeong et al., 2011; Kim et al., 2010), emulsions (Gianella et al., 2011), synthetic polymers (Bryson et al., 2009), polymeric micelles (Blanco et al., 2009; Kaida et al., 2010; Min et al., 2010; Nakamura et al., 2006; Shiraishi et al., 2009, 2010), and other nano-sized carrier systems (Ai, 2011; Moon et al., 2011; Pan et al., 2008; Sanson et al., 2011). Ultrasound is considered to be a preferable modality for theranostics because ultrasound has been well studied and developed for image diagnoses and local therapies such as ultrasound lithotresis and hyperthermia.

For theranostics of solid tumors, micron-sized bubbles (microbubbles) (Hernot and Klibanov, 2008; Schutt et al., 2003; Unger et al., 2004) have been actively studied because the bubbles provide strong contrasts in ultrasonic images, and because cavitation of microbubbles (Grishenkov et al., 2009) induced by ultrasound can effectively damage cells. Cells can be damaged by both jet-stream and heat that are generated in the bubbles' cavitation. In the design of microbubbles for tumor applications, the size of the microbubbles is a very important factor. Larger microbubbles can produce stronger ultrasound image contrasts. In contrast, smaller bubbles are preferred for efficient delivery into tumor tissues because the size of the trans-vascular passage from the bloodstream into the tumor interstitial space is of a diameter smaller than 1 μm. It is believed that the maximum diameter for efficient translocation into tumor tissues is 200–400 nm (Ishida et al., 1999; Litzinger et al., 1994; Nagayasu et al., 1996; Yuan et al., 1995). (In this diameter range, bubbles must be called nano-bubbles.) This is an essential dilemma concerning the size of bubbles used for

Abbreviations: PFC, perfluorocarbon; PFC5, perfluoropentane; PFC6, perfluorohexane; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; PEG-P(Asp(C7F9)*x*), poly(ethylene glycol)-b-poly(4,4,5,5,6,6,7,7,7-nonafluoroheptyl aspartate) block copolymer.

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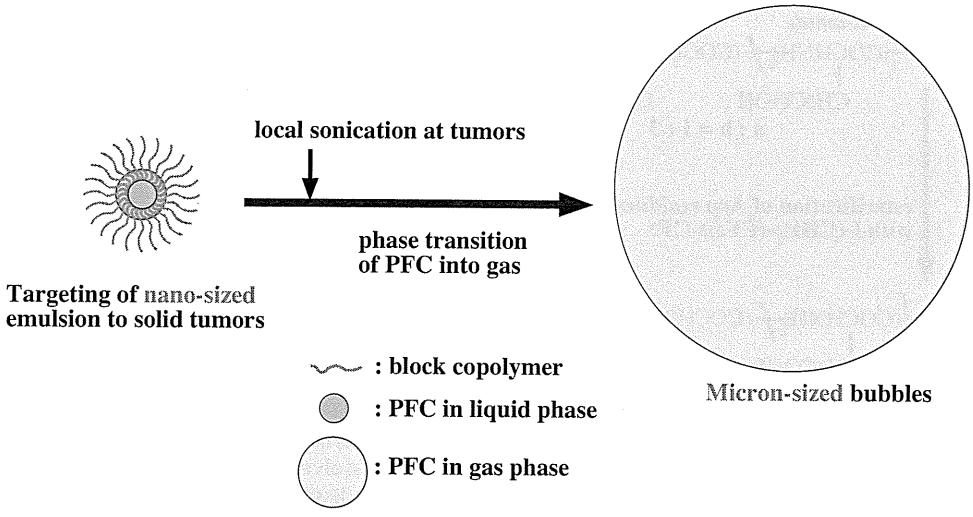

Fig. 1. Concept of phase-transition type nano-emulsion.

tumor theranostics. In order to resolve this dilemma, Kawabata et al. (Asami et al., 2009, 2010; Kawabata et al., 2005, 2010a,b) and Rapoport et al. (Mohan and Rapoport, 2010; Rapoport et al., 2007, 2009a, 2009b, 2010a,b, in press) examined nano-emulsions incorporating a specific kind of perfluorocarbon, as illustrated in Fig. 1. A boiling temperature of this perfluorocarbon (perfluoropentane, PFC5) is 29 °C, which is lower than normal human body temperature, but the integrity of these nano-emulsions is maintained owing to interfacial excessive pressure called Laplace pressure (Rapoport et al., 2009a). Upon ultrasound irradiation, the integrity of these nano-emulsions is broken, and this liquid perfluorocarbon exhibits a phase-transition into gas. Accordingly, the nano-emulsions change into microbubbles. Efficient delivery into tumor tissues is attained with the nano-emulsions, and then local sonication at the tumor tissues generates the microbubbles from the nano-emulsions, resulting in high imaging and therapeutic efficiencies. This phase-transition type nano-emulsion may be an ideal system for the theranostics of solid tumors.

Generally, preparations of smaller emulsions in a nano-meter range are more difficult because a higher power input is required in the emulsion preparations. (Tadros et al., 2004) Previously, we had prepared perfluorocarbon-containing emulsions by means of vigorous mechanical stirring with a magnetic stirrer and obtained emulsions of ca. 600 nm in diameter (Nishihara et al., 2009). In this paper, we have tried to obtain much smaller emulsions by means of ultrasound irradiation as well as high-pressure emulsification. Another important parameter for preparations of the phase-transition type nano-emulsion is temperature. A boiling temperature (29 °C) of perfluoropentane (PFC5) is close to the room temperature; therefore, preparations must be carried out at a low temperature and in a small scale for evasion of evaporation of PFC5 because heat generated in emulsification or sonication processes must be efficiently removed for the evasion. We want to find a facile preparation method that can be carried out at either room or a higher temperature, and that can be easily scaled up because the heat removal is a much less serious concern than the conventional method. Rapoport et al. (Rapoport et al., 2010b) reported preparations of nano-bubbles by means of ultrasound irradiation (with a probe type sonicator at 20 kHz) in ice-cold water. They obtained nano-emulsions of ca. 600 nm in diameter.

In this paper, we have tried to obtain very small nano-emulsions containing PFC5 by using an inexpensive bath-type sonicator (usually used as an ultrasonic cleaner) at room temperature or higher. For this emulsion preparation, we synthesized fluorinated block copolymers and optimized their compositions.

2. Materials and methods

2.1. Materials

We purchased perfluoropentane (PFC5) and perfluorohexane (PFC6) from Stream Chemicals (Newburyport, MA, USA) and Alfa Aesar (Ward Hill, MA, USA), respectively, and used them as received. We purchased 4,4,5,5,6,6,7,7,7-nonafluoroheptyl iodide from Sigma–Aldrich (Tokyo branch, Japan) and used it as received. We purchased reagent-grade solvents, dehydrated *N,N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and diethyl ether from Wako Chemicals (Tokyo, Japan), and used them as received. Poly(L-lactic acid)-grafted gelatin was prepared through a coupling reaction between a primary amine group of gelatin and a terminal hydroxyl group of the poly(L-lactic acid) by the use of disuccimidyl carbonate according to a published synthetic procedure. (Tanigo et al., 2010) Poly(ethylene glycol)-block-poly(L-lactic acid) block copolymer (PEG-b-PLA) was purchased from Sigma–Aldrich (Tokyo branch, Japan). The average molecular weights of the PEG block and the PLA block were 750 and 1,000, respectively.

2.2. Block copolymer synthesis

Poly(ethylene glycol)-*b*-poly(4,4,5,5,6,6,7,7,7-nonafluoroheptyl aspartate) block copolymers (PEG-P(Asp(C7F9)*x*)) were prepared by means of esterification of the aspartic units of poly(ethylene glycol)-b-poly(aspartic acid) block copolymer (PEG-P(Asp)) by the use of an iodinated compound, as shown in Fig. 2. PEG-P(Asp) was synthesized according to our previous paper (Yamamoto et al., 2007). A value *x* in the PEG-P(Asp(C7F9)*x*) formula denotes mol.% of the esterified units. This esterification reaction was carried out with a corresponding iodinated compound in the presence of a super base according to a previously reported procedure (Opanasopit et al., 2004; Yokoyama et al., 2004; Yamamoto et al., 2007) with a slight modification.

The starting material was poly(ethylene glycol)-b-poly(aspartic acid) block copolymer (PEG-P(Asp)). The average molecular weight of PEG was 5200 (n = 119 in Fig. 2), and the average number of Asp units per one chain was 26.0. The aspartate amide bond can be either α or β, and our group previously had reported that a ratio of α:β was 1:3 (=*a*:*b* in Fig. 2) (Yokoyama et al., 2004). PEG-P(Asp) (2.001 g, containing 6.33×10^{-3} mol Asp residue) was dissolved in 20 mL of DMF. To this mixture, was added both 4.904 g of 4,4,5,5,6,6,7,7,7-nonafluorohepty iodide (which is

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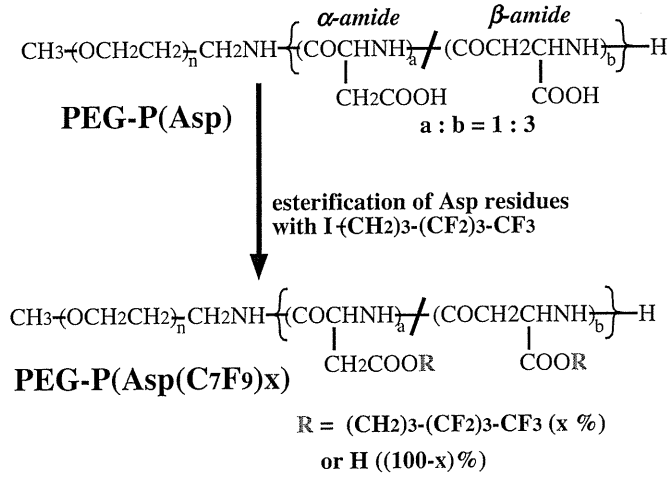

Fig. 2. Synthesis of the fluorocarbon-containing block copolymer PEG-P(Asp(C7F9)*x*).

2.00 mol. equivalents to the Asp residue, I-(CH2)3-(CF2)3-CF3 in Fig. 2) and 0.972 g of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, which is 1.01 mol. equivalents to the Asp residue). DBU is a very strong base, and can induce ionization in a carboxyl group of the aspartic acid residue in an organic solvent, DMF. The reaction mixture was heated at 50 °C for 16 h. An ester formed at the Asp residue through a nucleophilic substitution reaction of the ionized carboxyl group with I-(CH2)3-(CF2)3-CF3. After this 16-h reaction, the reaction mixture was poured into 200 mL of ice-cold diethyl ether for precipitation of the polymer. The precipitated polymer was filtered and washed with diethyl ether. The obtained polymer was dissolved in 20 mL of DMSO, to which was added 2.11 mL of 6 N hydrochloric acid. This acid works for removal of DBU from polymers. This polymer solution was dialyzed with a Spectra/Por 6 dialysis membrane (molecular weight cut-off is 1000) against DMSO for 2 days and against milliQ water for an additional 2 days, followed by freeze-drying. Yield was 2.436 g. To determine the contents of the fluorinated ester group of the polymer, we used ^{1}H NMR spectroscopy in DMSO-d_6 containing 3 v/v% trifluoroacetic acid. For this determination, we identified a peak area ratio between the methylene protons ($-COOCH_2\underline{CH_2}CH_2CF_2CF_2CF_2CF_3$) at 1.8 ppm of the ester group and the methylene protons ($-OC\underline{H_2}\underline{CH_2}-$) at 3.6 ppm of the PEG block. The esterification percentage (*x* in Fig. 2) was revealed to be 59%. The other compositions of block copolymers were synthesized according to the same method with various molar ratios of I-(CH2)3-(CF2)3-CF3 and DBU with respect to the aspartic acid residue. Table 1 lists all the compositions of the synthesized block copolymers.

Table 1
Compositions of PEG-P(Asp(C7F9)*x*).

Code	M.W. of PEG	Asp unit number (*n*)	Esterification degree (*x*%)
F-6%	5200	22.1	5.9
F-15%	5200	23.3	14.6
F-39%	5200	22.1	38.5
F-59%	5200	26.0	58.5
F-67%	5200	22.1	67.0

2.3. Preparation of PFC-containing nano-emulsions

We examined preparations of PFC5-containing nano-emulsions according to two methods using a high-pressure emulsifier and a bath-type sonicator.

2.3.1. Preparation with a high-pressure emulsifier

We dissolved PEG-P(Asp(C7F9)15) block copolymer by stirring it in distilled water at a concentration of 4.0 wt. % of the solution, and added perfluoropentane (PFC5) and perfluorohexane (PFC6) at each 1.25 vol.% of the solution. We vigorously stirred the solution with a homogenizer Polytron (Kinematica AG, Tokyo, Japan) at 25,000 rpm for 10 s. Then, we conducted emulsification using a high-pressure emulsifier EmulsiFlex-C5 CSC (AVESTIN, Inc., Ottawa, Ontario, Canada) at 4 °C for 6 min at ca. 50 MPa. We collected a white emulsion, and filtered it with a Sartorius Minisart (R) filter (1.2 μm pore, Sartorius AG, Göttingen, Germany).

2.3.2. Preparation with a bath-type sonicator

We dissolved PEG-P(Asp(C7F9)*x*) block copolymers in MilliQ water at a concentration of 1.0 to 4.0 wt.% of water. In case of a high ester content such as $x = 59$, we heated (up to ca. 40 °C) and sonicated the solutions until we obtained a transparent polymer solution. The polymer solution was transferred to a 1.5-mL glass vial that was sealed with a Teflon–silicon rubber cap (Chromacol auto-sampler vial 2-SV for HPLC; GL Science, Inc., Tokyo, Japan), and was cooled on ice. Then, we added perfluoropentane (PFC5) and perfluorohexane (PFC6) at 0.5–4.0 vol.% of water. We confirmed PFCs' position at the bottom of the solution. (Sometimes PFCs, whose densities are much greater than water's, did not go into the aqueous solution. Therefore, we shook the vial vigorously to allow PFC droplets to sink to the bottom by force of gravity.) Then, we sealed the vial with a cap, and applied sonication for 3 min with a bath-type sonicator Branson model 1510 (oscillating frequency at 42 kHz, max. power intensity: 90 W, Danbury, CT, USA). The temperature of the bath was kept constant with degassed cold and hot water. In all the sonication procedures, we had a constant water level in a sonicator bath and a fixed position of the vial in order to obtain sonication conditions that were as identical to one another as possible. Finally, we collected a supernatant by leaving unincorporated PFC droplets at the bottom.

Table 2
Effects of polymer composition and sample volume on PFC5 incorporation behaviors.

Run	Polymer	Sample volume (μL)	PFC5 concentration (vol.%)[a]	Cumulant average diameter (nm)[a]
1	F-6%	300	0.840 ± 0.097	261.2 ± 3.4
2	F-15%	300	0.948 ± 0.131	232.4 ± 14.5
3	F-39%	300	0.625 ± 0.074	198.4 ± 33.3
4	F-59%	300	0.669[b]	133.9[b]
5	F-67%	300	0.682 ± 0.060	222.8 ± 37.9
6	F-59%	300	0.682 ± 0.074	205.5 ± 15.8
7	F-59%	300	0.634 ± 0.361	173.5 ± 24.5
8	F-59%	700	1.110[b]	231.8[b]
9	F-59%	1200	1.792[b]	280.6[b]

[a] Average ± standard deviation ($n = 3$) except runs 4, 8, and 9.
[b] Average of two preparations.

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In order to measure amounts of the polymer chains that were not included in the PFC-emulsions, we carried out the following experiment. PFC-emulsion was prepared in the conditions of Run 4 of Table 2; polymer: F-59%, sample volume: 300 μL, polymer concentration: 4 wt.%, PFC5: 2 vol.%, PFC6: 2 vol.%, sonication at 40 °C for 3 min. The obtained emulsion was transferred into a 1.5 mL Eppendorf-type poly(propylene) tube and centrifuged at 13,200 rpm for 5 min with an Eppendorf centrifuge model 5415D (Eppendorf Co., Ltd. Japan, Tokyo, Japan). The emulsion was found to precipitate at the bottom. 200 μL of the supernatant was collected and freeze-dried. We calculated the polymer amounts that were not included in the PFC-emulsions by multiplying 1.5 (=300 μL/200 μL) to the freeze-dried polymer weight. As a control, we carried out the same experiment just only for the polymer (without addition of TFC5 nor TFC6).

2.4. Measurements

2.4.1. Dynamic light scattering (DLS)

The size of emulsions was measured with a dynamic light scattering (DLS) instrument, the DLS-7000 (Otsuka Electronics, Tokyo, Japan). DLS samples were prepared through appropriate dilution of the emulsions with commercial distilled water for internal injection (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan). The measurements were made at 25 °C, and scattering was observed at a 90° angle with respect to the incident beam. The cumulant average particle size and the particle size distribution from a non-negative least square method were determined by the use of software provided with the instrument.

2.4.2. Gas chromatography

We measured concentrations of PFC5 using two gas chromatograph systems as described below. In both cases, we successfully obtained clear separation of PFC5's peak from PFC6's peak, and carried out quantitative analyses using a standard sample of PFC5. Therefore, the two gas chromatograph systems gave us identical results. However, we only used the (2) system described below for blood samples because its pre-heating function was essential for measurements of blood samples.

2.4.2.1. Gas chromatograph system. We measured PFC5 using a gas chromatograph model G-6000 (Hitachi High-Technologies Corporation, Tokyo, Japan) equipped with a Gaskuropack 54 80/100 packed column (GL Sciences, Inc., Tokyo, Japan) and an FID detector at 200 °C. Carrier gas was nitrogen at a flow rate of 300 mL/min. 5 μL of a sample solution were injected into the gas chromatograph system with a micro syringe at 0 min. Column temperature was controlled in the following manner; 100 °C (0 min), raised at a rate of 5 °C/min until 130 °C (6 min), and then raised at a rate of 60 °C/min until 190 °C (7 min), followed by maintenance of 190 °C for 2 min. PFC5 and PFC6 were found to elute at 3.8 min and 6.4 min, respectively.

2.4.2.2. Gas chromatograph system. We measured PFC5 using a gas chromatograph system GC-2014 (Shimadzu Corp., Kyoto, Japan) equipped with an FID detector at 250 °C. We used two tandem-connected two columns: DB-WAX 127-7012 (Agilent Technologies Japan, Ltd., Tokyo, Japan) and RESTEK Rt-QBond 19741 (Shimadzu GLC Ltd., Tokyo, Japan). Carrier gas was helium at a flow rate of 20 mL/min. Either 100 or 544 μL of a sample solution were heated at 200 °C and injected with a headspace autosampler TurboMatrix Trap 40 (PerkinElmer Japan Co., Ltd., Yokohama, Japan). Column temperature was constant at 150 °C. PFC5 and PFC6 were found to elute at 3.6 min and 4.4 min, respectively.

2.5. Measurements of PFC5 concentration in blood

In vivo PFC5 concentration profiles in blood were evaluated in Balb/c female mice (6 weeks old). 100 μL of PFC-emulsion was intravenously administered via lateral tail veins. The emulsions' PFC5 concentrations ranged from 0.429 to 0.670 vol.%. Blood (44 μL) was collected with a heparinized blood-collecting glass tube, and mixed with 500 μL of heparin solution in a capped sample tube of the (2) gas chromatograph system.

3. Results

3.1. General characteristics of the emulsion-preparation method with a bath-type sonicator

In representative conditions, we successfully obtained PFC5-containing nano-sized emulsions having diameters of ca. 200 nm in considerably high PFC5 yields. Fig. 3(a) and (b) shows diameter distributions measured by means of dynamic light scattering (DLS) for PEG-P(Asp(C7F9)59) (F-59% in Table 1). In these conditions, we dissolved 12.0 mg of polymer in 300 μL water (4.0 wt.% solution), and put this polymer solution in a 1.5 mL glass vial, followed by additions of 6 μL (corresponding to 2.0 vol.% of water) of PFC5 and 6 μL of PFC6. Sonication was performed for 3 min in a bath-type sonicator at 40 °C. In the first three preparations (run 6 in Table 2), the cumulant diameter obtained was 205.5 ± 15.8 nm (the average ± standard deviation; $n = 3$), and Fig. 3(a) shows the weight-weighted diameter distribution of one preparation. Almost uniformly distributed emulsions were obtained, and the diameter of the emulsion droplets had a very small size about 200 nm. In this run 6, PFC5 concentrations were 0.682 ± 0.074 vol.%. These values are considered large enough for ultrasound images (Kawabata et al., 2005, 2010a,b). In another set of three preparations (on another day, run 7 in Table 2), we obtained a very similar average diameter, 173.5 ± 24.5 nm (the average ± standard deviation; $n = 3$) and PFC5 concentrations. The diameter distribution of one preparation of run 7 is shown in Fig. 3(b). These two figures exhibited a major peak at about 200 nm, while a minor peak was seen in a larger diameter side and a smaller diameter side, as shown in Fig. 3(a) and (b), respectively. This difference may result from a slight variation in sonication conditions such as the position of samples and the water level of the sonicator. These emulsions were obtained and measured without any purification process after the sonication, and a large majority of the emulsions in weight were found to have a diameter of about 200 nm. All these results clearly indicate that this sonication method brought about very small nanometer-sized PFC5-containing emulsions with considerably high PFC5 concentrations.

We measured a proportion of polymer incorporated in the PFC-emulsion out of the feed polymer amount. In these preparation conditions (run 7 in Table 2), 75.4 ± 2.6% ($n = 3$) of the feed polymer was found in a supernatant obtained after centrifugation. (All the PFC-emulsions were observed to precipitate in this centrifugation.) When this measurement was carried out for the polymer alone, 93.8 ± 2.0% ($n = 3$) of the feed polymer was found in a supernatant obtained after centrifugation. Therefore, 18.4% (=93.8%–75.4%) of the feed polymer was considered to be incorporated into the PFC-emulsions. Removal of the free polymer chain, that was not incorporated into the PFC-emulsion, was not examined in this study. The removal is difficult because the free polymer existed as a polymeric micelle was close to the PFC-emulsion in size. (If the free polymer existed as a single polymer chain, a difference in size between the free polymer and the PFC-emulsion would be so large to allow separation such as ultrafiltration.)

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