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Research paper

Induction of Th1 polarized immune responses by thiolated Eudragit-coated F4 and F18 fimbriae of enterotoxigenic *Escherichia coli*

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ABSTRACT

Diarrhea in newborn and weaned piglets is mainly induced by enterotoxigenic *Escherichia coli* (ETEC) with fimbriae F4 (K88) and F18 (F107). In this study, we evaluated F4 and F18 coated with thiolated Eudragit microspheres (TEMS) as a candidate for an oral vaccine. The average particle sizes of TEMS, F4-loaded TEMS, and F18-loaded TEMS were measured as 4.2 ± 0.75 μm, 4.7 ± 0.50 μm, and 4.5 ± 0.37 μm, respectively. F4 is more efficiently encapsulated than F18 in the loading with TEMS. In the release test, F4 and F18 fimbriae were protected in acidic circumstances, whereas most were released at pH 7.4 of intestine circumstances. Production of TNF-α and NO from RAW 264.7 cells was increased in a time-dependent manner after exposure to all groups, whereas only F4- or F18-loaded TEMS-stimulated IL-6 secretion. The levels of IFN-γ from mouse splenocytes after exposure to F4 or F18 were increased while IL-4 was not detectable. These results suggest that F4- and F18-loaded TEMS may effectively induce immune response with the efficient release of antigens to appropriate target sites.

1. Introduction

Most pathogens invade hosts via mucosal surfaces. Therefore, the induction of a mucosal immune response is necessary to prevent this invasion. Also, mucosal immunization can overcome the problems of parenteral immunization such as no induction of mucosal immune response, needle born infections, poor compliance, injection site pain, and local side effects from injections [1–3]. Of the mucosal immunization methods, oral vaccinations have received attention because of their easy and acceptable properties. However, the antigens for oral vaccinations must be able to resist the harsh environment of the gastrointestinal tract, including various gastric acids, hydrolytic enzymes, and degradation of the ingested antigens [4–7]. Therefore, various delivery systems have been developed to solve these limitations. Of these delivery methods, oral administration using mucoadhesive and/or pH-sensitive microspheres has successfully delivered protein drugs [8–12]. Recently, thiolated polymers—so-called thiomers—have been investigated as mucoadhesive polymers. The mucoadhesive property derives from covalent bonds between the reactive thiol groups of the polymer and the cysteine-rich subdomains of the mucin glycoproteins by thiol/disulfide exchange reactions and an oxidation process [13,14]. In this study, thiolated Eudragit microsphere (TEMS) as a thiomer was used as a coating material, which was synthesized by forming covalent bonds between the amino groups of the cysteine and the carboxylic groups of the Eudragit® L-100.

Diarrhea in newborn and weaned piglets is a severe problem that causes significant economic losses in the pig industry. Enterotoxigenic *Escherichia coli* (ETEC) is one of the major causes of diarrhea or edema disease in piglets. The bacteria bind with their fimbriae to specific receptors on porcine intestinal brush border epithelial cells (enterocytes), colonize the cell surface, and induce diarrhea by secreting heat labile and heat stable enterotoxins [15,16]. Therefore, adherence of the fimbriae to enterocytes might be considered the first step in ETEC infections. Previous studies identified that colonization begins after adhesion of the bacteria with their fimbriae to the small intestine and stops when antifimbrial antibodies can be detected in the intestinal lumen of the infected pigs [17–20]. Thus, fimbrial proteins are interesting antigens to be included in a vaccine against ETEC. F4 or F18 fimbriae have been mainly detected with ETEC causing post-weaning diarrhea [21,22]. Based on this knowledge, the biophysical and immune stimulating properties of F4- or F18-loaded TEMS were investigated in this study to evaluate its possibility of as a candidate for oral vaccines.

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2. Materials and methods

2.1. Materials

2.1.1. Polymers and reagents

L-cysteine hydrochloride monohydrate, fluorescein diacetate (FDA), tripolyphosphate (TPP), and lipopolysaccharide (LPS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Eudragit® L-100 was obtained from Röhm Pharma (Weiterstadt, Germany).

2.1.2. Bacterial strain

E. coli strains were isolated from suckling and weaned piglets with diarrhea from different farms in Korea and were identified by colony morphology and a biochemical and automatic bacteria identification system, Vitek 2 system (Hazelwood, MD, USA). F4 ac and F18 ab and ac genes in *E. coli* were confirmed by multiplex PCR amplification [23].

2.2. Preparation of fimbria proteins and microspheres

2.2.1. Purification of F4 and F18 fimbriae

The F4 fimbriae were purified as described by Wim van den Broek et al. [24]. Briefly, the bacteria were cultured in Trypton Soy Broth at 37 °C while shaking at 200 rpm for 18 h. Subsequently, the bacteria were collected by centrifugation at 4 °C, 7000 rpm for 20 min, and were washed in PBS. F4 fimbriae were isolated by homogenizing the bacterial suspension using a T10 basic Ultra Turrax (Janke & Kunkel, IKA Labortechnik, Staufen, Germany) at 24,000 rpm for 15 min. Larger fragments were removed by centrifugation at 8500 rpm for 20 min and the supernatant was further purified by a subsequent centrifugation at 10,000 rpm for 40 min at 4 °C. The solubilized F4 fimbriae were precipitated with 20% (w/v) ammonium sulfate and the pellet was dissolved in PBS and dialyzed overnight against ultra-pure H_2O. The F18 fimbriae were isolated by heat shock at 60 °C for 20 min, followed by a purification with two centrifugation steps and a precipitation step as for F4 [25]. The pellet, then, was dissolved in PBS and dialyzed overnight against ultra-pure H_2O.

2.2.2. Preparation of thiolated Eudragit (TE) and F4- or F18-loaded TEMS

The synthesis of thiolated Eudragit was performed according to a previously reported method [26]. F4- or F8-loaded TEMS were prepared according to a water-in-oil-in-water (W/O/W)-solvent evaporation method of a previous study with a little modification [27]. The F4 or F18 solution was mixed with Pluronic F-127 solution (1%) to form an internal aqueous phase (IAP) (W). The IAP was emulsified with an organic phase using an ultrasonic processor (Sonics, Vibra cells™) for 1 min to make the primary emulsion (W/O). The organic phase (O) consisted of 200 mg of thiolated eudragit (TE) dissolved in 5 ml of dichloromethane (DCM). The primary emulsion (W/O) was added to external aqueous phase (EAP) (W) of 1% PVA (polyvinyl alcohol) solution. The mixture was homogenized with T25 basic Ultra Turrax (Janke & Kunkel, IKA Labortechnik, Staufen, Germany) at 11,000 rpm for 4 min to form W/O/W emulsion, which was then stirred for 2–3 h at RT to allow the solvent to evaporate. F4- or F18-loaded TEMS were collected and washed 3 times with distilled water. The F4- or F18-loaded TEMS were lyophilized and stored at −70 °C. The unloaded TEMS were prepared using similar method without F4 or F18.

2.2.3. Morphology and size of microspheres

The particle sizes were measured using DLS-7000 (Otsuka Electronics. Ltd., Tokyo, Japan) with an argon laser beam at 488 nm at 20 °C. The particles were gold-coated using a coating chamber (CT 1500HF, Oxford Instruments, Oxfordshire, UK) to observe the microsphere morphology. The coated samples were observed using JSM5410LV field emission SEM (Tokyo, Japan).

2.2.4. Loading efficiency (%)

The loading efficiency of F4 or F18 fimbriae in TEMS was measured by quantifying the unloaded vaccine in the supernatant during the TEMS preparation washing time. Briefly, in each washing time, the supernatant was collected and the concentration of the unloaded vaccine in the supernatant was measured using the micro BCA protein assay method. The unloaded vaccine concentration was then subtracted from the initially loaded vaccine concentration to calculate the actual loading content of F4 or F18 in TEMS. For each sample, the loading content was measured by a micro BCA protein assay in triplicates. The general equation to calculate the loading efficiency (%) is as follows:

$$\text{Loading efficiency } (\%) = \frac{\text{total amount of F4 or F18} - \text{unloaded F4 or F18}}{\text{total amount of F4 or F18}} \times 100.$$

2.2.5. Circular dichroism spectroscopy

The changes in the secondary structure of the released F4 and F18 with respect to the native F4 and F18 were measured by CD spectroscopy (Chirascan plus, UK) with a quartz cylindrical cell. Solutions of native fimbriae and released fimbriae were scanned over the wavelength range 200–260 nm. Using the Chirascan plus software, the background solution (PBS 7.4) was subtracted from each protein spectrum.

2.3. In vitro studies

2.3.1. Release of F4 or F18 from F4- or F18-loaded TEMS

In vitro F4 or F18 release from F4 or F18-loaded TEMS was carried out as follows: 10 mg of F4- or F18-loaded TEMS were suspended in PBS (pH 7.4 and 2.0) separately into 1.5 ml microtubes and agitated up to 8 h at 37 °C with 100 rpm/min, using a shaking water bath. Aliquots (1 ml) were withdrawn from the released medium and replaced by an equal volume of fresh medium at each sampling time. The amount of released fimbriae proteins was determined as a cumulative release (%) with incubation time by the BCA protein assay method in triplicates.

2.3.2. Measurement of secreted cytokines and nitric oxide (NO)

A murine macrophage cell line, RAW 264.7, was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (50 μl/ml) and streptomycin (50 μg/ml) at 37 °C under 5% CO_2. After incubating the cells for 18 h in the 12 well plates containing 1×10^5 cells/ml, they were stimulated with 1 μg/ml of unloaded TEMS, F4-, F18-, F4-loaded TEMS, F18-loaded TEMS, and *E. coli* LPS (1 μg/ml, Sigma). LPS was used as positive control and unloaded TEMS and DMEM were used as negative controls. After stimulation, the secreted concentrations of TNF-α and IL-6 were measured using the enzyme-linked immunosorbent assay (ELISA) method (eBioscience Inc.). Production of NO in the culture medium was measured by measuring the nitrite accumulation with the Griess reaction. Briefly, 100 μl aliquots of the culture supernatants were incubated with same volume of the solution containing 1% sulfanilamide (Sigma) and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma) in 2.5% phosphoric acid at room temperature. After 10 min of incubation, the absorbance was measured at 540 nm and nitrite concentrations in each well were calculated based on the standard curve generated with sodium nitrite. For the IFN-γ and IL-4 measurement

assay, female ICR mice (5 weeks old) were purchased from Orient Bio Inc. (Kyunggi, Korea). All care and handling of animals were performed with the approval of Institutional Animal Care and Use Committees of Seoul National University. Splenocytes isolated from mice (1×10^6 cells/ml) were stimulated with 1 μg/ml of unloaded TEMS, F4-, F18-, F4-loaded TEMS, F18-loaded TEMS, and concanavalin A for 0, 1, 2, 3, and 4 days. The levels of IFN-γ and IL-4 in the culture supernatant were measured using the ELISA method (eBioscience Inc.).

3. Results

3.1. Characterization of microspheres

Unloaded TEMS, F4-loaded TEMS, and F18-loaded TEMS were observed by SEM micrographs (Fig. 1). The results indicated that the morphologies of unloaded TEMS, F4-loaded TEMS, and F18-loaded TEMS were observed as spherical shapes with a smooth surface (Fig. 1). The average particle sizes of unloaded TEMS, F4-loaded TEMS, and F18-loaded TEMS were measured as 4.2 ± 0.75 μm, 4.7 ± 0.50 μm, and 4.5 ± 0.37 μm, respectively (Fig. 2). The encapsulation efficiencies of F4-loaded TEMS and F18-loaded TEMS were 87.8 and 56.1%, respectively.

3.2. Release of F4 or F18 from F4- or F18-loaded TEMS in vitro

In vitro release profiles of F4 or F18 from F4- or F18-loaded TEMS up to 8 h are shown in Fig. 3. In the release study, around 70% of F4 and F18 were released within 8 h at pH 7.4, whereas less than 20% of F4 and F18 were released within 8 h at pH 2.0, respectively. Compared with F4, a higher percentage of F18 protein was released at both pH conditions. Moreover, Fig. 4 shows the CD spectra of fimbriae before and after microencapsulation. These results indicated that the encapsulation process did not provoke any structural change in F4 and F18.

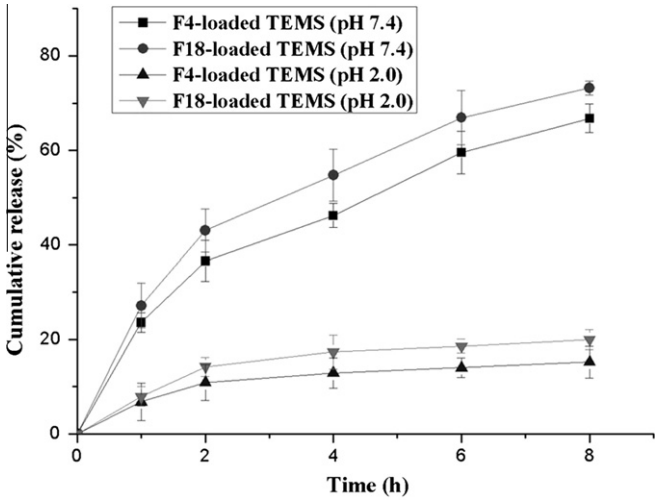

Fig. 3. Cumulative release (%) of the vaccine (F4 and F18) from TEMS in vitro (in PBS at pH 7.4 and KCl–HCl buffer 2.0; 37 °C with 100 rpm shaking).

3.3. Measurement of secreted cytokines and nitric oxide (NO)

TNF-α from RAW 264.7 cells was increasingly produced in a time-dependent manner after exposure to F4-, F18-, F4-, or F18-loaded TEMS and unloaded TEMS (Fig. 5). TNF-α inducing activity was increased by F4- or F18-loaded TEMS when compared with unloaded F4 and F18 even though TEMS itself showed the activity (Fig. 5). The production of IL-6 was increased time-dependently after exposure to only F4- or F18-loaded TEMS (Fig. 6). The concentration of TNF-α, IL-6, and NO from F18-loaded TEMS was higher than that from F4-loaded TEMS. Production of NO was increased time-dependently after exposure to all groups (Fig. 7). NO concentration in F18-loaded TEMS group was highest, followed by that in

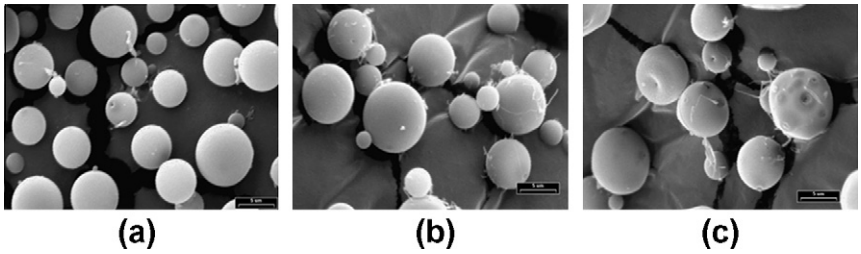

Fig. 1. SEM photographs of unloaded TEMS (a), F4-loaded TEMS (b), and F18-loaded TEMS (c) (2000X).

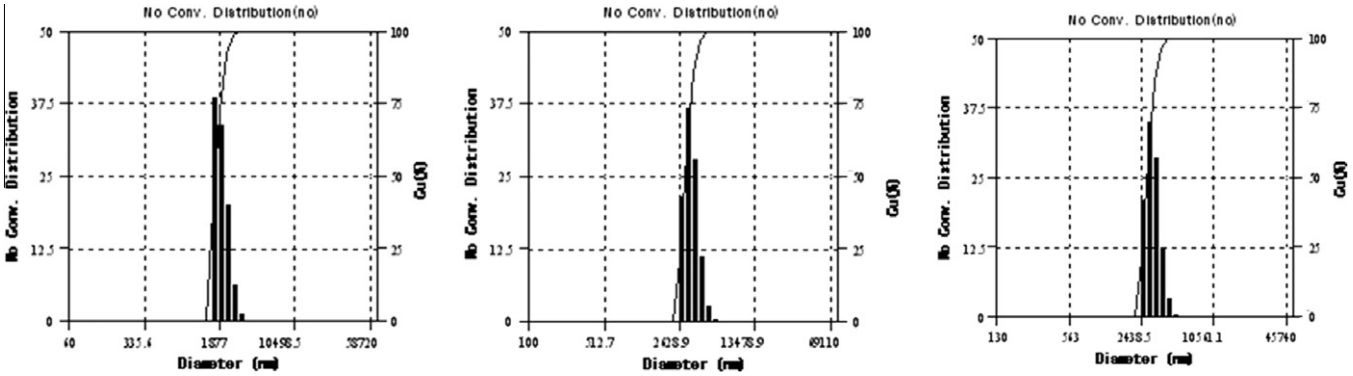

Fig. 2. Particle size distribution of unloaded TEMS (a), F4-loaded TEMS (b), and F18-loaded TEMS (c).

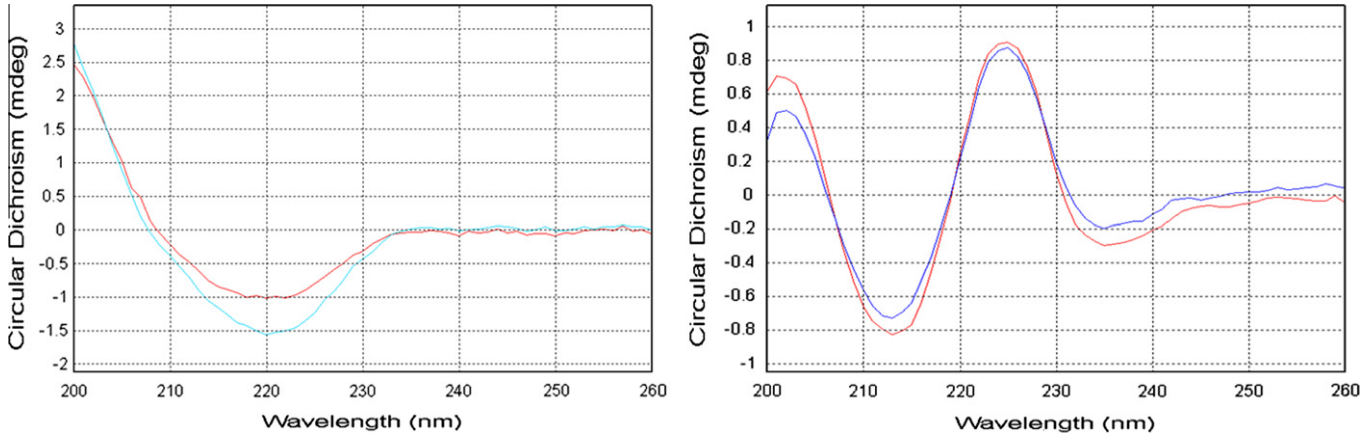

Fig. 4. Circular dichroism spectra of native fimbriae and released fimbriae from fimbriae-loaded TEMS. (a) Red: native F4, blue: released F4 from F4-loaded TEMS, (b) red: native F18, blue: released F18 from F18-loaded TEMS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

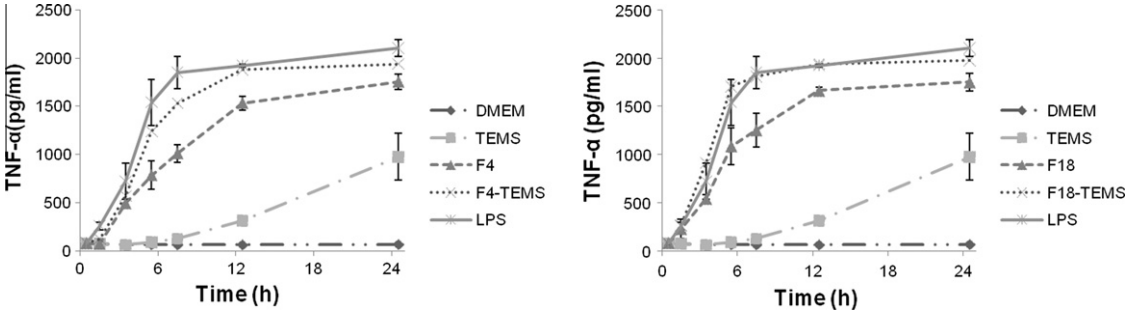

Fig. 5. Secretion of TNF-α from RAW 264.7 cells stimulated TEMS, F4, F4-loaded TEMS, F18, and F18-loaded TEMS.

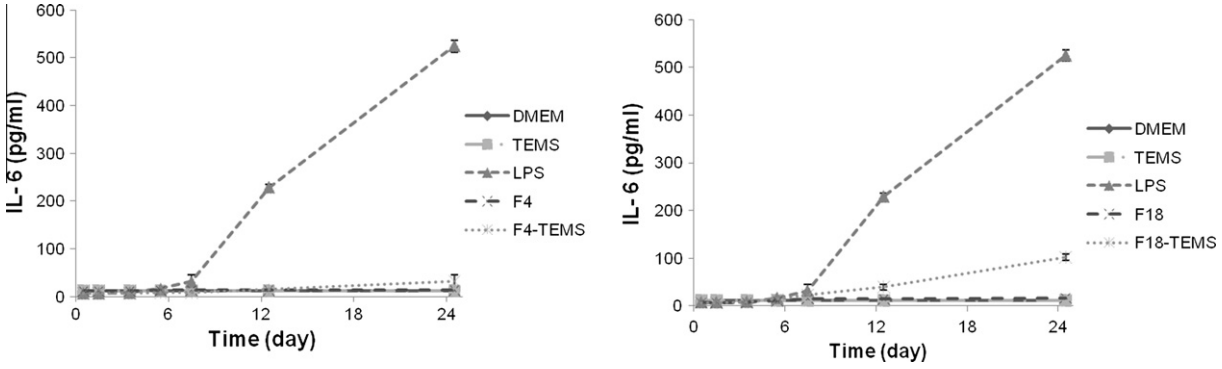

Fig. 6. Secretion of IL-6 from RAW 264.7 cells stimulated TEMS, F4, F4-loaded TEMS, F18, and F18-loaded TEMS.

F18 and TEMS group. The NO levels of F4-loaded TEMS group was similar to that of F4 group. Splenocytes stimulated with F4 and F18 produced slightly higher levels of IFN-γ than with F4- or F18-loaded TEMS (Fig. 8). However, the production of IL-4 from all groups was not detectable in this study (Fig. 9). *E. coli* LPS as a positive control of TNF-α, IL-6, and NO production and Concanavalin A as a positive control of IFN-γ and IL-4 production showed highest levels of these cytokines, whereas DMEM as a negative control of all cytokines and NO showed no production.

4. Discussion

The aim of this study is to develop an effective oral mucosal vaccine delivery system using thiolated Eudragit microspheres with mucoadhesive and pH-sensitive properties. F4 and F18 fimbriae of ETEC were purified and used as antigens. These antigens were coated with thiolated Eudragit and the particle sizes were estimated. Previous studies have already reported that an appropriate size of microspheres is quite important in targeting vaccines to mucosal associated lymphoid tissues (MALT) [28]. In the study, orally administered 1–10 μm microspheres were specifically taken up into the Peyer's patch lymphoid tissue of the gut, where those greater than or equal to 5 μm remained until 35 days. The initial step of the oral vaccination is the internalization of an antigen into M-cells in the Peyer's patches. Therefore, the effective delivery of the microparticles to the Peyer's patch is considered important for the elevation of immune responses. The average particle sizes of F4-loaded TEMS and F18-loaded TEMS were 4.7 ± 0.50 μm and

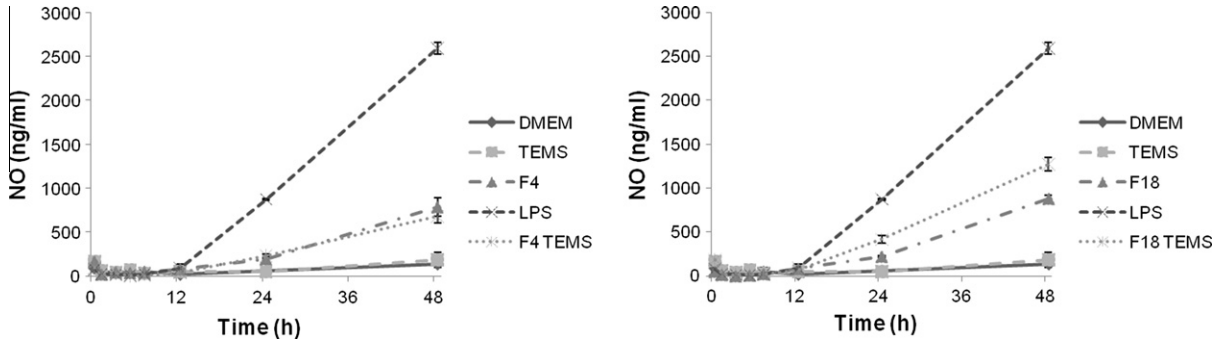

Fig. 7. Secretion of NO from RAW 264.7 cells stimulated TEMS, F4, F4-loaded TEMS, F18, and F18-loaded TEMS.

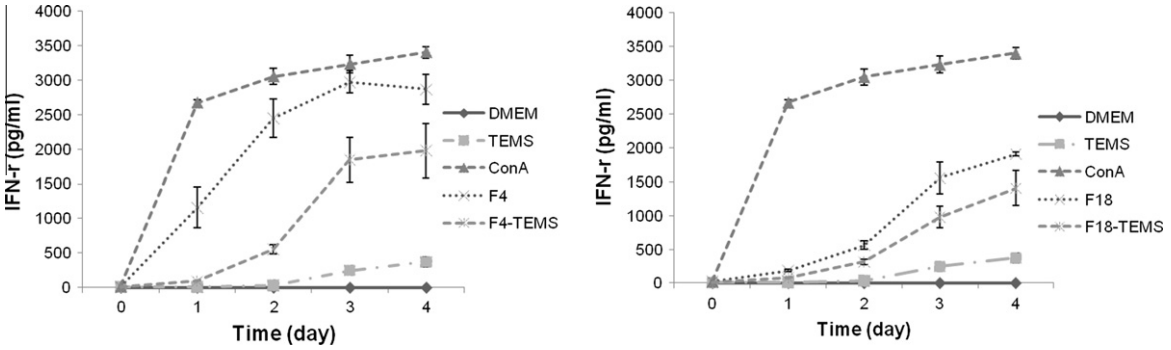

Fig. 8. Secretion of IFN-γ from mouse splenocytes stimulated TEMS, F4, F4-loaded TEMS, F18, and F18-loaded TEMS.

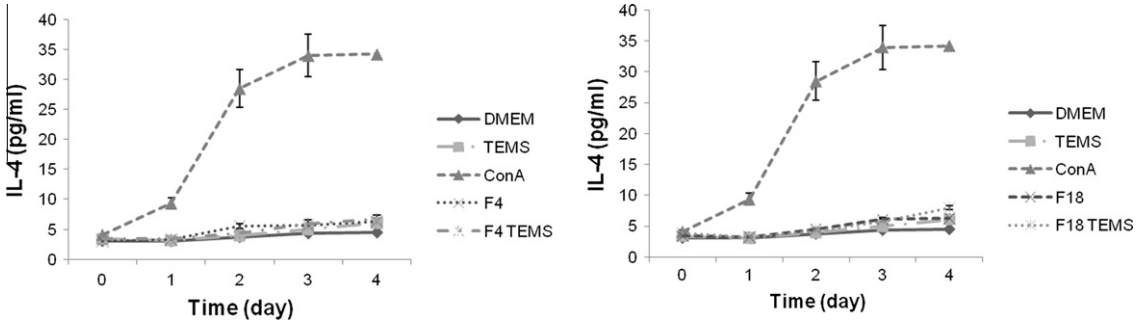

Fig. 9. Secretion of IL-4 from mouse splenocytes stimulated TEMS, F4, F4-loaded TEMS, F18, and F18-loaded TEMS.

4.5 ± 0.37 μm, respectively, and the size of both of these TEMS was around 5 μm. Therefore, these particles were expected to be effectively taken up into the Peyer's patch of the gut.

It has been reported that most TEMSs remained in the stomach at 0.5 and 1 h after oral administration to mice [26]. Therefore, most TEMSs were expected to be exposed to acidic solution for about 1 h. In this study, less than 10% of F4 and F18 were released within 1 h at pH 2.0 and most of the proteins were considered to be protected from the gastric solution. Even the released fimbriae proteins at pH 2.0 were considered to be stable up to 2 h according to a previous study [29]. The release of F4 and F18 from particles was increased rapidly at pH 7.4, and around 70% of fimbriae proteins were released within 8 h. Microspheres were shown to exist in the intestines of mice from 4 to 6 h after oral administration [26], and majority of proteins were expected to be exposed to the intestine as target organs after protection from gastric solution. The amount of F18 released from F18-loaded TEMS was higher than that of F4 in all graphs. The encapsulation efficiency of F18-loaded TEMS was lower than F4-loaded TEMS, suggesting that F18 dissolved faster than F4 from microspheres.

It is important to investigate the immune stimulating activity of F4-loaded TEMS and F18-loaded TEMS as an oral vaccine. We investigated the secretion of TNF-α, IL-6, and NO from RAW 264.7 cells as immune inducing indicators because the cells produce these materials more efficiently than others. Production of TNF-α from unloaded TEMS was increased although unloaded TEMS was a negative control (Fig. 5). Furthermore, F4- or F18-loaded TEMS stimulated more TNF-α and IL-6 secretion from RAW 264.7 cells than other groups (Figs. 5 and 6). It is suggested that TEMS has immune stimulating activity and it could become an adjuvant to enhance immunogenicity of vaccine. According to a previous study, Eudragit microparticles could preserve the biological functions of proteins without any adjuvants or stabilizers [30]. F18-loaded TEMS caused higher concentration of TNF-α, IL-6, and NO than F4-loaded TEMS. This could be because the encapsulation efficiency of F18-loaded TEMS was lower than F4-loaded

TEMS and more TEMS were contained in F18-loaded TEMS than F4-loaded TEMS. TEMS was considered to have its own immune stimulating activity, and F18-loaded TEMS released more cytokines and NO production than F4-loaded TEMS. TNF-α, IL-6, and NO levels were increased after stimulation of F4- or F18-loaded TEMS (Figs. 5–7). TNF-α is the principal mediator of the acute inflammatory response, and IL-6 stimulates the growth of B lymphocytes that have differentiated into antibody producers. NO is released by responses to activated macrophages. Thus, F4- or F18-loaded TEMS are considered to induce innate and adaptive immunity and protective immune response against systemic *E. coli* infection [31,32]. To investigate T cell-mediated immunity, we measured IFN-γ and IL-4 levels from mouse splenocytes stimulated with each group. IFN-γ and IL-4 are well-known indicators of Th1 and Th2 cell-mediated immunity, respectively. IFN-γ levels of all groups were increased time-dependently, while IL-4 levels were not detectable. This indicates that the processed F4 and F18 proteins triggered Th1 cells to secrete IFN-γ, but not IL-4. IL-4 is known to be inhibited by the Th1-derived cytokine IFN-γ [33]. Thus, these results imply that F4 and F18 could induce Th1 cell-mediated immunity.

Therefore, our results support that TEMS could be used as a delivery system for the effective induction of mucosal immunity for ETEC.

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