—Regular Articles—

# Preparation and *in Vitro* Evaluation of Chitosan-coated Alginate/Calcium Complex Microparticles Loaded with Fluorescein-labeled Lactoferrin

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An attempt was made to prepare FITC-labeled-lactoferrin (LF-FTC)-loaded microparticles, durable under gastrointestinal conditions, first by the combination of alginate/calcium complexation and emulsification-evaporation and next by treatment with chitosan solution. The obtained microparticles were examined for particle characteristics, *in vitro* release profiles and physical stability in solutions of pH 1.2 and 6.8. The obtained chitosan-coated alginate/calcium complex microparticles (Ch/Al/Ca-MP) showed almost uniform size of 1–2 $\mu$m and a spherical shape with a non-smooth surface. The content and recovery of LF-FTC in Ch/Al/Ca-MP fell as the concentration of chitosan solution used in chitosan coating increased. The release rate of LF-FTC was faster in Ch/Al/Ca-MP prepared with more chitosan at pH 1.2 and 6.8. Ch/Al/Ca-MP coated with 0.25 and 0.5% (w/v) chitosan solution showed good gradual release characteristics *in vitro*. Furthermore, they were durable at pH 1.2 and 6.8, though swelling and softening of the microparticles occurred at pH 6.8. It is suggested that alginate/calcium complex microparticles coated with 0.25–0.5% (w/v) chitosan solution would be useful for the intestinal delivery of LF.

**Key words**——lactoferrin; alginate/calcium complex; chitosan coating; *in vitro* release; physical stability

## INTRODUCTION

Lactoferrin (LF) is an iron-binding glycoprotein, expressed abundantly in milk, saliva, tears, other exocrine secretions, and polymorphonuclear leukocyte granules.[1–3] It has attracted much attention in the field of alternative medicine, because it exhibits various biological functions, including antibacterial, antiviral, antitumor, antiinflammatory and immunomodulatory effects.[4–9] Generally, many developed drugs can cause toxic side effects as well as having therapeutic efficacy, resulting in their limited use. On the other hand, since LF has no toxic side effects, it is expected to be a possibly useful therapeutic agent for host defense and the treatment of various refractory diseases. LF can act directly in diseased areas, and its minimal inhibitory concentrations (MICs) for bacteriostatic and/or bactericidal activities were reported.[10,11] In addition, LF shows effectiveness against diseases developing in the remote sites *via* LF receptor-mediated and immune defense systems *etc*.[8,9,12–14] For instance, oral administration of LF exhibits good effect against oral candidiasis and urinary tract infection or tumor.[15–17] However, for oral administration of LF, its degradation by peptic enzymes is a problem. Therefore, dosage forms to allow LF to reach target sites effectively are important to improve its efficacy. It was recently reported that oral pretreatment using LF-containing liposomes was more effective than that with LF alone for anti-inflammatory effects.[9] Also, enteric coating is effective for protection from peptic degradation and intestinal absorption.[18] Polymeric microparticles are considered to be effective to protect LF under stomach conditions, and are expected to enhance mucosal adhesion and penetration into the mucus layer.[19] Previously, we prepared chitosan microparticles loaded with LF, and characterized their *in vitro* properties.[20] Simple chitosan microparticles exhibited better particle characteristics and release profile than the microparticles prepared by sonication or by complexation with sulfate ions. However, such chitosan microparticles were considered to be problematic because they could dissolve, swell and degrade physically in gastric pH.[21] Therefore, in this study, we attempted to prepare chitosan-coated alginate/calcium complex microparticles in order to obtain microparticulate formulations of LF, which is physically durable in the stomach and intestine. Chitosan/alginate/calcium complex microparticles are known to be able

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to be produced by several methods.[22–25] Wittayaareekul *et al*. reported the two-step preparative method in which alginate-calcium complex was first produced and subsequently complex microparticles were further coated with chitosan by soaking in chitosan solution.[23] However, they were much larger than 1 $\mu$m or less in particle size, which is appropriate for longer mucoadhesion, or were not uniform. In the study, the preparation of smaller microparticles of around 1 $\mu$m in size was attempted by a combination of chitosan-alginate-calcium complexation and emulsification-evaporation technique. The obtained microparticles were examined for particle characteristics, *in vitro* release and physical states in JP15 1st and 2nd fluids.

## MATERIALS AND METHODS

**Chemicals** Bovine lactoferrin (LF) was donated by NRL Pharma Inc. (Kawasaki, Japan). Sodium alginate (Al–Na; 80–120 cP) and calcium chloride were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Daichitosan VL (81% deacetylation degree and extra low viscosity grade) was supplied by Dainichiseika Color & Chemicals Mfg. Co., Ltd. (Tokyo, Japan), and used as chitosan (Ch) throughout the experiment. Sorbitan sesquioleate (SO-15) was obtained from Nikko Chemicals Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

**Preparation of Microparticles** First, LF was labeled with fluorescein isothiocyanate (FITC). LF (100 mg) and FITC (10 mg) were dissolved in 10 ml of 0.1 M carbonate buffer, pH 8.4, and stirred for 4 h. The mixture underwent gel-filtration using a Sephadex 50 column, dialyzed against water and lyophilized, resulting in 81 mg of FITC-labeled LF (LF-FTC), which displayed the absorbance coefficient, E (1% (w/v), 1 cm), of 44.5 in JP15 2nd fluid.

Ten milliliters of aqueous solution containing Al-Na (150 mg) and 5 ml of aqueous solution containing LF-FTC (18 mg) were mixed, and added to 300 ml of liquid paraffin containing 1% (w/v) SO-15. The mixture was stirred at 1000 rpm for 1 h. Then, 3 ml of aqueous solution containing 300 mg of $CaCl_2$ were added, and stirring was continued under the same conditions for 15 min. The mixture underwent reduced pressure using a rotary evaporator under gentle stirring. After complete evaporation of water, n-hexane (300 ml) was added to the mixture, and stirred. The mixture was centrifuged to obtain microparticles named Al/Ca-MP.

Al/Ca-MP (50 mg) were added to 0.25, 0.5 or 1% (w/v) chitosan solution in 1% (v/v) aqueous acetic acid solution (20 ml), and stirred at 500 rpm for 1 h. The residue was washed twice with 20 ml of water. The final residue was obtained as chitosan-coated Al/Ca-MP, named Ch/Al/Ca-MP.

**Measurement of Particle Characteristics** Drug content was measured as follows. A certain amount of Al/Ca-MP was suspended in a certain volume of JP15 1st fluid, and stirred vigorously. After the mixture was centrifuged, the clear supernatant and solid portion were separated. The solid residue was dissolved by stirring in a certain volume of JP15 2nd fluid. The above clear supernatant in JP15 1st fluid and the solution in JP15 2nd fluid were examined spectrophotometrically at 446 nm and 494 nm, respectively, to determine the concentration of LF-FTC in both media, leading to the LF-FTC content in Al/Ca-MP.

In the preparation of Ch/Al/Ca-MP, the clear supernatants obtained by treatment with chitosan solution and after washing with water were examined spectrophotometrically to determine LF-FTC concentration in the media. The LF-FTC content in Ch/Al/Ca-MP was calculated by subtracting the amount in the media from that in the initial amount in Al/Ca-MP.

The microparticles were observed by scanning electron microscopy (SEM) using a JEOL JSM-5600LV scanning electron microscope (JEOL, Tokyo, Japan) after coating at approximately 10 nm in thickness with platinum. Their particle size and shape were investigated using SEM images. Parallel lines touching the opposite sides of each microparticle were made in the same direction, and the distance between parallel lines, called Green diameter, was measured. The Green diameter corresponds to the Feret diameter used by the Association of Powder Process Industry and Engineering, Japan. The measurement was performed for 150 microparticles chosen at random.

***In Vitro* Release Studies** Ch/Al/Ca-MP were suspended in JP15 1st and 2nd fluids at a concentration of 0.6 mg/ml, and shaken horizontally at 100 strokes per min at 37°C. At appropriate time points, the whole sample was centrifuged at 3000 rpm for 10 min, and the supernatant was measured spec-

trophotometrically to determine the LF-FTC concentration. After the measurement, the whole supernatant and the residue were mixed, and the incubation was re-started under the same condition. The release amount was calculated from the concentration of LF-FTC. The incubation time was defined as the time for the sample to be set in the thermostat bath at 37℃.

In addition, the sequential release test was conducted for Ch/Al/Ca-MP. Namely, Ch/Al/Ca-MP were suspended in JP15 1st fluid at a concentration of 0.6 mg/ml at 100 strokes per min at 37℃ for 1 h. The whole sample was centrifuged at 3000 rpm for 10 min, and the supernatant was analyzed spectrophotometrically to determine the released LF-FTC. The residue was then suspended in the same volume of JP15 2nd fluid, and the release test was continued for another 4 h. The release amount was determined in the same manner as above.

**Physical States of Microparticles in Media at Different pHs** Ch/Al/Ca-MP prepared at a chitosan concentration of 0.5% (w/v), named Ch0.5/Al/Ca-MP, were suspended in JP15 1st and 2nd fluids, and incubated at a concentration of 0.6 mg/ml at 100 strokes per min at 37℃ for 3 h. The suspensions were then observed visually and using an optical microscope and a fluorescence microscope to investigate their physical particle states. In addition, Ch0.5/Al/Ca-MP were examined for their particle states in sequential incubation using JP15 1st fluid for initial 3 h and JP15 2nd fluid for another 3 h, with incubation conditions in the same as above. After incubation in JP15 1st fluid and next in JP 2nd fluid, the suspension was observed visually and by optical and fluorescence microscopy.

Moreover, after the sequential release test stated above, the residue obtained by centrifugation of the incubated suspension was dried in a desiccator, and the dried sample was observed by SEM.

## RESULTS AND DISCUSSION

**Particle Characteristics** When Al/Ca-MP were prepared by the emulsification-evaporation method under the present conditions, their yield, drug content and size of Al/Ca-MP were obtained as shown in Table 1. Al/Ca-MP released LF-FTC in JP15 1st fluid to a fair extent. The Al/Ca-MP suspension in JP15 1st fluid was centrifuged, and the resultant precipitate dissolved easily in JP15 2nd fluid. These dissolution properties allowed measurement of the LF-FTC content in Al/Ca-MP. The used materials, including LF-FTC, were recovered in Al/Ca-MP at a high recovery ratio. As the used materials were water-soluble but hardly soluble in organic solvent, most of them were considered to be recovered in the microparticles, Al/Ca-MP. Al/Ca-MP were approximately spherical and their size was mainly in the range of 1–2 $\mu$m, with a mean diameter of 1.45 $\mu$m (Fig. 1).

Three kinds of Ch/Al/Ca-MP were prepared using 0.25, 0.5 and 1.0% (w/v) chitosan solution in 1% (v/v) acetic acid as the solution for chitosan coating, and they were named Ch0.25/Al/Ca-MP, Ch0.5/Al/Ca-MP and Ch1.0/Al/Ca-MP, respectively. Their yields, drug contents and sizes are summarized in Table 2. Ch/Al/Ca-MP hardly dissolved in aqueous media,

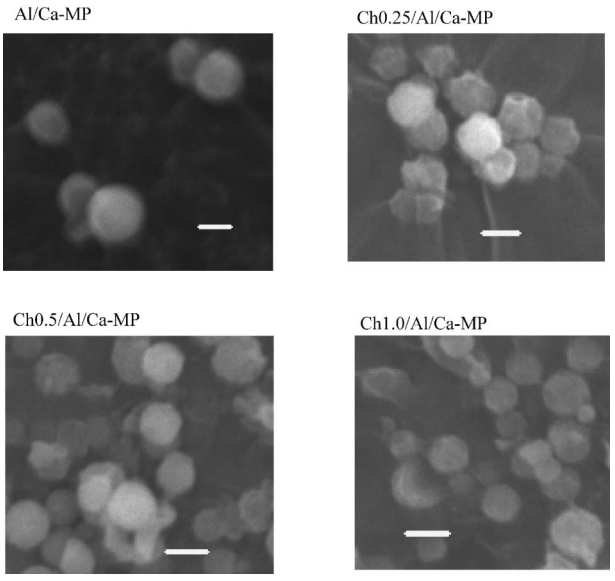


Fig. 1. SEM Images of Al/Ca-MP and Ch/Al/Ca-MP
The length of each white bar is 1 $\mu$m.

Table 1. Preparative Conditions, Yields and Particle Characteristics of Al/Ca-MP

| Al-Na (mg) | LF-FTC (mg) | $CaCl_2$ (mg) | Produced amount (mg) | Yield (%, w/w) | LF-FTC content (%, w/w) | LF-FTC recovery (%) | Particle size[a] ($\mu$m) |
|---|---|---|---|---|---|---|---|
| 150 | 18 | 300 | 439 | 94 | 2.6 | 64 | 1.45±0.30 |

*a*) The results are expressed as the mean±S.D. ($n$=150).

Table 2. Preparative Conditions, Yields and Particle Characteristics of Ch/Al/Ca-MP

| Ch/Al/Ca-MP | Al/Ca-MP (mg) | Ch solution volume (ml) | Produced amount (mg) | LF-FTC content (%, w/w) | LF-FTC recovery (%) | Particle size[a] ($\mu$m) |
|---|---|---|---|---|---|---|
| Ch0.25/Al/Ca-MP | 50 | 20 | 14.8 | 8.12 | 91.9 | 1.13±0.21 |
| Ch0.5/Al/Ca-MP | 50 | 20 | 13.2 | 6.91 | 69.8 | 1.12±0.21 |
| Ch1.0/Al/Ca-MP | 50 | 20 | 19.4 | 3.92 | 58.2 | 1.25±0.20 |

*a*) The results are expressed as the mean±S.D. ($n$=150).

including JP15 1st and 2nd fluids. Therefore, the content of LF-FTC was determined by subtracting the washed-out amount of LF-FTC from the initial amount of loaded LF-FTC. The amount of the obtained Ch/Al/Ca-MP was considerably less than that of the starting Al/Ca-MP. This was probably because Ca ion ($CaCl_2$), not associated with complexation, was washed out during the treatment with chitosan solution. As the chitosan concentration increased, the amount of obtained Ch/Al/Ca-MP tended to increase, which was probably because more complexation between alginate and chitosan should be formed with the increase in chitosan concentration. On the other hand, the content of LF-FLC in Ch/Al/Ca-MP decreased with the increase in chitosan concentration. Also, a recovery ratio of LF-FTC became lower by the treatment with chitosan solution of higher concentration. Addition of chitosan solution was considered to create the complex of chitosan and alginate and to cause some LF-FTC to be removed out because of the competition with chitosan for the complex formation with alginate. Therefore, more LF-FTC was considered to be lost with the chitosan solution of higher concentration. The mean particle size was 1.1–1.3 $\mu$m in all Ch/Al/Ca-MP, and the particle size range was from 0.8 $\mu$m to 1.5 $\mu$m. Furthermore, Ch0.25/Al/Ca-MP obviously exhibited rough surface (Fig. 1). Also, the other Ch/Al/Ca-MP seemed not to be completely spherical, though they were not observed clearly as compared with the surface of Ch0.25/Al/Ca-MP. As the surplus $CaCl_2$, not associated with complexation, was removed out in treatment with chitosan solution, which might make the surface rough. Chitosan-alginate complexation might cause the recovery of the smooth surface from the roughness. However, as the details were not grasped from Fig. 1, further examination, especially that of the surface conditions, will be needed to elucidate these considerations.

The effects of chitosan concentration on the particle size, yield, recovery and loading are shown in Fig. 2. As chitosan concentration increased, the amount of the product rose, but LF-FTC recovery and loading fell. Figure 3 is the proposed scheme of the formation of Al/Ca-MP and Ch/Al/Ca-MP. Namely, as discussed above, the materials were highly recovered in the preparation of Al/Ca-MP by the emulsification-evaporation method. However, in chitosan coating with chitosan solution, surplus $CaCl_2$ in Al/Ca-MP was considered to be lost by the dissolution, and chitosan could form a complex with alginate and remove LF-FTC from Al/Ca-MP to some extent due to its cationic charge. These were confirmed by the recovered amount and LF-FTC content and particle size (Table 2, Fig. 2).

***In Vitro* Release** As stated above, Al/Ca-MP was suspended in JP15 1st fluid, but the precipitate obtained by centrifugation of the Al/Ca-MP suspension dissolved easily in JP15 2nd fluid. Therefore, Al/Ca-MP could not be used as controlled release microparticles. On the other hand, Ch/Al/Ca-MP hardly dissolved in JP15 1st or 2nd fluids. Therefore, Ch/Al/Ca-MP were considered adequate as microparticles physically durable in the stomach and intestine. The *in vitro* release profiles of Ch/Al/Ca-MP in JP15 1st and 2nd fluids are shown in Fig. 4. LF-FTC was released gradually from all Ch/Al/Ca-MP at both pH 1.2 and 6.8. The release rate became slower with the decrease of chitosan concentration. Ch0.25/Al/Ca-MP released LF-FTC at 39% and 30% in JP15 1st and 2nd fluids, respectively, at 7 h. Ch0.5/Al/Ca-MP released 43% and 41% of LF-FTC in JP15 1st and 2nd fluids, respectively, at 7 h. For Ch1.0/Al/Ca-MP, 79% and 53% of LF-FTC were released in JP15 1st and 2nd fluids, respectively, at 7 h. The initial release was greater in JP15 1st fluid than in JP15 2nd fluid for each kind of microparticle. A sequential release test using JP15 1st fluid, followed by JP15 2nd fluid, was performed. The release pattern was obtained as described in Fig. 5. The release pro-

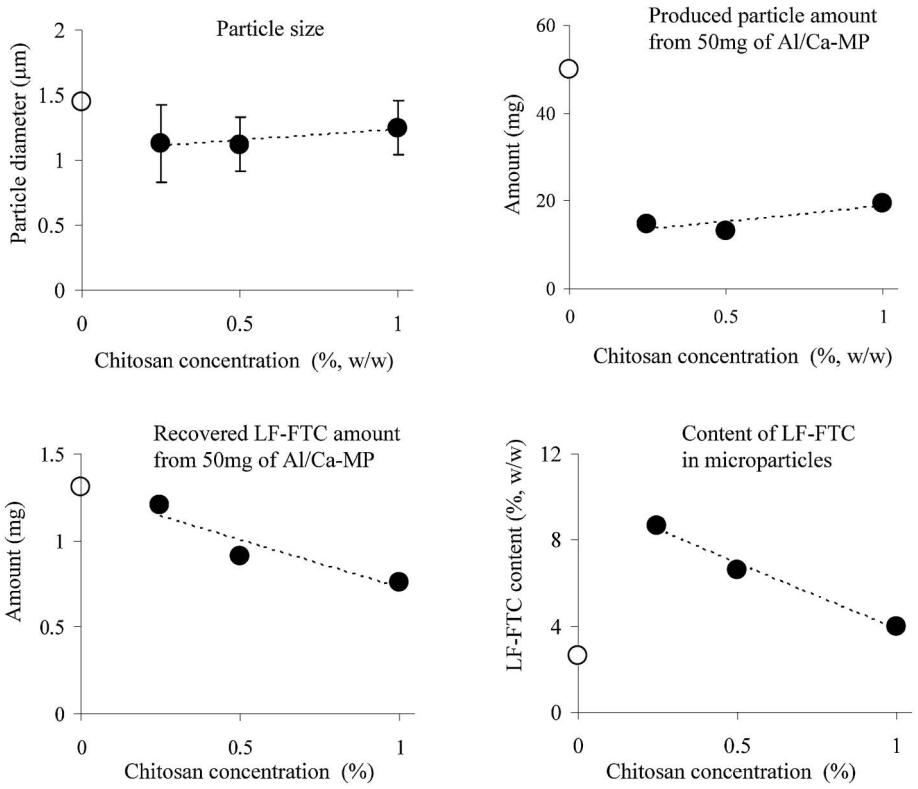


Fig. 2. Relationships of Particle Features before and after the Treatment with Chitosan Solution

Open and closed circles represent microparticles before and after treatment of chitosan solution, respectively. Particle size is expressed as the mean ± S.D. ($n$ = 150).

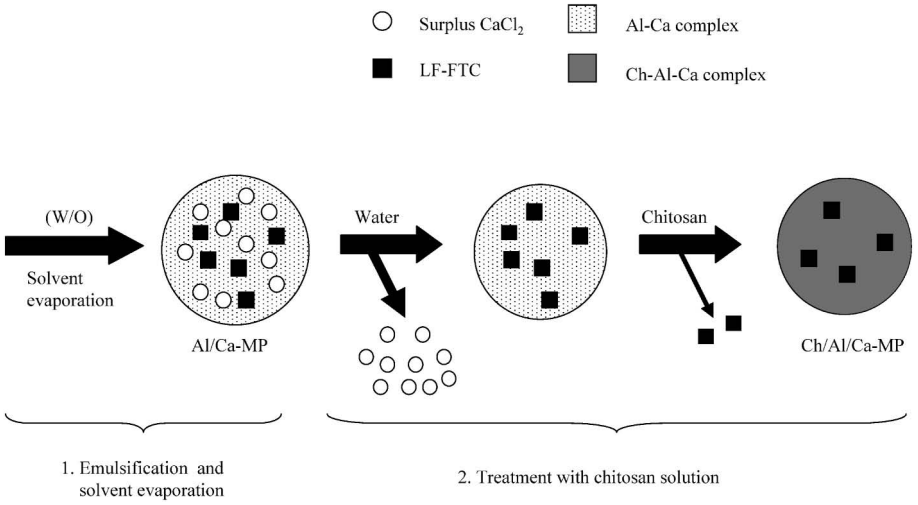


Fig. 3. Proposed Schematic Particle Formation and Structures of Al/Ca-MP and Ch/Al/Ca-MP during Their Preparation Processes

file was almost consistent with the profile expected from the results obtained by examination with JP15 1st and 2nd fluids separately (Fig. 4). These results suggested that Ch0.25/Al/Ca-MP and Ch0.5/Al/Ca-MP should be useful for sustained release. On the other hand, for Ch1.0/Al/Ca-MP, the initial release in JP15 1st fluid was too high to achieve a gradual release. At pH 1.2, chitosan and LF-FTC are highly ionized, while alginate is less ionized. LF-FTC, especially that located at the outer part, was considered to be released due to the hydration of chitosan and LF-FTC itself, ionic repulsion by chitosan and less interaction with alginate. These phenomena were considered to be caused more markedly in Ch/Al/Ca-MP

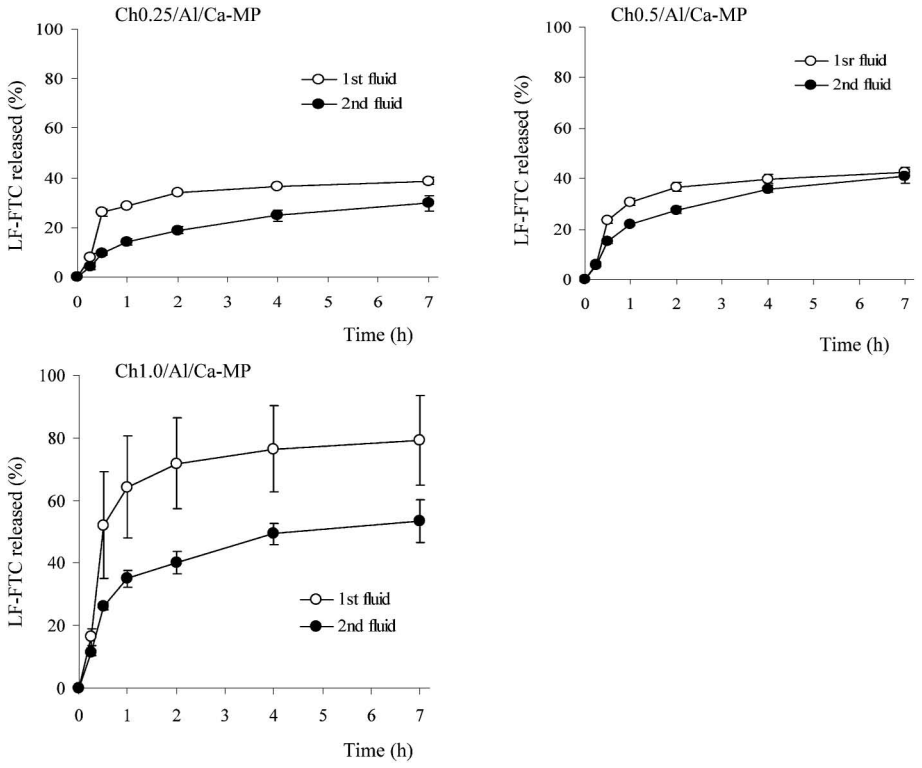


Fig. 4. Release Profiles of LF-FTC from Ch/Al/Ca-MP in JP15 1st and 2nd Fluids at 37℃ and 100 rpm
The results are expressed as the mean±S.D. ($n$=3).

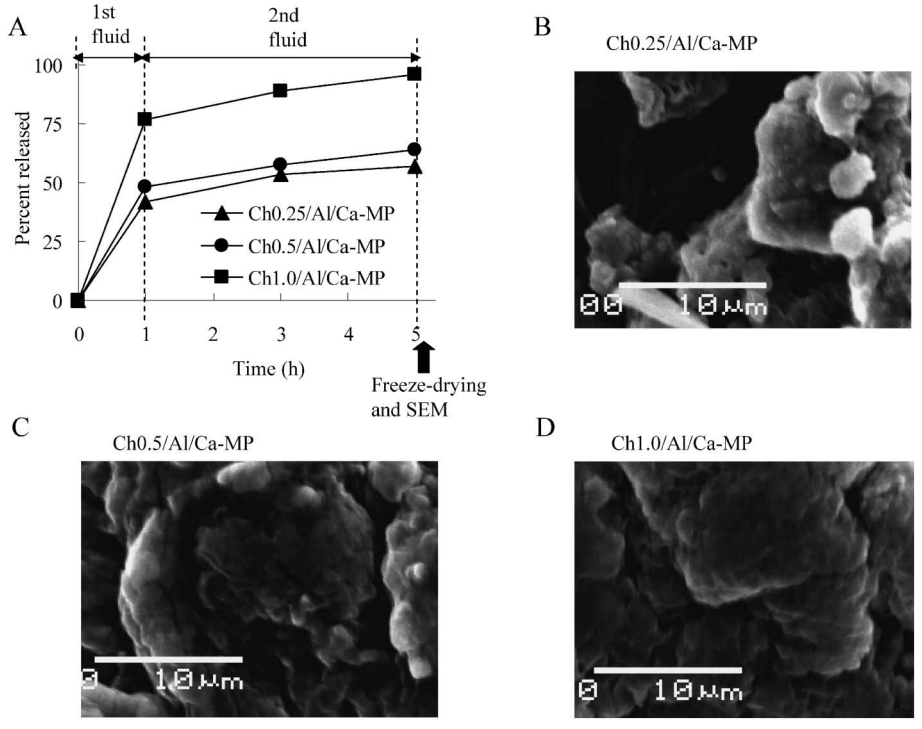


Fig. 5. Release Profiles of LF-FTC from Ch/Al/Ca-MP with the Incubation in JP15 1st Fluid Followed by JP15 2nd Fluid at 37℃ and 100 rpm (A) and SEM Images of Precipitates Obtained by Centrifugation after the Incubation (B, C and D)
The number of experiment=1.

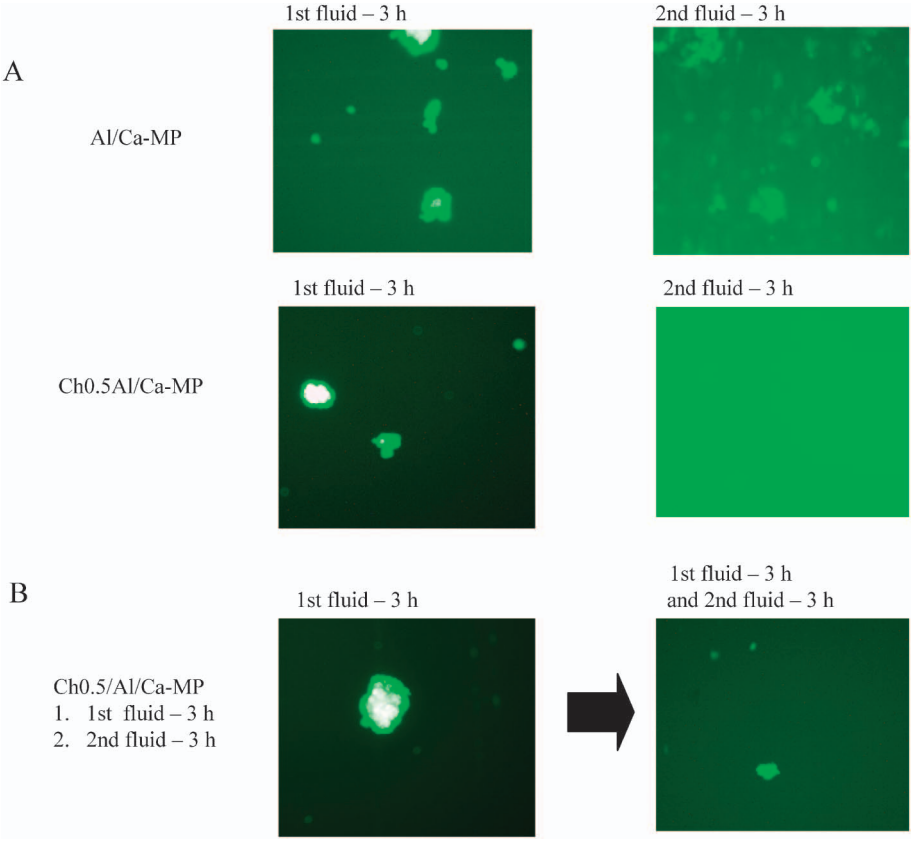


Fig. 6. Particle States of Al/Ca-MP and Ch/Al/Ca-MP with the Incubation in JP15 1st Fluid and JP15 2nd Fluid (A), and JP15 1st Fluid followed by JP 2nd Fluid (B) at 37°C and 100 rpm

with more chitosan like Ch1.0/Al/Ca-MP, resulting in a larger initial rapid release. As the pKa of amino groups of chitosan is approximately 6.3–7,[26] chitosan is partially cationized at pH 6.8, while alginate is highly anionized at this pH. Therefore, the hydration was considered to be caused throughout the particles at pH 6.8, leading to the gradual release of LF-FTC, but the release of LF-FTC located at the outer area might be suppressed due to incomplete ionization of chitosan, which also would be related to the retention of the ionic interaction between LF-FTC and alginate.

**Physical States at Gastric and Intestinal pH**

SEM images in Fig. 5 show the shape of residues obtained after the sequential release test. Since Ch/Al/Ca-MP were swollen and softened during incubation in JP15 2nd fluid, they were considered to be aggregated or entangled, resulting in the formation of a larger solid after the lyophilization.

Particle states with the incubation of Ch0.5/Al/Ca-MP in JP15 1st and 2nd fluids were observed by optical and fluorescence microscopy. In JP15 1st fluid, Ch0.5/Al/Ca-MP were not swollen or softened, that is, the core part was hard, resulting in clear fluorescence images (Fig. 6). In JP15 2nd fluid, LF-FTC, released to a certain extent, was observed as solution fluorescence and, at this time, microparticles were observed without being dissolved. For Al/Ca-MP, disintegration and dissolution were observed.

Thus, Ch/Al/Ca-MP were shown to be durable microparticles under both gastric and intestinal pH conditions. Given their release, Ch0.25/Al/-MP and Ch0.5/Al/Ca-MP were proposed to act as smaller microparticles providing the prolonged supply of LF in the gastrointestinal tract. In the next study, it will be attempted for the purpose of practical application to scale up the production of microparticles and to incorporate LF at a higher ratio.

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