

Formulation Study of Chitosan Microparticles Loaded with Lactoferrin

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Abstract

Lactoferrin (LF) is known to show various useful biological features such as antimicrobial, antiviral, antitumor and anti-inflammatory effects. However, orally administered LF undergoes enzymatic degradation in the gastrointestinal tract, resulting in extensive decrease of such functions. The purpose of this study is to develop a useful oral delivery system of LF. Also, chitosan (Ch) was adopted as a carrier because it is yielded abundantly and a safe polymer. LF-loaded Ch microparticles were produced by the emulsification-evaporation technique using sorbitan sesquioleate (SO-15) and sorbitan trioleate (SO-30) as surfactants. The resultant LF-loaded Ch microparticles were evaluated from the viewpoints of particle size, shape, drug content and *in vitro* release in the gastric and intestinal pHs. The selected LF-loaded microparticles (MS4) were coated with an enteric coating polymer, Eudragit L100 (EL-100). The resultant microparticles (E/MS4) were protected from the deformation in the gastric pH, and exhibited fairly appropriate release profiles of LF. Thus, enteric-coated MS4 was suggested to be possibly useful as an orally-taken delivery system of LF.

Keywords: Lactoferrin; Chitosan; Microparticle; Enteric coating; Oral delivery system

Introduction

Lactoferrin (LF) was found as a multifunctional iron-binding glycoprotein, and is widely contained in milk, saliva, tears, various exocrine secretions and neutrophil granules [1,2]. It exhibits antimicrobial [3,4], antiviral [5], antitumor [6], anti-inflammatory [7] and immunomodulation [8] activities. Recently, since it is a natural substance and very safe, such biological functions are to be of great interest in the medical fields. LF functions in various manners. It works against microbes and viruses not only by direct binding [9-14] but also by interaction with host cells [15] or activation of immune systems [16,17]. Recently, LF is used as a supplement in various countries.

Since LF is a glycoprotein, it is subjected to enzymatic degradation [18,19]. LF, injected intravenously, was eliminated rapidly from the blood circulation [20]. Therefore, it is usually taken orally at a fairly large dose because the oral dosing is conducted easily and safely. In addition, the LF receptor in the intestinal membrane is importantly related to the activation of gut-associated lymphoid tissue (GALT) [21,22]. Therefore, the delivery of intact LF to the intestinal part is essential to the sufficient expression of LF functions [23-25]. As LF is subjected to peptic degradation, its considerable amount is degraded before LF reaches the receptor [24,26]. The protection of LF from the gastric conditions has been reported to be important in the oral dosing of LF [24,25,27].

Chitosan (Ch) is a biopolymer produced by deacetylation of chitin, abundantly yielded from shells of crabs or shrimps and cell walls of fungi etc. [28]. As it is very safe in oral use, it is used as a food additive or supplement. As Ch is not water-soluble in neutral and basic pH, it is expected as an excipient for medical or pharmaceutical products [29,30]. As Ch is soluble in acidic aqueous solution, its solution can be applied to make various products. In addition, Ch is less or insoluble at intestinal pH, Ch is adequate to the delivery to oral mucosa or intestinal parts. We have tried to apply Ch as microparticulate delivery devices such as simple ones and complex-type ones [31-33]. In the previous paper, Ch microparticles loaded with LF were produced, and their possibilities toward topical application or oral use were evaluated *in vitro*, in which the simple formulation composed of Ch and LF was demonstrated to exhibit smaller size (4.9 μ m) and prolonged release

over 24 h. In the present study, Ch microparticles loaded with LF were newly produced in order to develop an oral delivery system to elevate intestinal or systemic immunity. A high LF content, sufficient LF release [2,27] and the particle size of submicron to a few micrometer size [34,35] will be better to achieve biological potential or intestinal localization and retention of LF. As to the present preparation, the effect of the LF/Ch ratio on the particle characteristics was examined in detail. Also, the influence of surfactant species (sorbitan sesquioleate or sorbitan trioleate) used in emulsification-evaporation process was investigated for the particle characteristics. The adequate Ch microparticles loaded with LF were selected from those preparations based on particle features such as particle size and LF content, and on *in vitro* release. Finally, the chosen Ch microparticles were enteric-coated with Eudragit L100 (EL-100) for oral dosing, and the utility of enteric-coated MS4 were evaluated *in vitro*.

Materials and Methods

Materials

Bovine lactoferrin (LF) was donated by NRL Pharma Inc. (Kawasaki Japan). Chitosan (Ch, MW 100,000 – 200,000, deacetylation degree = 80%) and liquid paraffin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sorbitan sesquioleate (SO-15) was obtained from Nikko Chemicals Co., Ltd. (Tokyo, Japan). Sorbitan Trioleate (SO-30) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Eudragit L100 (EL-100) was supplied by and used from Rohm GmbH Chemische Fabrik (Darmstadt, Germany) and used as an enteric coating polymer. A Pierce BCA Protein Assay kit was purchased from Thermo Fisher Scientific Inc. (Rockford, IL,

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USA), and used for the determination of LF concentration. All other chemicals used were of reagent grade.

Preparation of LF-loaded Ch microparticles

LF-loaded Ch microparticles were prepared by the W/O emulsification-evaporation method. The formulations in the preparation were shown in Table 1. LF and Ch, with their amounts shown in Table 1, were dissolved in 10 mL of 1% (v/v) acetic acid aqueous solution. The mixed solution was added drop by drop at a fairly rapid speed to 150 mL of liquid paraffin containing 1% (v/v) surfactant. The resultant mixture was stirred at 1000 rpm for 1.5 h and sonicated at 28 Hz (100 W) for 10 min. The resultant emulsion was evaporated with the reduced pressure at 40°C under stirring at 400 rpm until it became to be white suspension. n-Hexane was mixed to the suspension with the same volume, and the mixture was centrifuged at 3000 × g for 10 min. The precipitate was taken by discarding the supernatant, and washed several times with n-hexane, and dried in a desiccator.

Measurement of particle characteristics

LF-loaded Ch microparticles were investigated for particle size, shape, LF content and encapsulation efficiency. The microparticles were coated thinly (10 nm in thickness) with platinum using a JFC-1600 Auto Fine Coater made by JEOL (Tokyo, Japan), and observed using a JEOL JSM-5600LV scanning electron microscope, in which their photomicrographs were taken. The particle shape and surface structure were observed from each photo. The Green diameter was measured for 200 particles chosen at random based on the scale bar, and the mean size and deviation were calculated (n=200).

The microparticles (5 mg) were dissolved in 10 mL of 2% (v/v) acetic acid aqueous solution. The obtained solution (200 µL) was measured for the LF concentration using a BCA protein Assay kit, which was validated using LF standard samples dissolved in 2% (v/v) AcOH.

The LF content and encapsulation efficiency of the microparticles were calculated in the following equation.

Content (%) = $100 \times [\text{LF amount in sample microparticles (mg)}] / [\text{amount of sample microparticles (mg)}]$

Encapsulation efficiency (%) = $100 \times [\text{amount of incorporated LF (mg)}] / [\text{amount of added LF (mg)}]$

In vitro release from LF-loaded Ch microparticles

The 1st (pH 1.2) and 2nd (pH 6.8) fluids in Japanese Pharmacopoeia 16 were used as the artificial gastric and intestinal fluids. LF-loaded Ch microparticles (5 mg) was added to 10 mL of the 1st or 2nd medium, and incubated at 37°C under the condition of horizontal shaking at 100 rpm. The upper solution (200 µL) of the suspension was taken at 1, 3, 7, 24 and 48 h after the start of the incubation. The taken sample was centrifuged at 1300 × g for 10 min. The obtained supernatant was analyzed using a BCA Protein Assay kit to determination of the concentration of released LF, which was validated using LF standard samples dissolved in the 1st (pH 1.2) and 2nd (pH 6.8) fluids.

Enteric coating of LF-loaded Ch microparticles

The selected LF-loaded Ch microparticles, MS4, were coated with EL-100. MS4 was suspended to the methanol solution containing 200 mg of EL-100. The suspension was added drop by drop at a fairly rapid speed to 150 mL of liquid paraffin containing 1% (v/v) SO-15, while the dispersion solvent was being stirred at 600 rpm. The resultant emulsion was stirred for 1 h, and sonicated at 28 Hz (100 W) for 10 min. The

resultant emulsion was evaporated with the reduced pressure at 20°C under stirring at 400 rpm until it became to be white suspension. n-Hexane was mixed to the suspension with the same volume, and the mixture was centrifuged at 3000 × g for 10 min. The precipitate was taken by discarding the supernatant, and washed several times with n-hexane, and dried in a desiccator.

The obtained coated microparticles (E/MS4) were investigated for particle size, shape, drug content and *in vitro* release in the same manner as stated above.

Results and Discussion

Preparation and particle characteristics of LF-loaded Ch microparticles

LF-loaded Ch microparticles were prepared according to the formulations in Table 1. The microparticles, produced using SO-15 (HLB 4.5) and SO-30 (HLB 1.8) as surfactants, were shown in Figures 1 and 2 respectively. The particle size and shape were almost the same in MS1 – MS4. On the other hand, the particle size tended to be greater in MT3 and MT4, and their surface was not smooth in comparison with MT1 and MT2. In particular, the microparticles with large size had nearly round shape but showed the structure of laminated agglomeration. Table 2 summarizes the particle characteristics. The drug content and encapsulation efficiency were fairly high. The addition of LF at a high ratio tended to exhibit the better encapsulation efficiency.

In the previous paper, chitosan/alginate/Ca (II) complex microparticles loaded with LF was produced [32]. The complex microparticles showed LF content of 15-22% (w/w). Therefore, MS4 and MT4 exhibited much higher LF content than those complex microparticles. This is an advantage for the present microparticles because a fairly large dose of is needed to get sufficient function of LF.

| Formulation code | Formulation composition | | Surfactant in dispersion medium |
|------------------|-------------------------|---------|---------------------------------|
| | LF (mg) | Ch (mg) | |
| MS1 | 10 | 50 | 1% SO-15 |
| MS2 | 20 | 50 | 1% SO-15 |
| MS3 | 30 | 50 | 1% SO-15 |
| MS4 | 50 | 50 | 1% SO-15 |
| MT1 | 10 | 50 | 1% SO-30 |
| MT2 | 20 | 50 | 1% SO-30 |
| MT3 | 30 | 50 | 1% SO-30 |
| MT4 | 50 | 50 | 1% SO-30 |

Table 1: Formulation composition and used surfactant in the preparation of LF-loaded Ch microparticles.

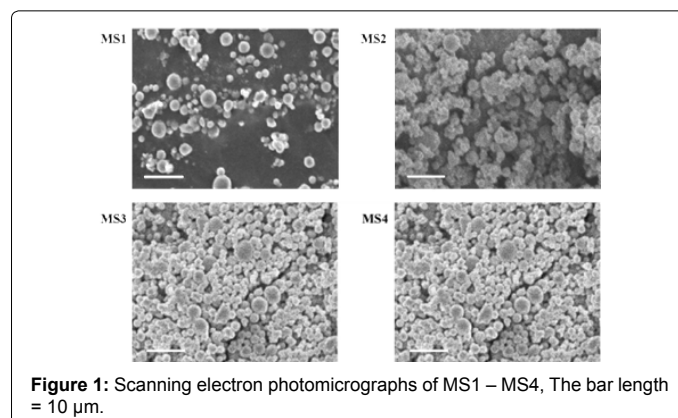


Figure 1: Scanning electron photomicrographs of MS1 – MS4, The bar length = 10 µm.

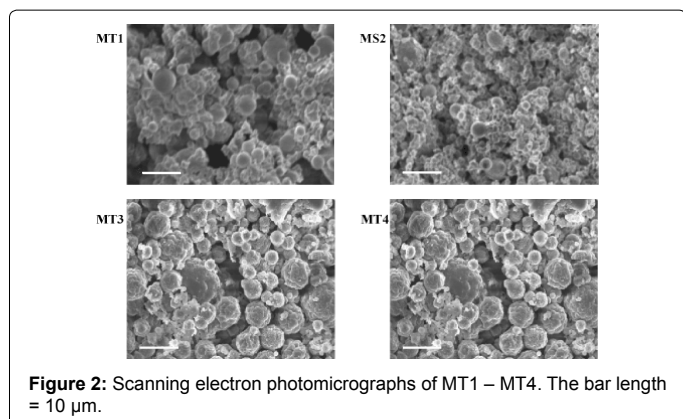


Figure 2: Scanning electron photomicrographs of MT1 – MT4. The bar length = 10 μ m.

| Formulation Code | Particle diameter (μ m) | Drug content (% w/w) | Encapsulation Efficiency (%) |
|------------------|------------------------------|----------------------|------------------------------|
| MS1 | 1.9 \pm 1.1 | 11.2 \pm 3.8 | 67.5 \pm 22.7 |
| MS2 | 2.0 \pm 0.5 | 18.9 \pm 3.0 | 66.2 \pm 10.6 |
| MS3 | 2.0 \pm 0.9 | 27.0 \pm 3.7 | 77.5 \pm 2.2 |
| MS4 | 1.9 \pm 0.5 | 38.0 \pm 4.5 | 75.9 \pm 9.1 |
| MT1 | 2.7 \pm 1.5 | 12.5 \pm 1.6 | 74.8 \pm 9.6 |
| MT2 | 2.7 \pm 1.0 | 22.4 \pm 0.7 | 78.4 \pm 2.4 |
| MT3 | 3.6 \pm 2.2 | 27.2 \pm 5.9 | 72.5 \pm 15.8 |
| MT4 | 3.4 \pm 2.5 | 44.2 \pm 2.4 | 88.4 \pm 4.8 |

Table 2: Particle characteristics for LF-loaded Ch microparticles. The results are expressed as the mean \pm S.D. (n=200 for particle diameter, n= 3 for drug content, encapsulation efficiency).

Microparticles, with a size of submicron or near size, appear to show better localization and retention in the intestinal membrane [34,35]. From the viewpoints of the particle size, smooth surface structure and LF content, MS4 was considered to be a best formulation.

In vitro release features of LF-loaded Ch microparticles

The release profiles of LF were investigated in the incubation using 1st fluid (pH 1.2) and 2nd fluid (pH 6.8). The initial rapid release was observed at pH 1.2 for all the MS and MT microparticles, in which the influence of the LF/Ch ratio on the release rate was greater in MT microparticles than MS ones. After the initial rapid release, the LF release was very slow. The release profiles were similar among MS1 – MS4. The initial rapid release was suppressed at pH 6.8, in which it was less than 40% for all the MS and MT microparticles. LF was released very slowly after initial release.

MS4, chosen from the particle characteristics, exhibited nearly 80% release at pH 1.2 in the initial period, and then hardly released LF until 48 h after the start of the incubation. At pH 6.8, MS4 released LF at approximately 30% initially, and released LF gradually to approximately 50% at 48 h after the start of the incubation.

Enteric coating of LF-loaded Ch microparticles

Considering these *in vitro* release features as stated above, it was proposed that the initial release at pH 1.2 had to be suppressed. This was considered to be because the acidic aqueous solution could permeate the Ch matrix easily. In addition, ss Ch is subjected to dissolution in the acidic aqueous condition, the microparticles should be coated with some enteric coating substance to protect their structure.

In this study, MS4, chosen from the above some reasons, was coated using EL-100 by the W/O emulsification-solvent evaporation method.

The coated microparticle SEM photo is shown as Figure 3 (E/MS4, 0 h). The particle diameter was $4.3 \pm 2.4 \mu$ m (mean \pm S.D., n=200), and their surface was smooth; the increase in size was considered to be due to the coating by EL-100. The LF content was more than 22% (w/w), which suggested that MS4 could be incorporated in E/MS4 at a higher ratio than EL-100. In addition, the *in vitro* release was estimated to be 9-15%, 20-39% and 29-50% at 1 h, 24 h and 48 h after the incubation at pH 1.2 at 37°C. The slightly faster release was observed at pH 6.8. These suggested that E/MS4 could be produced at a fairly high LF content, and that the initial release at pH 1.2 could be suppressed.

MS4 and E/MS4 were examined for the change in particle size and shape in the incubation at pH 1.2. At the specified time points after the incubation in the 1st fluid at 37°C, the microparticles were taken after the centrifugation at 1300 x g for 10 min. Then, the precipitate was washed briefly with water, and dried in a desiccator. The dried sample was observed by SEM as stated above. The resultant SEM micrographs are shown in Figure 4. The particle shape and surface structure of MS4 changed more with time, and particle structure was broken completely. As Ch is subjected to swelling and dissolution in acidic aqueous solution, MS4 was considered to undergo the deformation or dissolution. On the other hand, the particle size and shape of E/MS4 were scarcely changed even at 24 h after the start of the incubation, indicating that MS4 could be protected with EL-100 from the deformation at pH 1.2.

Conclusions

LF-loaded Ch microparticles were prepared with different formulations. Ch microparticles with high load of LF (approximately 40% (w/w)) could be produced at the LF/Ch ratio of 1:1 (w/w) by the

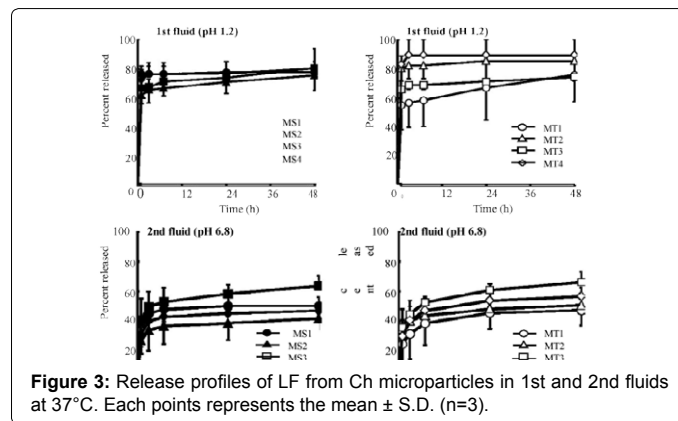


Figure 3: Release profiles of LF from Ch microparticles in 1st and 2nd fluids at 37°C. Each points represents the mean \pm S.D. (n=3).

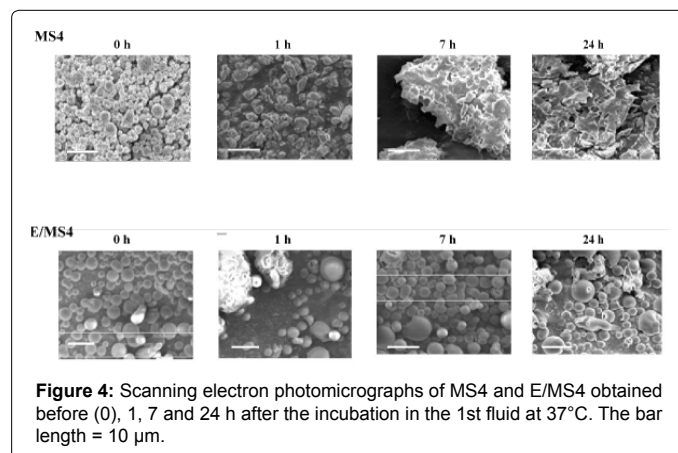


Figure 4: Scanning electron photomicrographs of MS4 and E/MS4 obtained before (0), 1, 7 and 24 h after the incubation in the 1st fluid at 37°C. The bar length = 10 μ m.

O/W emulsification-evaporation method. Furthermore, LF-loaded Ch microparticles, with a size of one to a few μm and smooth surface, could be obtained using SO-15 as a surfactant. The LF-loaded Ch microparticles with high LF showed similar release profiles among all the formulations; LF was released initially at pH 1.2 at a fairly large extent, and the initial rapid release was suppressed at 30-50% at pH 6.8. After the initial period, LF was released slowly in both pH conditions. Based on those particle features and release profiles, the formulation MS4, produced at LF/Ch ratio of 1:1 (w/w) using 1% (w/w) SO-15, was chosen. Furthermore, EL-100 coated MS4 (E/MS4) were found to have fairly high LF content and to show the suppression of the initial release at pH 1.2 and gradual release of LF. Thus, enteric-coated MS4 was suggested to be possibly useful as an oral delivery system of LF.

Author's Contribution

Hiraku Onishi and Kei-ich Watanabe carried out studies design, experiment and data interpretation. Hiraku onishi also wrote the manuscript. Yoshihary Machida participated in data interpretation.

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