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ORIGINAL ARTICLE

Preparation of chitosan/alginate/calcium complex microparticles loaded with lactoferrin and their efficacy on carrageenan-induced edema in rats

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Abstract

Background: Although lactoferrin (LF) possesses useful functions such as antitumor, antiviral, and anti-inflammatory acitivities, it is subject to gastric digestion, resulting in the reduction of efficacy. Therefore, it is important to develop a system delivering LF efficiently to intestinal mucosa or gut-associated lymphoid tissue. *Method:* Chitosan/alginate/calcium complex microparticles containing LF at a high loading were prepared using alginate, LF, and calcium chloride at the ratio of 6:3:8 (w/w). The release test was performed using Japanese Pharmacopoeia, Fifteenth Edition (JP15) first fluid (pH 1.2) for initial 2 hours, followed by JP15 second fluid (pH 6.8) for another 5 hours. Furthermore, the in vivo efficacy was evaluated from anti-inflammatory effect using rats with carrageenan-induced edema, in which dosing was performed intragastrically at 50 mg LF eq./kg 5, 3, and 1 days before carrageenan injection. *Results:* Microparticles have 20 – 30 % (w/w) LF content and 1 – 3 mm size. Nearly 60 % of LF was released at pH 1.2 at the first 1 hour, and then slowly released up to 80 % at 7 hours. Suppressive effect against the edema was greater in the order of microparticles LF solution control (saline). Initial burst of LF from microparticles was not associated with their promoted efficacy. *Conclusion*: Chitosan/alginate/calcium complex microparticles are suggested to be useful for promotion of efficacy of LF at oral administration

Key words: *Carrageenan-induced edema; chitosan/alginate/calcium complex microparticle; lactoferrin; release profile; suspension state*

Introduction

Lactoferrin (LF) is an iron-binding glycoprotein, expressed in milk, saliva, tears, and many other exocrine secretions, neutrophil granules, and so on. As LF exhibits many biological functions such as antibacterial[1,2], antiviral[3], antitumor[4], anti-inflammatory[5,6], and immunomodulatory activities[7–9], its application is expected in the medical field. In addition, its high safety and host defense potential based on immunomodulation attract much attention for the treatment of severe or refractory diseases. Such effects are generally achieved by oral administration, but LF is known to be subject to gastric digestion. Therefore, recently, protection of LF from gastric harsh conditions has been attempted by various techniques such as microencapsulation and enteric coating[10,11]. Liposomes encapsulating LF were reported to improve the anti-inflammatory effect[12], which was suggested to be because liposomes should protect LF from gastric digestion and/or assist the function of LF on the intestinal sites including the LF receptors. Accordingly, polymeric microparticles were also considered to be useful for the improvement of LF functions, because they are available for the protection, controlled release, and delivery to intestinal sites[13,14].

Although we developed chitosan (Ch) microparticles 1 containing LF, which enabled the prolonged release of LF[15], Ch microspheres could swell, disrupt, and dissolve in the gastric pH conditions[16], suggesting that further processing such as coating should be required to maintain the microparticulate state. Therefore, the MP, not subject to such swelling, disruption, and

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dissolution, are more desirable because they can be produced simply and keep the LF content high. In our previous studies, Ch/alginate/calcium microparticles containing LF (Ch/Al/Ca-MP) were developed by the combination of their complexation[17–20] and the emulsification–evaporation technique, and the particle characteristics and in vitro release properties and physical states of the MP in Japanese Pharmacopoeia, Fifteenth Edition (JP15) first (pH 1.2) and second (pH 6.8) fluids were investigated[21]. The MP showed the LF content of 4–9% (w/w), dependent on the formulation, but their size was almost the same, hardly dependent on the formulation. The release rate was faster with an increase in the concentration of Ch solution used in the coating (the second complexation). However, that LF content was considered to be too low to use for the in vivo application, because LF was generally used in a fairly large amount (several dozen mg/kg – several hundred mg/kg) in the treatment of many diseases. Therefore, in this study, the goal is to produce MP with more LF content to potentially improve efficacy. Namely, Ch/Al/Ca-MP were prepared in a novel formulation, referring to the previous results[21]. The resultant Ch/Al/Ca-MP were examined for their particle characteristics and release properties. Furthermore, their efficacy was evaluated from the in vivo effect using rats with carrageenan-induced edema.

Materials and methods

Materials

Bovine LF was donated by NRL Pharma Inc. (Kawasaki, Japan). Daichitosan VL (81% of deacetylation degree, extra low viscosity grade) was supplied by Dainichiseika Color & Chemicals Mfg. Co., Ltd. (Tokyo, Japan) and used as Ch in this study. Sodium alginate (Al-Na; 80–120 cP grade) and calcium chloride ($CaCl_2$) were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Also, λ-carrageenan, degraded, was obtained from Wako Pure Chemicals Industries and used as carrageenan to induce the paw edema. Sorbitan sesquioleate (SO-15) was purchased from Nikko Chemicals Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

Animals

Male Wistar rats (180–200 g or 250–260 g) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan) and bred on the breeding diet MF supplied by Oriental Yeast, Co., Ltd. (Tokyo, Japan) with water ad libitum at 23 ± 1°C and relative humidity of 60 ± 5%. They were used for the experiments soon after purchase. The experimental protocol was approved by the Committee on Animal Research of Hoshi University, Japan. The animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, Japan.

Preparation of microparticles

Al-Na (300 mg) and LF (150 mg) were dissolved in 20 mL of water, and the resultant solution was gradually added for several minutes to the 400 mL of liquid paraffin containing 1% (w/v) sorbitan sesquioleate, which was stirred at 1000 rpm. After the mixture was stirred at the same speed for 1 hour, 4 mL of 10% (w/v) $CaCl_2$ was added, and the resultant mixture was stirred for 15 minutes. Then, the mixture was stirred gradually under the reduced pressure at 37°C using a rotary evaporator. After the complete evaporation of water, stirring and evaporation were stopped, and the 400 mL of *n*-hexane was added to the mixture, and the resultant mixture was centrifuged at 1500 × *g* for 10 minutes to precipitate MP. The precipitate was washed with *n*-hexane three times and dried in a desiccator to yield Al/Ca microparticles containing LF (Al/Ca-MP).

Al/Ca-MP (800 mg) were suspended in 340 mL of 0.5% (w/v) Ch solution, and the suspension was stirred gently for 1 hour. Then, the mixture was centrifuged at 1500 × *g* for 10 minutes to separate the microparticles. The precipitate was washed with water three times and dried in a desiccator to yield Ch/Al/Ca microparticles containing LF (Ch/Al/Ca-MP). The supernatants obtained in the separation and washing were kept for the measurement of the amount of LF that was lost from Al/Ca-MP, which was necessary for the determination of the LF content of Ch/Al/Ca-MP.

Characterization of microparticles

Ch/Al/Ca-MP were thinly (10 nm in thickness) coated with platinum using a JFC-1600 Auto Fine Coater made by JEOL (Tokyo, Japan) and observed with a JSM-5600LV scanning electron microscope produced by JEOL, when their photomicrographs were taken. The particle shape and size and the size distribution were examined by measuring the Green diameters of 150 microparticles chosen at random from the photomicrographs.

The LF content was investigated as reported previously[21]. Briefly, first, the LF content in Al/Ca-MP was examined as follows. A certain amount of Al/Ca-MP was suspended in a certain volume of JP15 first fluid, stirred vigorously, and centrifuged to separate the clear supernatant and solid portion. The solid portion was dissolved by stirring in a certain volume of JP15 second fluid. The above clear supernatant in JP15 first fluid

and the solution in JP15 second fluid were measured spectrophotometrically at 280 nm to determine their concentration of LF. The LF amount in Al/Ca-MP was calculated from the concentration and the solution volume.

Next, the LF content in Ch/Al/Ca-MP was examined in the following manner. The clear supernatant obtained after the treatment of Al/Ca-MP with Ch solution and that got after subsequent wash with water were examined spectrophotometrically at 280 nm to determine LF concentration in those media. The LF content in Ch/Al/Ca-MP was calculated by subtracting the LF amount in the media from the initial LF amount in the used Al/Ca-MP.

In vitro release study

Ch/Al/Ca-MP (10 mg) were suspended in 3 mL of JP15 first fluid and shaken horizontally at 100 strokes/min at 37°C. At 1 hour, the incubated sample was centrifuged at 1500 × *g* for 10 minutes, 100 μL of the supernatant was withdrawn, and the fresh first fluid (100 μL) was supplemented to the incubated sample. After the mixture was resuspended by simple vortex mixing, it was incubated in the same way, and the supernatant (100 μL) of the sample was similarly withdrawn at 2 hours. Then, the whole remaining supernatant was discarded, and 3 mL of JP15 second fluid was added to the residue. After the residue was suspended by simple vortex mixing, the incubation of the suspension was continued at the same condition. At 1, 3, and 5 hours after the medium was changed to JP15 second fluid, the supernatant (100 μL) of the sample was taken. At each sampling, the fresh JP15 second fluid (100 μL) was supplemented. Each supernatant withdrawn was analyzed using a Pierce® BCA protein assay kit made by Thermo Fisher Scientific Inc. (Rockford, IL, USA).

In vivo experiment

In the first experiment, LF solution in saline and Ch/Al/Ca-MP suspension in saline were prepared and used as samples, in which saline was used as control. The samples were intragastrically administered to rats at 50 mg LF eq./kg (0.5–1 mL saline) using a Teflon tube 5, 3, and 1 days before the carrageenan injection (total dose = 3 × 50 mg LF eq./kg). One day after the last dosing, 0.1 mL of 1% (w/v) carrageenan solution in saline was injected into the footpad of the right hind paw, subcutanously. Immediately before and 0.5, 1, 2, 3, 4, 5, and 7 hours after the carrageenan injection, the volume of the right hind paw, that is, the volume of the region from its top joint to its tip, was determined by immersing it into water and subsequently measuring the increase of the water volume. The extent of the edema was calculated from the ratio of the volume to that immediately before carrageenan injection[22–24].

In the second experiment, Ch/Al/Ca-MP underwent incubation in JP15 first fluid for 1 hour to remove the effect of the initial rapid release. The resultant microparticles, named Ch/Al/Ca-MP (1 hour treated), were used as a tested sample with no initial burst. The efficacy was investigated in the same manner. Namely, Ch (1 hour treated) suspension in saline, LF solution in saline, and saline alone (0.5–1 mL) were administered intragastrically at 50 mg LF eq./kg in the same dosing schedule, and the volume of the right hind paw injected with carrageenan was measured in the same manner.

For statistical analysis, each sample was compared for the extent of edema using ANOVA followed by the Dunnett's post hoc test, and the significant difference was set as $P < 0.05$.

Results and discussion

Particle characteristics

Formulation and particle characteristics of Al/Ca-MP are shown in Table 1. Most of the used substances were found to be recovered in Al/Ca-MP. Namely, the yield, which was calculated as the ratio of the product amount to the initial total amount (850 mg), was nearly 100%. LF was also recovered in Al/Ca-MP as observed from the LF content. This was probably because the used substances minimally dissolved in the used organic solvents (i.e., liquid paraffin and *n*-hexane).

Ch/Al/Ca-MP were prepared by the complexation of Ch and Al with the immersion of Al/Ca-MP in Ch solution[17–20]. The particle characteristics of Ch/Al/Ca-MP are shown in Table 2. As Ch/Al/Ca-MP did not dissolve in JP15 first (pH 1.2) or second (pH 6.8) fluids, the drug content was determined by subtracting the washed-out LF amount from the initial LF amount in

Table 1. Preparative conditions, yields, and particle characteristics of Al/Ca-MP.

Al–Na (mg)	LF (mg)	$CaCl_2$ (mg)	Produced amount (mg)	Yield (%, w/w)	LF content (%,w/w)	LF recovery (%)
300	150	400	835 ± 36	98 ± 4	15.8 ± 2.8	86 ± 16

Yield was calculated as the ratio of the product amount to the total amount of the used substances. LF recovery was the ratio of the incorporated LF amount to the used LF amount. The results are expressed as the mean ± S.D. ($n = 3$).

Table 2. Preparative conditions, yields, and particle characteristics of Ch/Al/Ca-MP.

Ch/Al/Ca-MP	Al/Ca-MP (mg)	0.5% Ch solution volume (mL)	Produced amount (mg)	Yield (%, w/w)	LF content (%, w/w)	LF recovery (%)	Mean particle size (μm)
Ch/Al/Ca	800	340	295 ± 5	38 ± 2	22 ± 5	52 ± 9	1.65 ± 0.15

Yield was calculated as the ratio of the product amount to the total amount of the used substances. LF recovery was the ratio of the incorporated LF amount to the used LF amount. The results are expressed as the mean ± SD (n = 3).

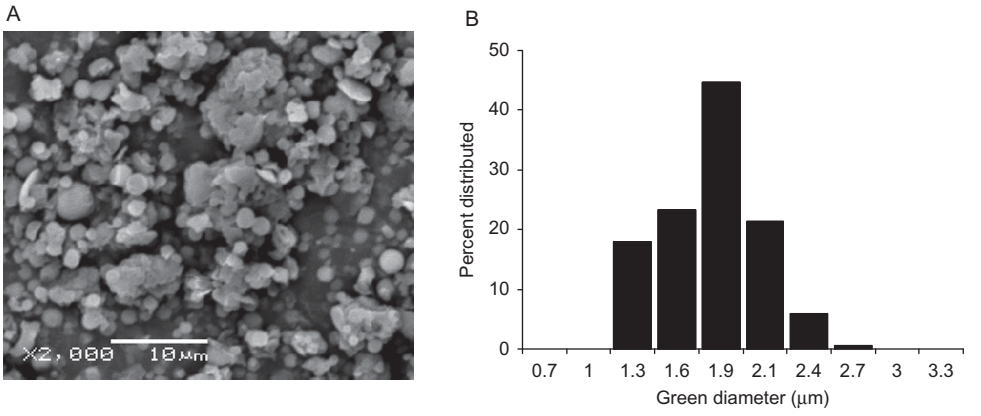

Figure 1. (A) SEM image and (B) particle size distribution of one lot of Ch/Al/Ca-MP. n = 150 for b.

Al/Ca-MP. The yield, which was calculated as the ratio of the product amount to the used Al/Ca-MP amount, was nearly 40% (w/w). The low yield was considered because excessive Ca ion ($CaCl_2$), not associated with complexation, was washed out in the treatment with Ch solution. The LF recovery, defined as the ratio of the amount of the recovered LF to that of the initial LF, was approximately 50%. The content of LF in Ch/Al/Ca-MP was 20–30% (w/w), with the mean of 22% (w/w), which was three times higher or more as compared to the content of the previous microparticles[16]. The microparticles were nearly spherical and the size ranged from 1 to 3 μm, with the mean diameter of 1.65 μm (Figure 1). Thus, Ch/Al/Ca-MP could be obtained as microparticles with high LF content and small particle size.

In vitro release

A sequential release test was performed using JP15 first fluid for the initial 2 hours and JP15 second fluid for another 5 hours. The release profile is shown in Figure 2. The release profile was similar to that reported previously. Approximately 60% of LF was released at 1 hour, and then LF was released gradually at both pH values 1.2 and 6.8. Approximately 80% of LF was released at 7 hours (2 hour at pH 1.2 and 5 hours at pH 6.8). The initial rapid release was considered to be because of LF located around the surface, and the LF located inside the network of the polymers was released slowly because of their barrier function. Sarmento et al.

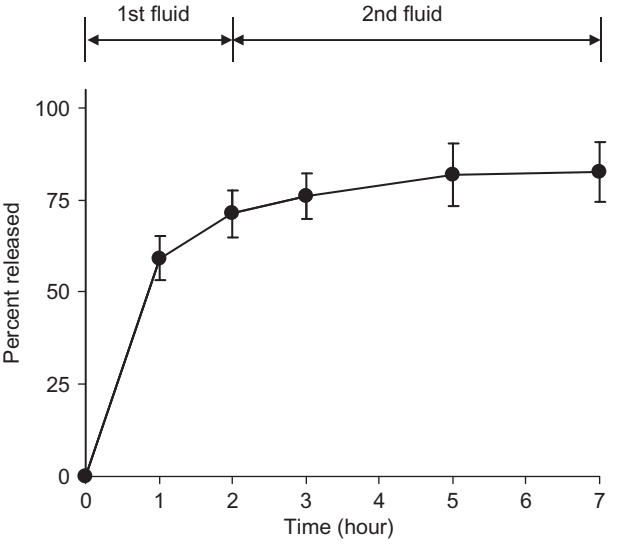

Figure 2. Release profiles of LF in the sequential incubation in JP15 first fluid (pH 1.2) for 2 hours and subsequently in JP15 second fluid (pH 6.8) for 5 hours. Each point represents the mean ± SD (n = 3).

reported an insulin-loaded microparticles prepared by complexation with Al, Ca, and Ch[25], in which the microparticles exhibited a similar release pattern to that in our study although insulin and LF are different in electric charge properties. Namely, it was reported that about half of insulin was released rapidly at pH 1.2 within 1 hour, and then the insulin was released slowly at both pH values 1.2 and 6.8.

The physical state of Ch/Al/Ca-MP was observed in the in vitro release test. Ch/Al/Ca-MP were well suspended in JP15 first fluid, in which they appeared to be rigid, not swollen. In that condition, Ch is ionized and undergoes hydration at pH 1.2, but Al is less ionized and not subject to hydration, leading to the rigid state of the inside of the microparticles. On the contrary, at pH 6.8, Al is ionized and Ch is also ionized to a certain extent, based on their pK_a. Therefore, the whole microparticles were hydrated and swollen, resulting in the physically soft particle state. However, Ch/Al/Ca-MP hardly dissolved throughout the in vitro release study. These properties were almost the same as those of the previously studied microparticles[21]. Ch/Al/Ca-MP were suggested to keep the microparticulate state in the gastrointestinal tract. In addition, the complex microparticles were prepared using fluorescein isothiocyanate (FITC)-labeled Ch (FITC-Ch) instead of Ch, and checked for the physical stability in the aqueous suspension. They showed yellow color because of the formation of FITC-Ch/Al/Ca complex. They were incubated in both JP15 first and second fluids (4 hours), and in JP15 first fluid (2 hours) followed by JP15 second fluid (2 hours), and then centrifuged at 1500 × *g* for 10 minutes. The precipitate was yellow and solid in JP15 first fluid and softened in JP15 second fluid, similar to the results in the above released studies (data not shown). These supported that Ch/Al/Ca-MP should have good physical stability in the gastrointestinal condition.

Efficacy against carrageenan-induced edema

The anti-inflammatory potential of Ch/Al/Ca-MP was evaluated from the inhibitory effect against carrageenan-induced edema in rats. The volume of the right hind paw was measured for the evaluation of the in vivo effect (Figure 3). In control, the paw was swollen gradually after carrageenan injection, and the volume reached maximum at 4 hours, when it was 1.53-fold the initial one. The paw volume decreased gradually after 4 hours. Ch/Al/Ca-MP tended to suppress the edema better than LF solution. Namely, for LF solution, the paw volume increased up to 1.36-fold the initial one at 4 hours. As to Ch/Al/Ca-MP, the paw volume became 1.25-fold the initial one at 3–4 hours. Overall, Ch/Al/Ca-MP exhibited better suppression of the edema and the faster recovery from the edema. These results suggested that Ch/Al/Ca-MP should improve the efficacy of LF. The features of the microparticles such as protective effect, better accessibility to the intestinal mucosal membrane, and prolonged release might be associated with the promotion of the efficacy.

To elucidate whether the initial burst was associated with the promoted efficacy or not, Ch/Al/Ca-MP treated so that the LF contributing to the initial rapid release could be removed, named Ch/Al/Ca/MP (1 hour treated), were examined for the efficacy in the same method. It was ensured by measuring the LF amount washed out in the medium that the initial burst portion of LF hardly remained in Ch/Al/Ca/MP (1 hour treated). Considering the remaining LF amount, Ch/Al/Ca-MP (1 hour treated) was administered at 50 mg LF eq./kg. The results are shown in Figure 4. In control, the

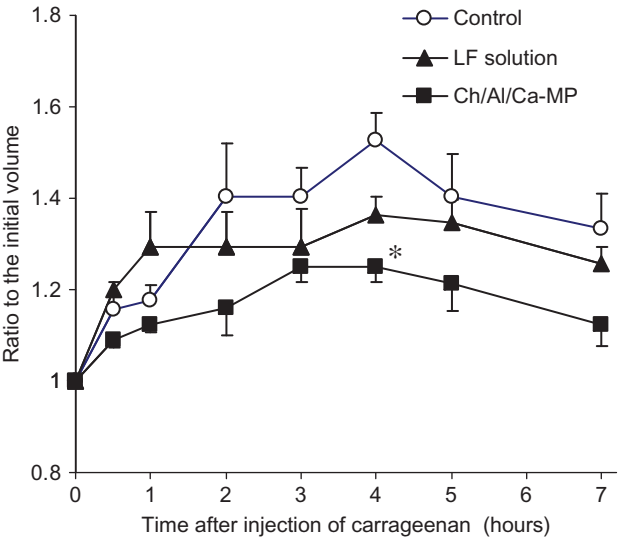

Figure 3. Change in right hind paw volume after subcutaneous injection of carrageenan to the footpad of the right hind paw in rats (250–260 g). Samples were administered intragastrically at 50 mg LF eq./kg 5, 3, and 1 days before carrageenan injection (total dose = 3 × 50 mg LF eq./kg). The results are expressed as the mean ± SE (n = 3). *P < 0.05 versus control (Dunnett's post hoc test).

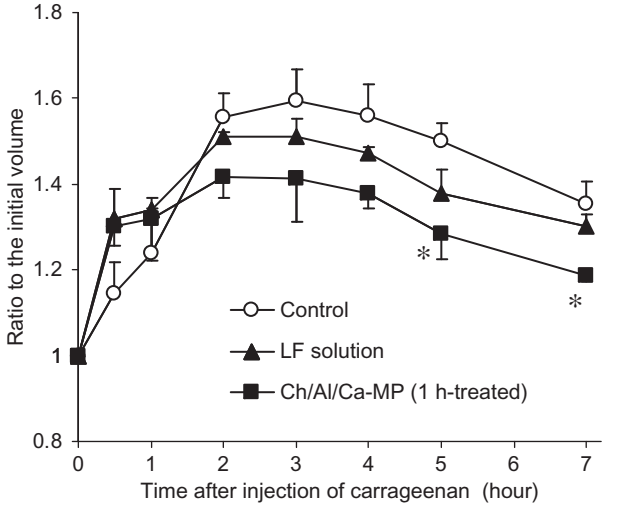

Figure 4. Change in right hind paw volume after subcutaneous injection of carrageenan to the footpad of the right hind paw in rats (180–200 g). Samples were administered intragastrically at 50 mg LF eq./kg 5, 3, and 1 days before carrageenan injection (total dose = 3 × 50 mg LF eq./kg). The results are expressed as the mean ± SE (n = 3). *P < 0.05 versus Control (Dunnett's post hoc test).

paw volume was a little larger than that in Figure 3. One of the reasons was considered to be due to the difference in the age in weeks of the animals used. However, overall, the change in the paw volume was similar to that in Figure 3. Ch/Al/Ca-MP (1 hour treated) tended to suppress the edema better than LF solution and exhibited significantly better suppression of the edema at the latter period. The results are basically similar to those in Figure 3, suggesting that the initial burst should not be importantly associated with the promotion of the efficacy. As stated above, the protective effect, better accessibility to the intestinal mucosal site, and prolonged release by the microparticles were considered to provoke the promotion of the efficacy. In the future, the usefulness of Ch/Al/Ca-LF will be elucidated more clearly by further detailed studies such as a dose dependency for effectiveness.

Conclusion

Ch/Al/Ca-MP with the LF content of 20–30% (w/w) could be produced in the novel formulation using the combination of emulsification–evaporation and subsequent coating with Ch solution. The microparticles were suspended at both pH values 1.2 and 6.8, and their suspension state was maintained even after the medium was changed from JP15 first fluid (pH 1.2) to JP15 second fluid (pH 6.8), suggesting Ch/Al/Ca-MP should keep the microparticulate state in the gastrointestinal tract. Ch/Al/Ca-MP showed an initial rapid release of approximately 60% and subsequent slow release. It was demonstrated that the initial burst was not associated with the improvement of the antiinflammatory effect. The present Ch/Al/Ca-MP are suggested to be useful for the improvement of the LF efficacy against the inflammation.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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