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Research paper

Alginate/chitosan microcapsules for in-situ delivery of the protein, interleukin-1 receptor antagonist (IL-1Ra), for the treatment of dextran sulfate sodium (DSS)-induced colitis in a mouse model

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ABSTRACT

Targeted delivery of bioactive compounds such as proteins to the colon has numerous advantages for the therapeutic treatment of inflammatory bowel disease. The present study sought to fabricate alginate/chitosan microcapsules containing IL-1Ra (Alg/Chi/IL-1Ra MC) via a single-step electrospraying method. Two important factors of efficacy were measured—the pH-responsiveness of the microcapsule and the in-vitro drug release profile. The DSS-induced colitis mouse model was used to evaluate the therapeutic effect of the Alg/Chi/IL-1Ra microcapsules, with results showing the protective effect of the Alg/Chi microcapsules for the passage of IL-1Ra through the harsh environment of the upper gastrointestinal tract. This effect was owing to the pH-sensitive response of the microcapsule, which allowed the targeted release of IL-1Ra in the colon. DAI evaluation, colon length, colon tissue morphology, histologic damage scores and relative protein concentrations (MPO, TNF-α and IL-1β) demonstrated that the Alg/Chi/IL-1Ra microcapsules alleviated DSS-induced colitis in mice. The present study thus demonstrates a practical means of oral delivery of proteins, in-situ colon release, and a promising application of IL-1Ra in the treatment of autoimmune and inflammatory diseases.

1. Introduction

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is an inflammatory disorder of the gastrointestinal tract. Possible triggers of the disease include an inflammatory response caused by an infection with a specific pathogen or virus, or having a defective mucosal barrier. The precise mechanism for the development and progression of IBD remains unclear, however, there has been increasing evidence suggesting that the imbalance between pro-inflammatory and anti-inflammatory cytokines leads to disease progression and tissue destruction [1,2]. The theory that IBD is the result of dysfunctional immunoregulation manifested by excessive production of mucosal cytokines allows the development of therapeutic measures for IBD treatment [3].

Colon biopsies from patients with IBD have shown an overexpression of interleukin-1 (IL-1) which is produced in increased quantities from mononuclear cells in areas of actively inflamed colonic mucosa. This supports the hypothesis that IL-1 is acting in a pro-inflammatory manner exacerbating disease activity (Fig. 1) [4,5,3]. IL-1 has been known be expressed at higher levels in colonic biopsies from patients with IBD, and produced in increased quantities from mononuclear cells in areas of actively inflamed colonic mucosa [6]. Many studies have assessed the role of IL-1 in chronic colon inflammation and their results have led to therapies based on the neutralization of specific IL-1 molecules [7]. It has also been suggested that the downregulation of IL-1 may be useful for the treatment of patients with ulcerative colitis [8].

Recombinant IL-1 receptor antagonist (recombinant IL-1Ra, anakinra) is 1 of 3 FDA-approved biologics available clinically for blocking the IL-1 pathway, It is used as a second line treatment to manage symptoms of rheumatoid arthritis [9]; a soluble decoy receptor, Rilonacept and a monoclonal antibody toward IL-1, Canakinurmab are the remaining blockers. They are approved by FDA for auto-inflammatory diseases treatment and autoinflammatory cryopyrin-associated periodic syndromes (CAPS) treatment, respectively [10]. Although these biologics are available IL-1 blockers as subcutaneous injections, they have not yet to be assessed in human trials as IL-1 blocking/modulating agents for IBD.

Although there are several biologics available for the treatment of IBD, their therapeutic potential is affected by a decrease in response,

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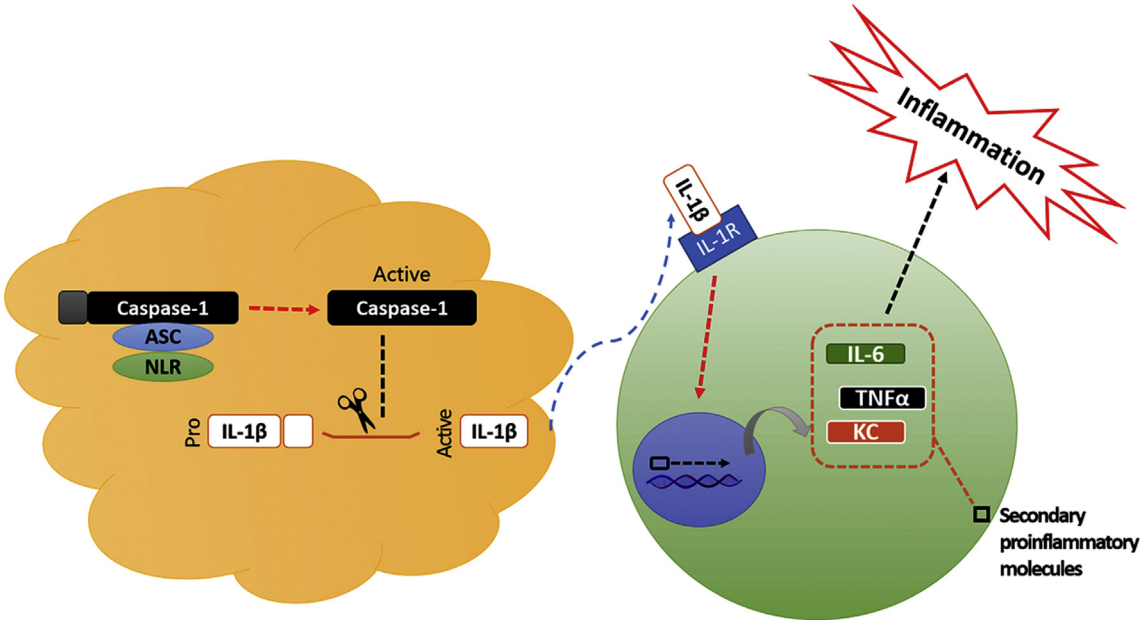

Fig. 1. IL-1β production and inflammation. (ASC: apoptosis-associated speck-like protein containing CARD, NLR: NOD-like receptor, KC: keratinocyte-derived chemokine).

neutralizing antibody generation and severe side effects induced by the parenteral high dose administration (e.g. subcutaneous or intravenous injection) [11–14]. Improving the efficacy of therapeutics and enabling localized treatment require the use of a drug delivery system that will locally deliver and release molecules at the target area (e.g. intestinal mucosa), function as a protection barrier from the stomach's harsh conditions (i.e. its low pH environment) and overcome other limitations of conventional drug formulations [15–17]. For the treatment of IBD, targeted delivery to the colon has numerous advantages and the ability to deliver molecules of interest in-situ avoids systemic delivery which tends to result in a number of adverse and toxic effects [18–20].

The combination of the carboxyl group of alginate with the amino group of chitosan is widely used, and as natural polymers, they are suitable for the fabrication of drug delivery systems. When compared to other delivery methods, they pose advantages such as good biocompatibility, the ability to respond to various environmental stimuli, biodegradability, and the least likely to exert toxic effects [21–23]. Alginate has the unique property of forming stable gels in mild conditions and aqueous media by the addition of multivalent cations [24–26]. The pH sensitivity and enzymatic degradability make chitosan-based hydrogels a good candidate for drug release systems as they are able to adjust to pH changes for oral drug delivery in IBD [27]. Wang et al. [28] have reported such investigation where alginate/chitosan microspheres were formulated and loaded with icariin, and induced by trinitrobenzene sulfonic acid (TNBS)/ethanol for the treatment of mucosal injury in rats. However, few studies have been reported focusing on the pH-sensitive alginate/chitosan microcapsules as loading systems for protein drugs geared to treating IBD via gastrointestinal drug delivery.

In the present study, alginate/chitosan microcapsules with IL-1Ra (Alg/Chi/IL-1Ra MC) were fabricated via a single-step electrospraying method with an optimized preparation parameter. After microcapsule preparation, the pH-responsiveness and in-vitro drug release profile were evaluated in the artificial solutions: simulated gastric fluid, simulated intestinal fluid and normal saline. The therapeutic efficacy of Alg/Chi/IL-1Ra MC was evaluated via intragastric administration in a DSS-induced colitis mouse model. It is intended that the study will provide a practical method for oral delivery of proteins, in-situ colon release and IL-1Ra application in the treatment of autoimmune and inflammatory diseases.

2. Materials and methods

2.1. Animal and bacterial strain

50 female *Balb/c* mice (18 ± 2 g) were obtained from the Laboratory Animal Center of Jiangsu University. All the experimental procedures were conducted in accordance with the institutional guidelines for the care and use of laboratory animals at Jiangsu University, Zhenjiang, China and the National Institutes of Health Guide for Care and Use of Laboratory Animals.

The plasmid, pET30a, and host bacteria, BL21(DE3), for IL-1Ra protein recombinant expression were purchased from Merck Biosciences.

2.2. Chemicals

The alginate (450 mPa·S) and Chitosan (200 mPa·S) were obtained from Qingdao Bright Moon Seaweed Group and Shanghai Qiming Biotechnology, Co., Ltd., respectively. Dextran sulfate sodium (DSS) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

All other regents were chemical pure and from Sinopharm Chemical Reagent Co., Ltd.

2.3. IL-1Ra preparation

The IL-1Ra protein (GenBank: AAH09745.1) was obtained by His-tag fusion protein expression. The pET30a/IL-1Ra Vector was constructed and transferred into BL21(DE3) *E. coli* cells to form the engineering bacteria. After IPTG induction, the IL-1Ra protein was captured via cell lysis with Ni-IDA resin (GenScript®) and eluted using 250 mM imidazole. The purified protein was identified by SDS-PAGE, Western blot and Nano LC-ESI-MS/MS (supplementary materials).

2.4. Microcapsule fabrication

Alg/Chi/IL-1Ra microcapsules were fabricated via a single-step electrospraying method with the following optimized preparation parameter: alginate viscosity = 450 mPa·S, protein concentration = 10 mg/mL, voltage = 24 kV, speed = 0.2 mL/h, distance from

the syringe tip to reception solution surface = 11 cm). The electrospraying device used in the experiment consisted of a high voltage apparatus with positive and negative poles connected to a syringe containing the therapeutic protein, 2% alginate solution, a conductive reception tank with 1 g/L chitosan and 1% calcium chloride ($CaCl_2$) solution. With the supply of high voltage and a micro-injection pump operating at constant speed, small and highly-charged droplets were formed and dispersed radially due to Coulomb repulsion. Microcapsule formation occurred through the interaction of alginate and Ca^{2+} in the tank and solidification on the surface. The particles were allowed to harden for at least 2 h before washing twice with distilled water and collected from the suspension by centrifugation and lyophilization.

2.5. Particle size and shape

The Alg/Chi/IL-1Ra microcapsules were fabricated with the optimized conditions mentioned previously. The size and shape of the 3 batches were observed using an optical microscope and the diameter value for 100 microcapsules from 5 microscopic fields randomly measured from each batch. The arithmetic mean value and standard deviation (SD) were calculated using this value. Alg/Chi/IL-1Ra microcapsules were lyophilized and the morphology was observed by scanning electron microscope (SEM).

2.6. Drug loading efficiency

The enveloped protein in the microcapsules was extracted and used to determine the drug loading efficiency and to calculate the drug release ratio. The microcapsules were suspended in phosphate buffer (pH 7.4) and shaken for 24 h. Protein release followed where ultrasonication was used to destroy the membrane of the particles in the suspension. The supernatant containing the protein was obtained by centrifugation and protein concentration determined using the BCA method (BCA kit, Beyotime Biotechnology, Shanghai, China). Loading efficiency (L) was calculated using Eq. (1) below.

$$L\% = \frac{W_p}{W_a} \tag{1}$$

where W_p is the weight of the protein drug in the microsphere, and W_a is the weight of the protein drug added.

2.7. Swelling ratio and in-vitro drug release in 3 media types

The swelling ratio of the microcapsules was required to describe the pH-responsiveness in various body fluids. The microcapsules were suspended in 20 mL of 3 different artificial solutions: simulated gastric fluid (HCl solution, pH 1.2, containing 2 g/L NaCl), simulated intestinal fluid (pH 7.5 phosphate buffer) and normal saline (0.9% NaCl solution). The suspensions were incubated at 37 °C with gentle shaking.

The diameter values of the microcapsules were recorded at the time points: 0, 20, 40, 60, 80 and 100 min. The swelling ratio (SR) was calculated using Eq. (2) below.

$$SR = \frac{D_t}{D_0} \tag{2}$$

where D_t is the average diameter of the microcapsules at each time point, and D_0 is the initial average diameter of the microcapsules.

For in-vitro drug release evaluation, microcapsules were incubated in the simulated body fluids mentioned above, with 1 mL sample of supernatant tested every 20 min for 240 min. The total volume of 20 mL was maintained in each sample through the addition of blank medium. The protein concentration was quantified using a BCA kit and the cumulative release calculated using Eq. (3) below.

$$CR = \frac{20C_i + V\sum C_{i-1}}{m} \times 100\% \tag{3}$$

where C_i is the protein concentration in the medium (mg/mL), V is 1 mL of sample and m is the total drug content in the microcapsules.

2.8. DSS-induced colitis mouse model and the administration of the microcapsules

Dextran sulfate sodium (DSS)-induced colitis in mice has been recognized as a model for studying human ulcerative colitis.

50 female *Balb/c* mice (18 ± 2 g) were kept in stainless cages with a 12 h light/dark cycle, a temperature of 23 ± 2 °C, and humidity of 55 ± 5%. During the acclimatization period, all animals were raised on a regular diet and water, however, after this period, they were randomly divided into five groups (n = 10): Normal group (N), Model group (M), Alg/Chi/IL-1Ra microcapsules group (IMT), Alg/Chi/BSA microcapsules group (BMT) and unencapsulated IL-1Ra group (IT). The protocol described by Li [29] was largely used to implement the colitis model. During the first 7 days, the N group was raised with regular diet and double distilled water (DDW) while the remaining 4 groups were provided with 5% dextran sulfate sodium (DSS, MW 36,000–50,000; MP Biomedicals, Santa Ana, CA) solution *ad libitum*. For the following 8 days, saline solution was administered intragastrically (i.g.) to mice in the N and M groups, while the remaining 3 groups were supplied with Alg/Chi/IL-1Ra microcapsules, Alg/Chi/BSA microcapsules and unencapsulated IL-1Ra intragastrically, respectively. Saline solution and microcapsules were applied to the animals once a day from day 8. The final dosage was kept to 1 mg of protein per kg of mice body weight. Body weight and defecation were monitored daily over the period of study with animal groupings and treatments listed in Table 1. On day 15, mice were euthanized by cervical dislocation. The colon was immediately collected and the length measured. Sections of the colon were stored at −80 °C for enzyme and cytokine analysis while segments for pathological observation were fixed in formalin. All the experimental procedures were conducted in accordance with the institutional guidelines for the care and use of laboratory animals at Jiangsu University, Zhenjiang, China.

2.9. Disease activity index (DAI) evaluation

The DAI score was followed as per Muran's method [30]. The body weight loss, hematochezia and stool property were observed and scored from 0 to 4; the addition of these points were applied to evaluate disease severity.

2.10. Colon tissue morphology and histologic damage scores

The degree of IBD severity was also evaluated based on histological

Table 1
Animal grouping and treatment.

No.	Group name	Treatment
1	Blank control group or Normal group (N)	DDW + Saline solution
2	IBD control group of Model group (M)	5% DSS + Saline solution
3	Alg/Chi/IL-1Ra microcapsules group (IMT)	5% DSS + Alg/Chi/IL-1Ra microcapsules
4	Alg/Chi/BSA microcapsules group (BMT)	5% DSS + Alg/Chi/BSA microcapsules
5	Unencapsulated IL-1Ra group (IT)	5% DSS + Unencapsulated IL-1Ra

observations of hematoxylin and eosin (H&E) stained images of the colon [31]. Approximately 4 mm of the middle section of the colon was removed and rinsed with saline water (0.9%, w/w) and formaldehyde solution (1:9, 0.01 M phosphate buffer, pH 7.4). Fixed tissues underwent routine processing for paraffin embedding, and dyed with H&E staining. The sections were observed under an optical microscope and photographs taken to record the colon histological damage scores (HDS) according to the criteria as per Delelman's method [32].

2.11. Enzyme and cytokine analysis on intestinal samples

The crude enzyme and cytokine expressed in the colon were extracted mainly according to previously described methods [33]. Tissues were briefly homogenized and centrifuged to collect the supernatant. Protein concentrations were measured using a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) and adjusted to 2.5 or 5 mg/mL by dilution with lysis buffer; the concentrations of myeloperoxidase (MPO), interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNFα) were determined. The enzyme and cytokine concentrations in the extract were quantified using a myeloperoxidase assay kit (Jiancheng Bioengineering Institute, Nanjing, China), mouse IL-1β ELISA kit (Beyotime Biotechnology, Shanghai, China) and mouse TNFα ELISA kit (Beyotime Biotechnology, Shanghai, China).

2.12. Serum cytokine analysis

Blood samples were collected from the eyeballs of mice, and were placed in plastic tubes at 4 °C for 2 h. The serum samples were then separated by centrifugation for 10 min at 3000 rpm, and stored at −20 °C until analysis. Protein concentrations quantitative methods were as same as "Enzyme and cytokine analysis on intestinal samples".

2.13. Immunohistochemistry

In order to detect the IL-1Ra protein released from the microcapsules, immunohistochemistry experiment was operated. The samples were obtained as described in "Colon tissue morphology and histologic damage scores". The immunohistochemistry technical service was offered by Shanghai Gefan Biotechnology Co., Ltd. Briefly, in this experiment, the molecules with a his-tag (like recombinant IL-1Ra in this study) can react with his-tag antibody plus horseradish peroxidase labeled secondary antibody, and be stained brown by DAB in a immunochemical reaction.

2.14. Statistical analysis

Sample means were compared using the one-way ANOVA test. Differences where $p < 0.05$ was considered statistically significant while those at $p < 0.01$ were considered extremely significant from a statistical standpoint.

3. Results

3.1. IL-1Ra expression and purification

As shown in Fig. 2A, the purity of the extracted protein was above 98% with its molecular weight close to 20 kDa. In addition, the results of the western blot performed using his-tag antibody binding (Fig. 2B) and Nano LC-ESI-MS/MS (supplementary materials), proved that the purified protein's amino acid sequence was consistent with the IL-1Ra protein.

3.2. Alg/Chi/IL-1Ra microcapsules preparation

A total of 3 batches of the Alg/Chi/IL-1Ra microcapsules were prepared and the morphology and size observed using an optical microscope (Fig. 3). According to the image, the round microcapsules were complete and smooth appearance. The average diameters of these microcapsules were 437 ± 41 μm, 447 ± 43 μm and 443 ± 36 μm respectively, with subsequent drug loading efficiencies of 85.4%, 82.3% and 80.7%. The morphology of the Alg/Chi/IL-1Ra microcapsules was shown in the SEM image (Fig. 4). According to the SEM result, the microparticle was integrity yet shrunken in the lyophilization process.

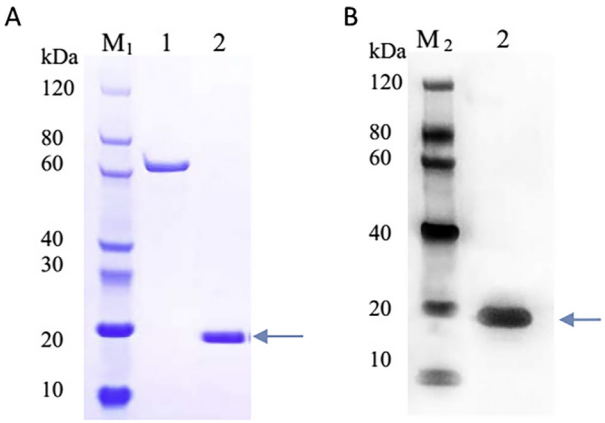

Fig. 2. Purified IL-1Ra protein (A. SDS-PAGE and stained with Coomassie brilliant blue; B. Western blotting using anti-His antibody.). Lane 1: BSA (1.0 μg), Lane 2: IL-1RA protein (1.5 μg), M1: SDS-PAGE marker, M2: Western blot marker. Arrowed bands: IL-1Ra. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. pH-responsiveness of the microcapsule

According to the swelling ratio results (Fig. 5), a range of reactions was observed when the microcapsules were placed in different solutions. In the first solution, pH 1.2 simulated gastric fluid (Fig. 5A), the shape of the particle was stable, with a small degree of swelling. The microcapsules in 0.9% NaCl solution swelled at a constant rate for the first 60 min before leveling for the remaining time (Fig. 5B). In the pH 7.5 simulated intestinal fluid, however, the microcapsules swelled rapidly between 0 and 40 min resulting in a loss in their integrity (Fig. 5C) and an undetectable swelling ratio. As shown in Fig. 5C and D, the material in the wall of the microcapsules had disintegrated into fragments during the late period of the test resulting in a thick, opaque medium.

3.4. In-vitro drug release

Fig. 6 demonstrates the cumulative release results. Owing to the microcapsule's morphological integrity, there is a small number of protein for release into the simulated gastric fluid medium. Given the slight swelling of the microcapsules in saline solution and an approximate cumulative release of 5.0% in 240 min totally. The drug release curve in the simulated intestinal fluid group increased rapidly and reached 50.4% in the first 40 min, and the number was above 80% in 120 min. There was a slow growth from 140 to 240 min, and a percentage relatively close to the final 86.2% cumulative release rate; these results as well as the swelling ratio results revealed the pH response ability of the Alg/Chi microcapsules. Therefore, the fabricated Alg/Chi/IL-1Ra microcapsule has demonstrated the potential for use as an oral administration and a protective barrier to prevent protein destruction while passing through the stomach to its intestinal target.

3.5. Disease activity index (DAI) evaluation

From the first day of DSS exposure to the last day of the experiment,

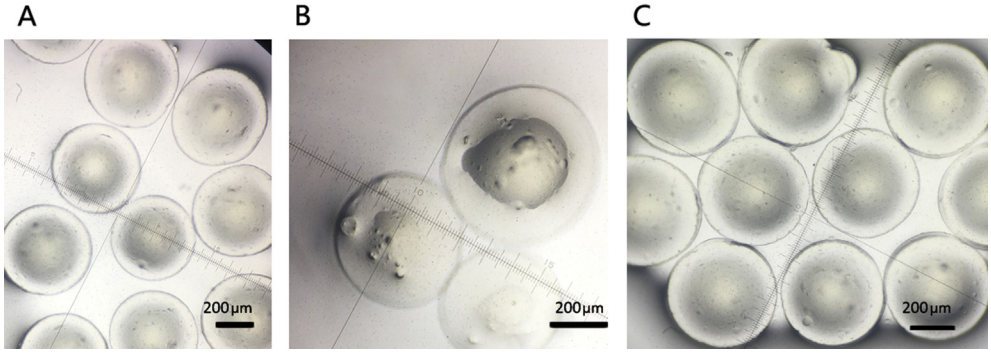

Fig. 3. Microscope images of Alg/Chi/IL-1Ra Microcapsules (3 batches).

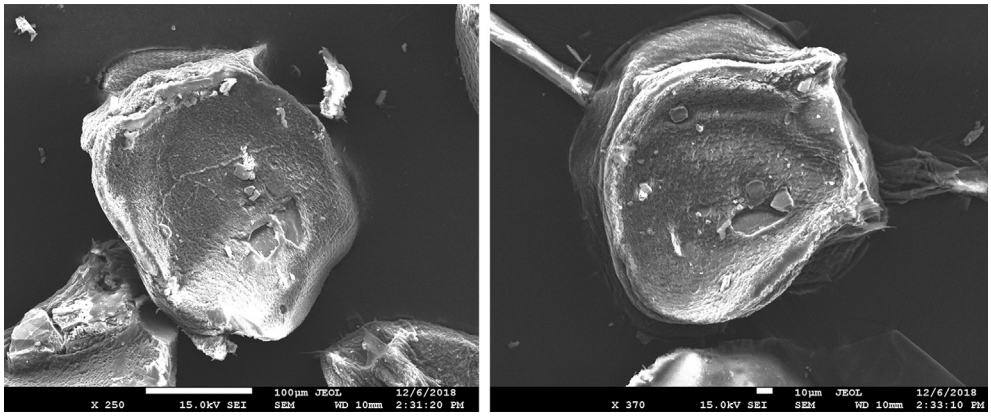

Fig. 4. Scanning electron microscope (SEM) images of lyophilized Alg/Chi/IL-1Ra Microcapsules.

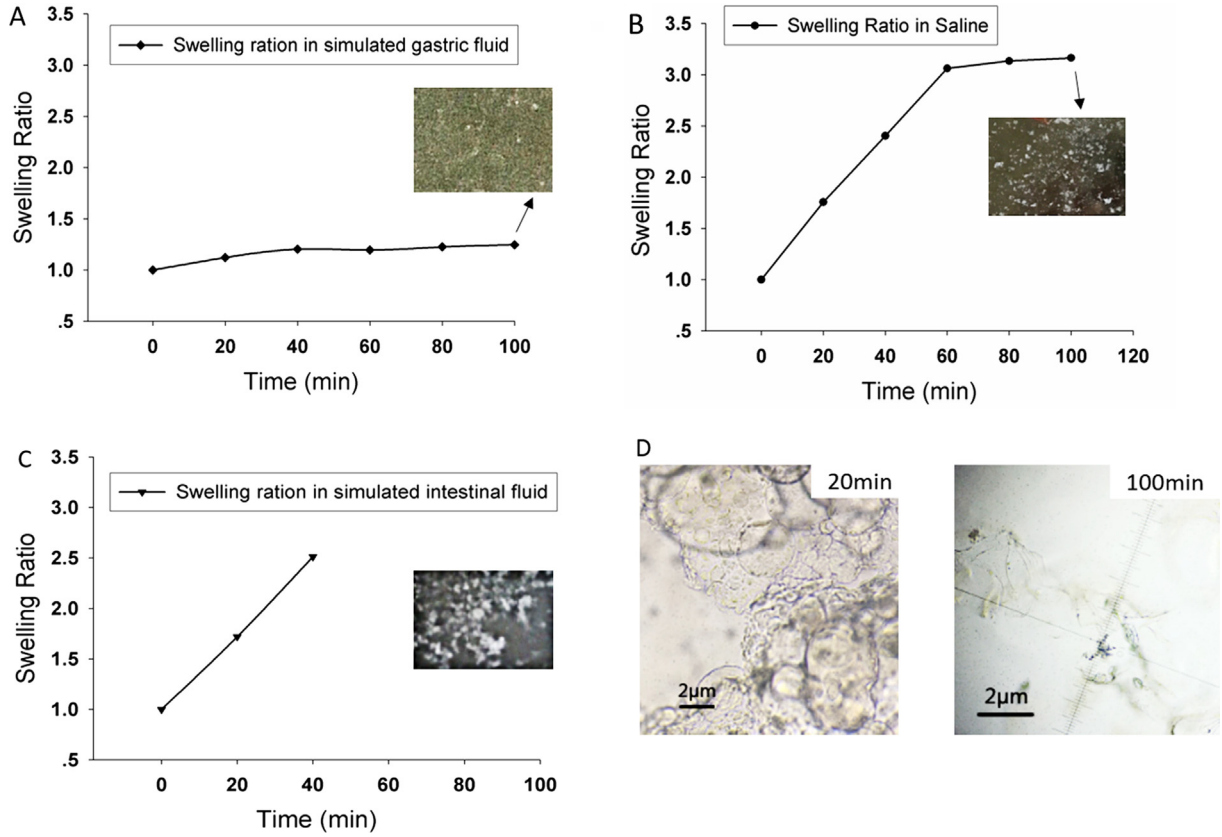

Fig. 5. Swelling ratio of Alg/Chi/IL-1Ra Microcapsules in simulated gastric fluid (A), 0.9% NaCl solution (B) and simulated intestinal fluid (C). D: Microscope image of Alg/Chi/IL-1Ra Microcapsules fragment in simulated intestinal fluid at time point of 20 min and 100 min.

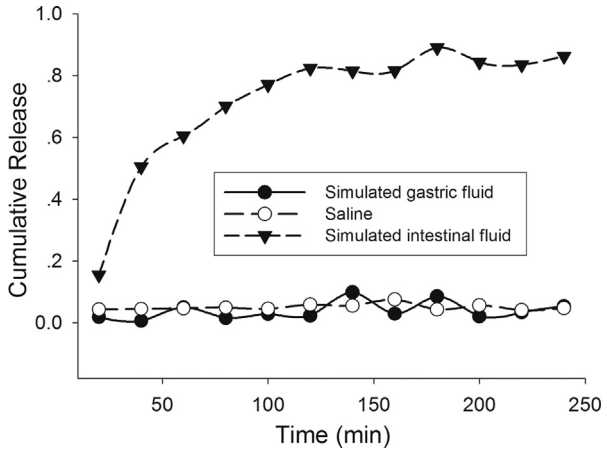

Fig. 6. Cumulative release curve of Alg/Chi/IL-1Ra Microcapsules.

weight loss, stool consistency and fecal bleeding of the animals were monitored and scored daily to calculated the DAI (Fig. 7A and D) where a greater score meant greater disease severity. According to the data, the DSS exposure period (the first 7 days) saw an increase in DAI in each group with symptoms including weight loss, loose stool and occult fecal bleeding or gross bleeding (Fig. 7C); the DAI of the N group was 0 (data not shown). In the treatment period (from day 8 to day 15), the DAI declined on account of DSS removal, but in varying degrees (Fig. 7A). The DAI of the M group maintained the highest score in the last 6 days, a value above 2. The BMT and IT groups had a very close falling range, however, the DAI had no significant difference when compared to the M group. The steepest decline occurred in the IMT group, which almost decreased to the lowest value on the final day. When the DAI value of the M group was compared to the value of the IMT group, significant difference ($p < 0.05$) or extremely significant difference ($p < 0.01$) was seen during the last 6 days (Fig. 7D).

The colon length of these groups was also recorded to evaluate the therapeutic effect of the fabricated microcapsules (Fig. 7-B). The mice in the N group had the longest colon, reflecting their organ integrity. In contrast, the colon length of mice in the M group was the lowest of the 5 groups, a result of the colon destruction due to lack of the therapeutic agent. The mice in the IMT group mice had the second longest colon of the 5 groups and showed extreme significant difference ($p < 0.01$) when compared to the M group. Interestingly, the IT group had the third longest colon length with a significant difference when compared to the M group. The body weight was illustrated as a line graph as Fig. 7-E. It can be observed that all of the mice weight in M, IMT, IT and BMT groups have started being recovered from day 9 or 10, because of the DSS withdrawal in day 7. But besides N group, the IMT group has the highest recovery level.

3.6. Colon tissue morphology and histologic damage scores

Upon observing the image of the HE-stained colon tissue for the N group, the sections of crypt structure in the mucosal layer, the submucosa, and the muscular layer appeared normal (Fig. 8). A large amount of inflammatory cell was seen to infiltrate the submucosa of the M group while crypt abscesses and crypt damages were observed (Fig. 8). In the IMT group, re-epithelialization can be seen as well as crypt formation indicating regeneration. In the IT and BMT groups, inflammatory cell infiltration and crypt damages were observed in addition to mucosa and submucosa irregularities (Fig. 8). This indicated that Alg/Chi/IL-1Ra microcapsules promoted the proliferation of crypt cells and restored the injured mucosa.

The M group had the highest colon histological damage score while the IMT group had the lowest (Fig. 9), with extreme significant difference between the two groups. The result indicated the alleviation effect of Alg/Chi/IL-1Ra microcapsules on colon inflammation caused by colitis.

3.7. Related enzyme and cytokines in colon tissue and serum

The concentrations of the inflammatory indicators MPO, IL-1β and

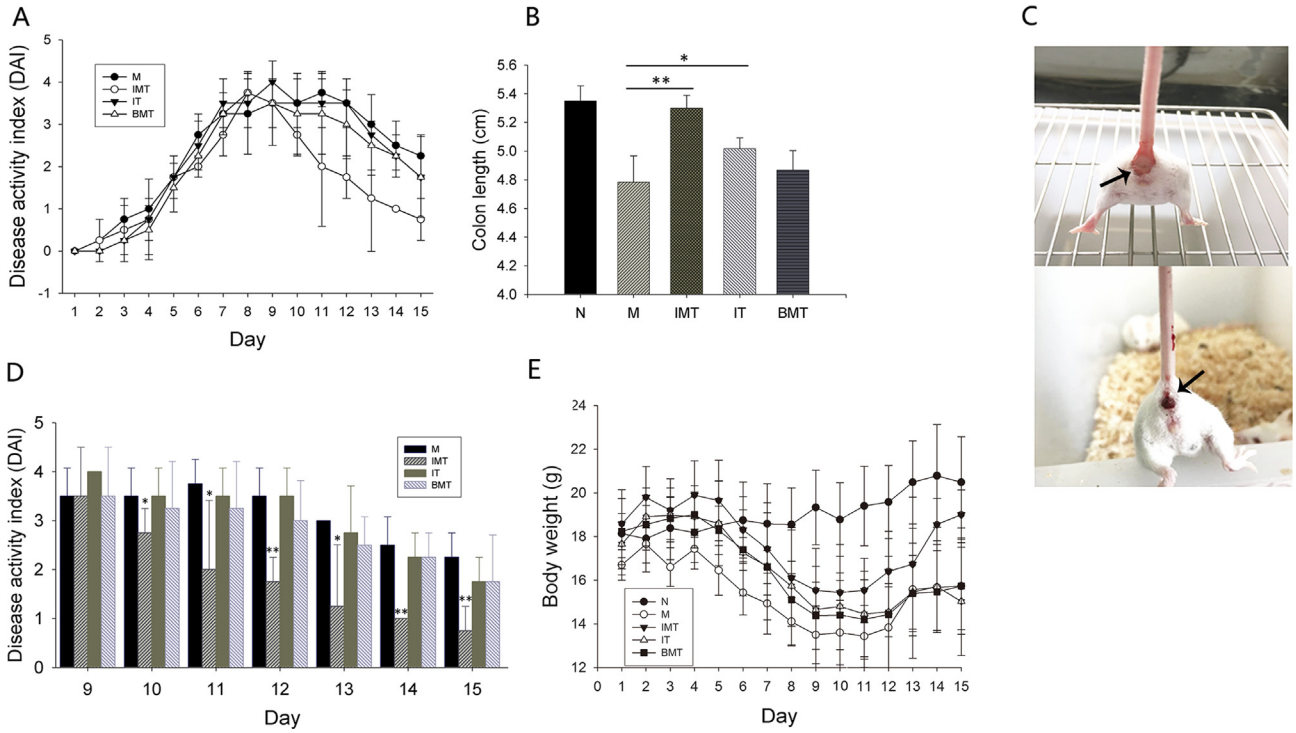

Fig. 7. Disease activity index (DAI) of mice (A: day 1–15, D: day 9–15); colon length (B); hematochezia (arrows) of M group mice at day 7 (C) and body weight (E: day 1–15). *significant differences ($p < 0.05$), **extremely significant differences ($p < 0.01$).

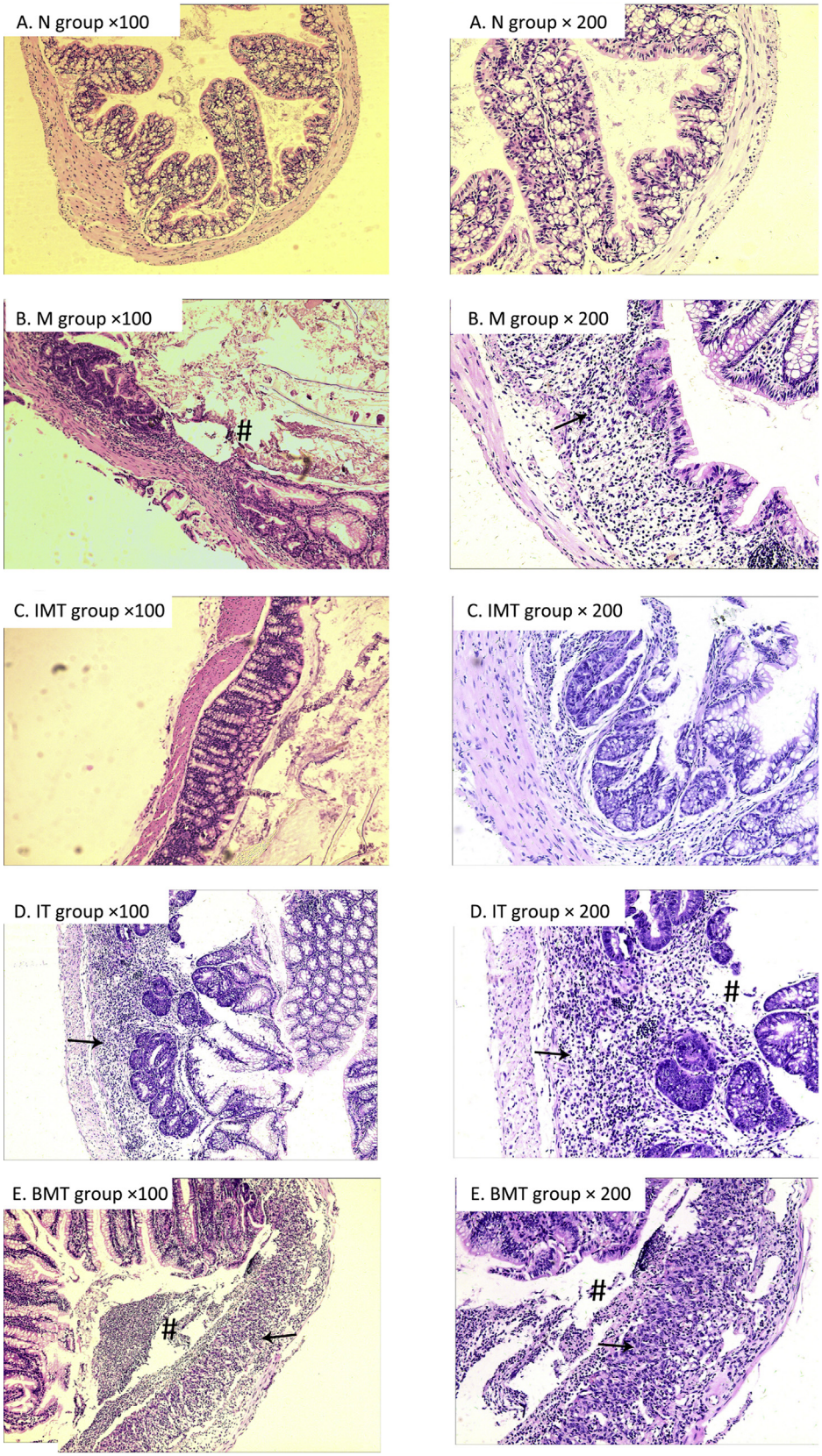

Fig. 8. Image of HE-stained colon of mice (A: Blank control group or Normal group (N), B: IBD control group of Model group, C: Alg/Chi/IL-1Ra microcapsules group, D:Alg/Chi/BSA microcapsules group, E: Unencapsulated IL-1Ra group). Inflammatory cell infiltration into the submucosa (arrows), crypt tissue damage (hash).

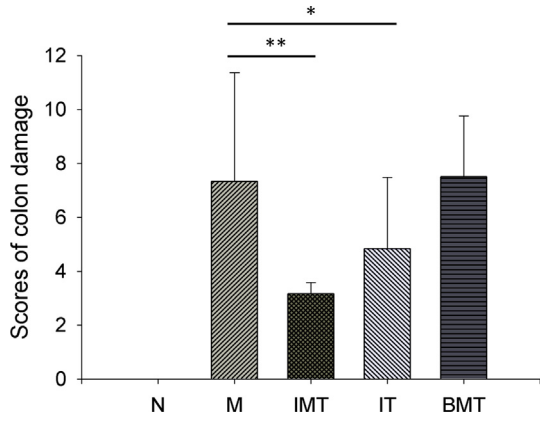

Fig. 9. Score of colon damage. *significant differences ($p < 0.05$), **extremely significant differences ($p < 0.01$).

TNFα, in the colon were detected, and the results are shown in Fig. 10. In the N group, the enzyme and cytokine concentrations were at a basic level with the M group having the highest, indicating that the mice in the M group had developed colon inflammation. The IMT group mice had the lowest index of the 3 treatment groups (IMT, IT and BMT), in addition to showing extreme significant difference in MPO and IL-1β concentrations when compared to the M group.

The TNFα and IL-1β concentration in serum were also being detected and shown in Fig. 11A and B, respectively. In M group, both TNFα and IL-1β have the highest concentrations amongst 5 groups. IMT group show the lowest serum TNFα and IL-1β concentration besides N, but doesn't show the significant difference compared to M.

3.8. Immunohistochemistry

As shown in the Fig. 12 result, the IMT group has an obvious positive his-tag result (brown color) while the N group and BMT group were negative (blue color). The his-tag-positive result indicated that there was IL-1Ra protein being released in the colon, since the IL-1Ra protein applied to mice was a recombinant one with his-tag on its −COOH terminal.

4. Discussion

Since the number of IBD patients has been increasing, novel, therapeutic strategies are in great demand. Targeted delivery of therapeutics to the colon is particularly advantageous for the treatment of this disease well as other local diseases affecting the colon. This form of delivery improves the efficacy of therapeutics, reduces systemic toxicity and enables localized treatment [19]. To achieve the goal of effective oral administration and in-situ treatment, numerous strategies have been utilized in previous research including recombinant lactic acid bacteria expression system [15], colon target delivery nanoparticle, microspheres of siRNA or antisense oligonucleotide [19,34], and edible ginger-derived nanoparticles [35]. Given the explicit mechanism and effective pharmaceutical potency, protein compounds are major forces in treating autoimmune diseases. However, owing to the harsh environment of the upper digestive tract, oral administration of this bioactive compound has proven difficult. This makes it an imperative and crucial task to target the colon so that treatments can be developed for UC using targeted drug delivery systems [27].

Chitosan blended with hydrogel-based alginate forms microcapsules that can be effectively used for drug delivery to the colon [27]. As these

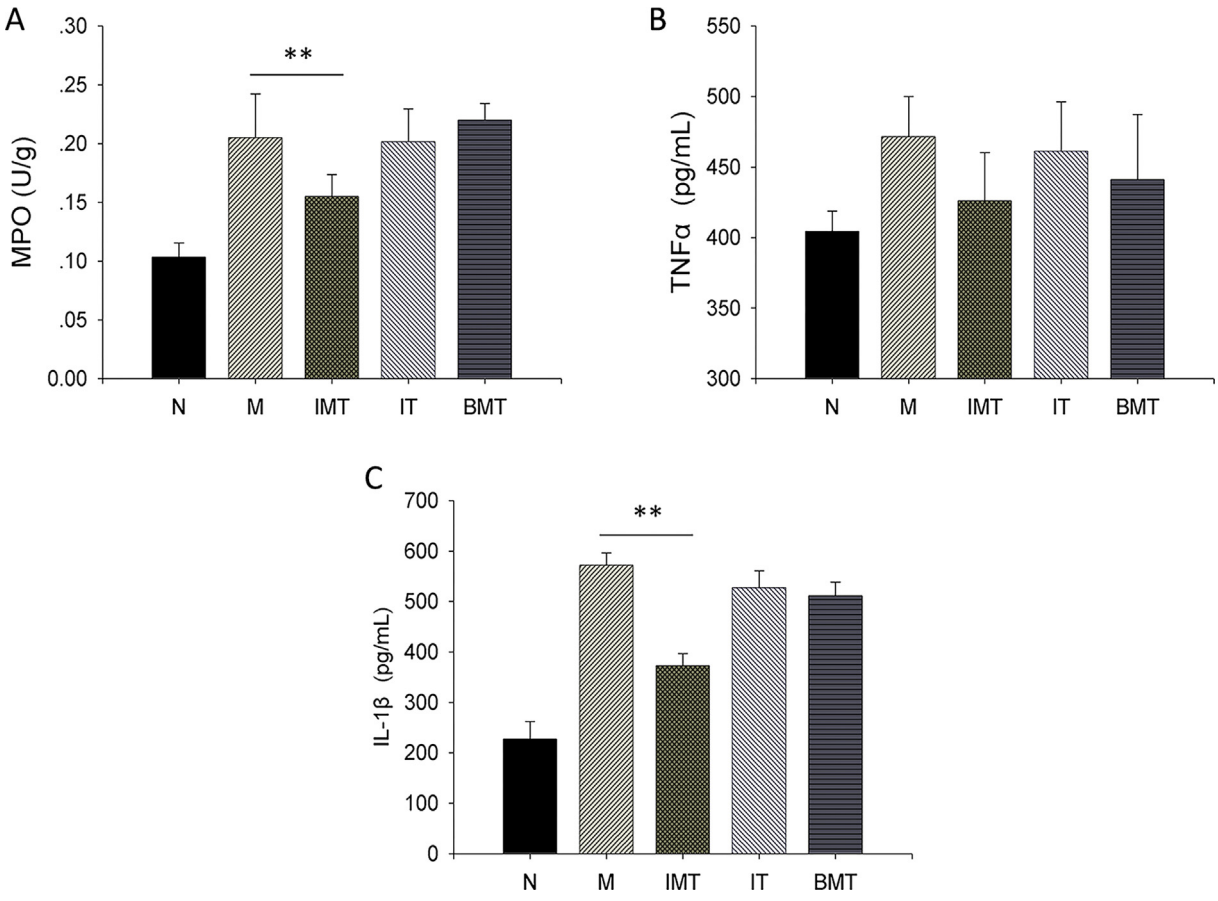

Fig. 10. Enzyme and cytokines concentration in colon tissue (A: Myeloperoxidase, B: Interleukin 1β, C: Tumor necrosis factor α). **extremely significant differences ($p < 0.01$).

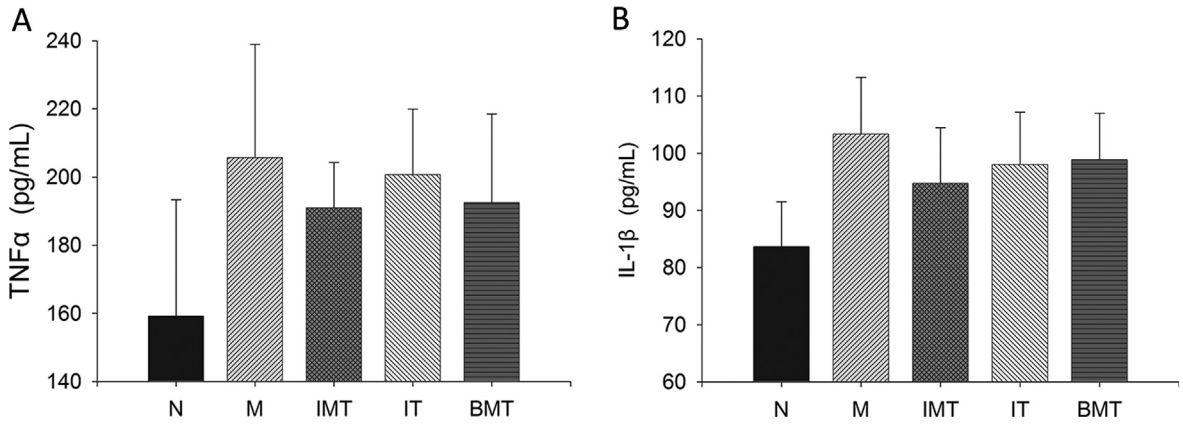

Fig. 11. Serum cytokines concentration (A: Tumor necrosis factor α, B: Interleukin 1β).

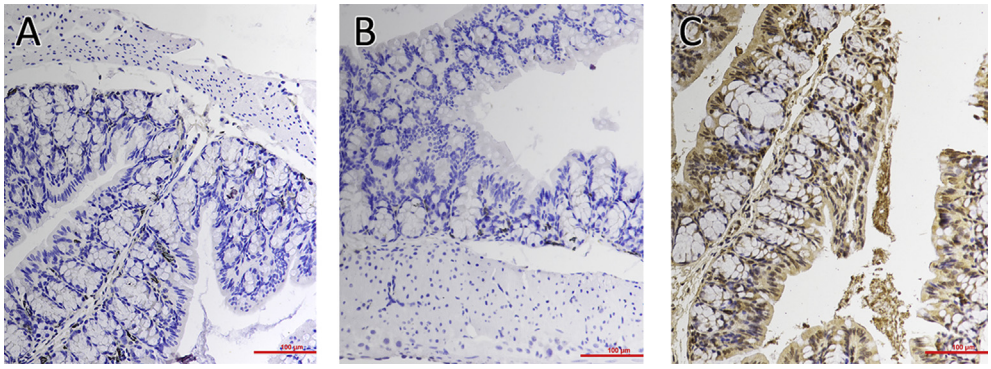

Fig. 12. Immunohistochemistry images (A: N group, B: BMT group, C: IMT group).

polymers are oppositely charged polyelectrolytes, combination occurs at the carboxyl group of alginate and the amino group of chitosan. Chitosan is positively charged due to its amine groups ($-NH_3^+$) and alginate is an anionic polysaccharide based on its carboxyl group ($-COO^-$). Several methods such as encapsulation, emulsion polymerization, polymerization, complex coacervation, solegel and spray draying can be used for combining the two polymers [21], however, the use of emulsifiers, mechanical forces and extreme reaction conditions in addition to the time required, capsule size limitations and difficulty in controlling size, make these techniques problematic in the study of the protein's activity and stability. Electrospraying is a highly versatile method used to fabricate fibers or microparticles with diameters ranging from μm to nm [23,36,37]. The potential for large scale production, the simplicity of the process and the mild reaction conditions associated, make this technique an efficient method to produce an appropriate size and uniformed microcapsule containing active protein compounds [36,38].

In this study, a novel IL-1Ra-loading chitosan/alginate microcapsule was fabricated for in-situ release of a therapeutic protein to treat DSS-induced colitis in mice. Assessment of the swelling ratios and in-vitro drug release profiles in various media demonstrated the pH-sensitivity and pH-responsiveness of the fabricated microcapsules. In low pH solution, the microcapsules displayed stability and resistance from surface leak, while in a high pH environment, they were prone to leakage from the pores and cracks caused by microcapsule swelling. This is as a result of chitosan being less positively charged in a weak alkaline solution and having less electrostatic interaction with alginate, allowing alginate to absorb water and swell. Moreover, the acidic environment does not disturb the stability of the alginate carrier, whereas in the intestine, the solubility of alginate is allowed [39]. The microcapsule wall is therefore seen to protect the IL-1Ra while travelling through the gastric environment as digestion or degradation is prevented. This allows for in-situ delivery and release in the intestinal mucosa.

This in-situ therapeutic effect was also tested in mice with DSS-induced colitis. The unenveloped IL-1Ra and BSA-loading Alg/Chi microcapsule groups were compared to the Alg/Chi/IL-1Ra group.

According to the results of DAI, colon length, colon tissue morphology and histologic damage scores, the Alg/Chi/IL-1Ra microcapsules alleviated the therapeutic effect in DSS-induced colitis mice. In addition, the effect was seen to be related to localized colon-released IL-1Ra, instead of the microcapsule wall material.

The related enzyme/cytokine concentration was determined for further evaluation (MPO, TNF-α and IL-1β in Fig. 8A–C). MPO is a peroxidase enzyme expressed by neutrophil granulocytes, which can function as a biomarker in inflammation. The imbalance in production of inflammatory cytokines like TNF-α and IL-1β contributes to immune dysfunction, mediates inflammation of the tissues and causes organ damage [40,41]. The IMT group had the lowest enzyme/cytokine concentration of the 3 treatment groups which was consistent with the study results above. When it comes to serum cytokines concentration, MT, IT and BMT groups were all lower than M group, and IMT was the lowest. However, according to the statistical analysis, the difference between M and IMT was not significant. Hence, it was possible to infer that instead of blood absorption, the impact of the IL-1Ra released from microcapsules on the IBD mice was mainly an effect on the colon tissue locally.

Interestingly, in some results (e.g. colon length and scores of colon damage), the IT group (i.e. IL-1Ra lacking a microcapsule wall) experienced some degree of alleviation when compared to the M group, however, this extent was incomparable to the IMT group. It can therefore be inferred that small amounts of IL-1Ra remained active during travel through the upper gastrointestinal tract, however, most of the protein was damaged during inactivation. Combined with the immunohistochemistry experiment result, the IL1-Ra in Alg/Chi/IL-1Ra

microcapsule can be released in colon. These experiments proved pH-sensitive microcapsule offers a good protection for protein traveling through the harsh conditions of the stomach while allowing this macromolecular therapeutic agent to be colon-targeted and released as an in-situ therapy in DSS-induced colitis animals.

5. Conclusion

In conclusion, Alg/Chi/IL-1Ra microcapsules with unified sizes and integrated shapes were fabricated via a single-step electrospraying method. The pH-responsiveness of the microcapsule and *in-vitro* drug release profile in simulated body fluids ranging in pH were evaluated, and the property of pH-sensitivity verified. The therapeutic effect of microcapsules administered orally were tested on DSS-induced colitis mice. Generally, combined colitis alleviation effect results with in-suti IL-1Ra detection by immunohistochemistry, the *in-vivo* results indicated that the Alg/Chi microcapsules ensured that the IL-1Ra was protected from the harsh environment of the upper gastrointestinal tract by providing a pH-sensitive response and colon-targeted IL-1Ra release. Alg/Chi/IL-1Ra microcapsules can paly a role as an oral administration therapeutic agent for DSS-induced colitis in a mouse model. Based on the study, the in-situ therapy using a therapeutic, bioactive macromolecule can be used as a target specific delivery system. However, further investigations into the detailed mechanisms, as well as dose-effect relationship are required to confirm the use of Alg/Chi/IL-1Ra microcapsule as an IBD therapeutic agent.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.02.011.

References

[1] M.F. Neurath, Cytokines in inflammatory bowel disease, Nat. Rev. Immunol. 14 (2014) 329–342.
[2] U.P. Singh, N.P. Singh, E.A. Murphy, R.L. Price, R. Fayad, M. Nagarkatti, P.S. Nagarkatti, Chemokine and cytokine levels in inflammatory bowel disease patients, Cytokine 77 (2016) 44–49.
[3] V. Casini-Raggi, L. Kam, Y.J. Chong, C. Fiocchi, T.T. Pizarro, F. Cominelli, Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation, J. Immunol. 154 (1995) 2434–2440.
[4] J.R. Lukens, J.M. Gross, T.D. Kanneganti, IL-1 family cytokines trigger sterile inflammatory disease, Front. Immunol. 3 (2012) 315.
[5] C. Cai, L. Wu, C. Li, P. He, J. Li, J. Zhou (2011).
[6] M.H. McLean, M.F. Neurath, S.K. Durum, Targeting interleukins for the treatment of inflammatory bowel disease-what lies beyond anti-TNF therapy? Inflamm. Bowel Dis. 20 (2014) 389–397.
[7] E. Voronov, R.N. Apte, IL-1 in colon inflammation, colon carcinogenesis and invasiveness of colon cancer, Cancer Microenviron. 8 (2015) 187–200.
[8] Y. Arai, H. Takanashi, H. Kitagawa, I. Okayasu, Involvement of interleukin-1 in the development of ulcerative colitis induced by dextran sulfate sodium in mice, Cytokine 10 (1998) 890–896.
[9] C. Kaiser, A. Knight, D. Nordstrom, T. Pettersson, J. Fransson, E. Florin-Robertsson, B. Pilstrom, Injection-site reactions upon Kineret (anakinra) administration: experiences and explanations, Rheumatol. Int. 32 (2012) 295–299.
[10] E. Dhimolea, Canakinumab, mAbs 2 (2010) 3–13.
[11] A. Papa, G. Mocci, M. Bonizzi, C. Felice, G. Andrisani, G. Papa, A. Gasbarrini, Biological therapies for inflammatory bowel disease: controversies and future options, Exp. Rev. Clin. Pharmacol. 2 (2009) 391–403.
[12] G.R. Lichtenstein, Comprehensive review: antitumor necrosis factor agents in inflammatory bowel disease and factors implicated in treatment response, Therap. Adv. Gastroenterol. 6 (2013) 269–293.
[13] W.J. Sandborn, State-of-the-art: Immunosuppression and biologic therapy, Dig. Dis. 28 (2010) 536–542.
[14] W.J. Sandborn, S.B. Hanauer, Antitumor necrosis factor therapy for inflammatory bowel disease: a review of agents, pharmacology, clinical results, and safety, Inflamm. Bowel Dis. 5 (1999) 119–133.
[15] L.G. Bermudez-Humaran, J.P. Motta, C. Aubry, P. Kharrat, L. Rous-Martin, J.M. Sallenave, C. Deraison, N. Vergnolle, P. Langella, Serine protease inhibitors protect better than IL-10 and TGF-beta anti-inflammatory cytokines against mouse colitis when delivered by recombinant lactococci, Microb. Cell Fact. 14 (2015) 26–36.
[16] R. Martín, F. Chain, S. Miquel, J.M. Natividad, H. Sokol, E.F. Verdu, P. Langella, L.G. Bermúdez-Humarán, Effects in the use of a genetically engineered strain of Lactococcus lactis delivering in situ IL-10 as a therapy to treat low-grade colon inflammation, Human Vacc. Immunotherap. 10 (2014) 1611–1621.
[17] B. Khor, A. Gardet, R.J. Xavier, Genetics and pathogenesis of inflammatory bowel disease, Nature 474 (2011) 307–317.
[18] M.M. Patel, Micro/nano-particulate drug delivery systems: a boon for the treatment of inflammatory bowel disease, Exp. Opin. Drug Deliv. 13 (2016) 771–775.
[19] S. Hua, E. Marks, J.J. Schneider, S. Keely, Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: selective targeting to diseased versus healthy tissue, Nanomedicine 11 (2015) 1117–1132.
[20] D. Prosperi, M. Colombo, I. Zanoni, F. Granucci, Drug nanocarriers to treat autoimmunity and chronic inflammatory diseases, Semin. Immunol. 34 (2017) 61–67.
[21] M.K. Moghaddam, S.M. Mortazavi, T. Khayamian, Preparation of calcium alginate microcapsules containing n-nonadecane by a melt coaxial electrospray method, J. Electrostat. 73 (2015) 56–64.
[22] F.Z. Wang, L. Chen, D.S. Zhang, S.M. Jiang, K. Shi, Y. Huang, R. Li, Q.W. Xu, Methazolamide-loaded solid lipid nanoparticles modified with low-molecular weight chitosan for the treatment of glaucoma: vitro and vivo study, J. Drug Target. 22 (2014) 849–858.
[23] J. Liu, H.X. Li, D.Q. Chen, X.A. Jin, X.L. Zhao, C. Zhang, Q.N. Ping, In vivo evaluation of novel chitosan graft polymeric micelles for delivery of paclitaxel, Drug Deliv. 18 (2011) 181–189.
[24] L.A. Luztonó, D. Zaldivar-Silva, L. Peña, M.L. Dias, Alginate microparticles as oral colon drug delivery device: a review, Carbohydr. Polym. 168 (2017) 32–43.
[25] H. Li, W.F. Dong, J.Y. Zhou, X.M. Xu, F.Q. Li, Triggering effect of N-acetylglucosamine on retarded drug release from a lectin-anchored chitosan nanoparticles-in-microparticles system, Int. J. Pharm. 449 (2013) 37–43.
[26] H.F. Liu, Y. He, P. Ke Zhaoying, Preparation of ambroxol hydrochloride carboxymethyl chitosan microphores without burst release, African J. Pharm. Pharmacol. 5 (2011) 1063–1069.
[27] M. Sohail, M.U. Mudassir, S. Minhas, Z. Khan, M. de Hussain, S.A. Matas, S. Shah, M. Khan, K. Ullah Kousar, Natural and synthetic polymer-based smart biomaterials for management of ulcerative colitis: a review of recent developments and future prospects, Drug Deliv. Transl. Res. (2018).
[28] Q.S. Wang, G.F. Wang, J. Zhou, L.N. Gao, Y.L. Cui, Colon targeted oral drug delivery system based on alginate-chitosan microspheres loaded with icariin in the treatment of ulcerative colitis, Int. J. Pharm. 515 (2016) 176–185.
[29] L. Cai, R. Liqun, Replication of Animal Models for Human Diseases, People's Medical Publishing House Co. Ltd, Beijing, China, 2008.
[30] M. Murano, K. Maemura, I. Hirata, K. Toshina, T. Nishikawa, N. Hamamoto, S. Sasaki, O. Saitoh, K. Katsu, Therapeutic effect of intracolonically administered nuclear factor kappa B (p65) antisense oligonucleotide on mouse dextran sulphate sodium (DSS)-induced colitis, Clin. Exp. Immunol. 120 (2000) 51–58.
[31] T. Kuda, T. Hishi, S. Maekawa, Antioxidant properties of dried product of 'habanori', an edible brown alga, *Petalonia binghamiae* (J. Agaradh) Vinogradova, Food Chem. 98 (2006) 545–550.
[32] L.A. Dieleman, M.J. Palmen, H. Akol, E. Bloemena, A.S. Pena, S.G. Meuwissen, E.P. Van Rees, Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines, Clin. Exp. Immunol. 114 (1998) 385–391.
[33] J.J. Kim, M.S. Shajib, M.M. Manocha, W.I. Khan, Investigating intestinal inflammation in DSS-induced model of IBD, J. Vis. Exp. 60 (2012) e3678.
[34] G. Monteleone, M.F. Neurath, S. Ardizzone, A. Di Sabatino, M.C. Fantini, F. Castiglione, M.L. Scribano, A. Armuzzi, F. Caprioli, G.C. Sturniolo, F. Rogai, M. Vecchi, R. Atreya, F. Bossa, S. Onali, M. Fichera, G.R. Corazza, L. Biancone, V. Savarino, R. Pica, A. Orlando, F. Pallone, Mongersen, an Oral SMAD7 antisense oligonucleotide, and Crohn's disease, N. Engl. J. Med. 372 (2015) 1104–1113.
[35] M.Z. Zhang, E. Viennois, M. Prasad, Y.C. Zhang, L.X. Wang, Z. Zhang, M.K. Han, B. Xiao, C.L. Xu, S. Srinivasan, D. Merlin, Edible ginger-derived nanoparticles: a novel therapeutic approach for the prevention and treatment of inflammatory bowel disease and colitis-associated cancer, Biomaterials 101 (2016) 321–340.
[36] H. Liu, S. Shi, J. Cao, L. Ji, Y. He, J. Xi, Preparation and evaluation of a novel bioactive glass/lysozyme/PLGA composite microsphere, Drug Dev. Ind. Pharm. 41 (2014) 458–463.
[37] M.M. Wang, Y.W. Wang, E. Omari-Siaw, S.L. Wang, Y. Zhu, X.M. Xu, Reduced burst release and enhanced oral bioavailability in shikimic acid-loaded polylactic acid submicron particles by coaxial electrospray, J. Pharm. Sci. 105 (2016) 2427–2436.
[38] S. Agarwal, J.H. Wendorff, A. Greiner, Use of electrospinning technique for biomedical applications, Polymer 49 (2008) 5603–5621.
[39] M.J. Cardoso, R.R. Costa, J.F. Mano, Marine origin polysaccharides in drug delivery systems, Marine Drugs 14 (2016) 34–60.
[40] B.R. Lauwerys, F.A. Houssiau, Involvement of cytokines in the pathogenesis of systemic lupus erythematosus, Cytok. Chemok. Autoimm. Dis. 520 (2003) 237–251.
[41] E. Tackey, P.E. Lipsky, G.G. Illei, Rationale for interleukin-6 blockade in systemic lupus erythematosus, Lupus 13 (2004) 339–343.