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Note

Novel preparation of enteric-coated chitosan-prednisolone conjugate microspheres and in vitro evaluation of their potential as a colonic delivery system

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Abstract

After chitosan-succinyl-prednisolone conjugate (Ch-SP) was synthesized, conjugate microspheres (Ch-SP-MS), Eudragit L100-coated Ch-SP-MS and Eudragit S100-coated Ch-SP-MS, were prepared under novel preparative conditions. Namely, sonication was utilized to prepare finer Ch-SP-MS, and the addition ratio of Eudragit was reduced to yield Eudragit-coated Ch-SP-MS with higher drug content. Ch-SP-MS and Eudragit-coated Ch-SP-MS had mean sizes of 1.3 μm and approximately 30 μm, respectively, and showed prednisolone (PD) contents of 4.6% (w/w) and approximately 3% (w/w), respectively. Morphological changes of all the types of microparticles in different pH media were observed by scanning electron microscopy and confocal laser scanning microscopy. Both methods gave similar results. Both types of Eudragit-coated Ch-SP-MS protected Ch-SP-MS from morphological change at pH 1.2, and regenerated Ch-SP-MS fast at pH 6.8 and 7.4. For all types of microparticles, release of PD was suppressed at pH 1.2, but caused gradually at pH 6.8. These particle characteristics and in vitro behaviors demonstrated that the present Eudragit-coated Ch-SP-MS were considered potentially suitable for in vivo or practical application as a specific delivery system of PD to IBD sites.

Keywords: Chitosan-prednisolone conjugate microspheres; Eudragit-coated microparticles; Particle characteristics; Morphological change; Drug release; Specific delivery

1. Introduction

In the treatment of inflammatory bowel disease (IBD), 5-aminosalicylic acid (5-ASA) and steroidal or non-steroidal anti-inflammatory drugs are frequently administered orally to the patients [1]. Administration of these drugs at a large and frequent dose for a long period causes significant and prolonged absorption of the drugs from the small intestine, often leading to toxic side effects [2]. Therefore, the specific delivery of drugs to diseased parts has been developed. For example, salazosulfapyridine, a prodrug of 5-ASA, [3] and Pentasa, acting as a delayed release system of 5-ASA, [4] are clinically available; however, they are not necessarily satisfactory, and more improved systems are expected.

Recently, a chitosan capsule containing 5-ASA was demonstrated to display an excellent effect against 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats [5]. Also, micro- or nano-particulate dosage forms have been found to be effective to deliver drugs to the intestine, Peyer's patches or colon [6–9]. Small particles can penetrate the mucus layer more deeply, and reach the diseased sites well [7]. In these systems, biocompatible and biodegradable polymers are often used [5,8,9], and their degradation by enzymes of bacteria and macrophages in the diseased region can accelerate drug release.

Chitosan is a biocompatible and biodegradable polymer, and is considered to be useful as a material for oral drug delivery systems due to its safety [10]. In order to

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produce microparticulate system with the ability to show the gradual drug release and efficient delivery, we synthesized a chitosan-succinyl-prednisolone conjugate (Ch-SP) as a macromolecular prodrug of PD, and prepared the microspheres (Ch-SP-MS) using Ch-SP [11,12]. Although Eudragit coating of Ch-SP-MS was attempted previously [12], the size of Ch-SP-MS and the drug content of Eudragit-coated Ch-SP-MS were not necessarily satisfactory. In this study, novel preparation conditions were examined to obtain refined Ch-SP-MS and their Eudragit L100- or S100-coated Ch-SP-MS. All the microparticles were evaluated based on particle size, morphology and drug release.

2. Materials and methods

2.1. Materials

Prednisolone (PD) and prednisolone 21-hemisuccinate (SP) sodium salt (SP-Na) were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Chitosan (Ch) (viscosity grade = 1000 (5 g/l, 20 °C), deacetylation degree = 80% (mol/mol)), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and fluorescein isothiocyanate (FITC) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Eudragit L100 and Eudragit S100 were obtained from Rohm GmbH Chemische Fabrik (Darmstadt, Germany). Sorbitan sesquioleate (SO-15) was purchased from Nikko Chemicals Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

2.2. Preparation of chitosan microspheres

Ch-SP conjugate microspheres (Ch-SP-MS) and FITC-labeled chitosan microspheres (FITC-Ch-MS) were prepared. Ch-SP conjugate (Ch-SP) was prepared as follows [11,12]. Ch (120 mg) was dissolved in water by adjusting the solvent pH to 3 with a 1 M HCl aqueous solution. SP-Na (40 mg) was dissolved in 5 ml of water, and added to the Ch solution. The pH of the mixture was adjusted to pH 5.5 with a 1 M NaOH aqueous solution. EDCI (200 mg) dissolved in 5 ml of water was added to the solution containing Ch and SP. The reaction mixture was stirred at 900 rpm under ice cooling for the first 5 h and at room temperature for 19 h. Further, 5 ml of water containing 200 mg EDCI was added, and the mixture was stirred at 900 rpm at room temperature for another 24 h. The reaction mixture was added to a fourfold volume of acetone to precipitate Ch-SP. Ch-SP was washed with a mixture of acetone and water (4:1, v/v), suspended in 60 ml of water, and lyophilized to obtain the Ch-SP powder.

FITC-labeled chitosan (FITC-Ch) was prepared in the following manner. Ch (500 mg) was dissolved in water by adjusting the pH to 3 with a 1 M HCl aqueous solution. After the pH was adjusted to 6.5 with a 1 M NaOH aqueous solution, FITC (15 mg) was added. The resultant mixture was stirred for 24 h in the dark, and the pH was adjusted to 9 with a 1 M NaOH aqueous solution. The precipitate was collected after centrifugation at 3000 rpm for 5 min, washed by repeating dissolution at pH 3 (HCl aqueous solution) and subsequent precipitation at pH 9 (NaOH aqueous solution), dispersed in water, and lyophilized to obtain FITC-Ch powder.

Ch-SP-MS were prepared by emulsification and subsequent evaporation using Ch-SP. Ch-SP (50 mg) was dissolved in a 1% (v/v) acetic acid aqueous solution, and emulsified in 150 ml of liquid paraffin containing 1% (w/v) SO-15 at 1200 rpm at 70 °C. Stirring was continued at 70 °C for 20 min, and at 80 °C for 10 min. Then, the emulsion was sonicated at 80 °C under 28 kHz (100 W) for 10 min using an ultrasonicator VS-100III SUNPAR (IUCHISEIEIDO, Japan). The resultant mixture was stirred at 400 rpm at 100 °C for 1 h. After the mixture was cooled to room temperature, the same volume of *n*-hexane was added, and the mixture was centrifuged at 3000 rpm for 5 min to precipitate the product. The product was washed with *n*-hexane, and dried in a desiccator to obtain Ch-SP-MS. FITC-Ch-MS were prepared in the same manner as Ch-SP-MS, except that FITC-Ch was used instead of Ch-SP.

2.3. Eudragit coating of chitosan microspheres

Eudragit L100 or S100 (130 mg) was dissolved in 1 ml of methanol, and Ch-SP-MS (130 mg) was added. The methanol suspension was dropped in 50 ml of liquid paraffin containing 2% (w/v) SO-15, which was stirred at 600 rpm at 40 °C. Stirring at 40 °C was continued under reduced pressure to evaporate methanol completely. Then, the mixture was cooled to room temperature, and the same volume of *n*-hexane was added, and the resultant mixture was centrifuged at 3000 rpm for 5 min to precipitate the product. The product was washed with *n*-hexane, and dried in a desiccator to obtain Eudragit L100-coated Ch-SP-MS (Ch-SP-MS/EuL) or Eudragit S100-coated Ch-SP-MS (Ch-SP-MS/EuS). Eudragit L100-coated FITC-Ch-MS (FITC-Ch-MS/EuL) or Eudragit S100-coated FITC-Ch-MS (FITC-Ch-MS/EuS) were prepared in the same manner except that FITC-Ch-MS were used instead of Ch-SP-MS.

2.4. High performance liquid chromatography (HPLC)

HPLC assay was used for the determination of PD in the sample solution. The HPLC system consisted of an LC-10AS pump, an SPD-10A spectrophotometric detector, a C-R7 chromatopac, an SCL-10A system controller, an SIL-10A autosampler and a CTO-10A column oven (Shimadzu Corp., Japan). The detector was set at 246 nm, and the column oven was set at 30 °C. A Spelcosil LC-18-DB column (4.6 mm in inner diameter × 150 mm in length, particle size 3 μm; SUPELCO, USA) was used as an analytical column. A 22% (w/v) 2-propanol aqueous solution containing 0.1% (w/v) trifluoroacetic acid was used as the mobile phase, and the flow rate was 1.0 ml/min. The absolute calibration curve method was applied to quantification analysis.

2.5. Measurement of particle size and drug content

The particle size and its distribution were investigated using scanning electron microscopy (SEM) photomicrographs. The particle size and size distribution were determined by measuring the Green diameters of 160 microparticles chosen at random.

The drug contents of Ch-SP, Ch-SP-MS, Ch-SP-MS/EuL and Ch-SP-MS/EuS were analyzed by referring to the previous study [11,12]. Briefly, the sample (2 mg) was added to a 0.1 M NaOH aqueous solution (10 ml), incubated for 10 min at 45 °C and centrifuged at 3000 rpm for 5 min. The UV absorbance of the supernatant was measured at 246 nm using a Beckman DU 640 spectrophotometer to determine the amount of PD. The obtained amount was used for calculation of the PD content.

2.6. Morphological analysis of microparticles in different pH media

SEM and confocal laser scanning microscopy (CLSM) were used as techniques for the morphological analyses. In SEM observations, powder samples were thinly coated with platinum using a JEOL JFC-1600 Auto Fine Coater and observed using a JEOL JSM-5600LV scanning electron microscope, and micrographs were taken. For CLSM analysis, powder or aqueous suspension was observed using a BIO-RAD Radiance 2100 confocal laser scanning microscope.

Morphological changes were examined as follows. The microparticles were added to 5 ml of Japanese Pharmacopoeia (JP) 14 1st fluid (HCl aqueous solution containing 0.2% (w/v) NaCl, pH 1.2), JP14 2nd fluid (phosphate buffer containing 0.05 M KH_2PO_4, pH adjusted to 6.8 with NaOH) or phosphate-buffered saline (pH 7.4) (PBS) at a concentration of 50 μg PD eq./ml. Each mixture was incubated by horizontal shaking at 100 rpm at 37 °C. At 0.25, 1.5 and 4 h after the start of incubation, a certain amount of the suspension was withdrawn, and centrifuged at 3000 rpm for 5 min. The precipitate was washed three times with water, dried in a desiccator, and observed by SEM. The particle size was measured for 160 microparticles chosen at random from SEM photomicrographs. At the same time, the particle surface, shape and status were observed.

In addition, FITC-Ch-MS, FITC-Ch-MS/EuL and FITC-SP-MS/EuS were examined for their morphological change under the same conditions. Sampling was performed with the same timing, and the sampled suspensions were observed by CLSM. The particle size, shape and status were investigated.

2.7. In vitro drug release experiments

Microparticles were put into 5 ml of JP14 1st fluid (pH 1.2) or JP14 2nd fluid (pH 6.8) at a concentration of 50 μg PD eq./ml. Each mixture was incubated by horizontal shaking at 100 rpm in a water bath warmed to 37 °C. At appropriate time points, the mixture was centrifuged at 3000 rpm for 5 min, and the aliquot sample (100 μl) was withdrawn from the supernatant, and stored in a freezer at −20 °C until analysis. After each sampling, the incubation mixture was gently stirred, and the incubation was continued. The incubation time was defined as the time for the mixture to be immersed in a water bath at 37 °C. Each sample was diluted to a threefold volume by addition of the HPLC mobile phase, and analyzed for PD by HPLC to determine the amount of released PD.

3. Results and Discussion

3.1. Particle characteristics

Ch-SP was synthesized by EDCI coupling, which is a reagent for amide formation. The structural features of Ch-SP were shown previously [11,12]; that is, the ^1H NMR spectra of Ch-SP in D_2O/D_3PO_4 showed that the conjugate was composed of Ch and SP, and HPLC analysis demonstrated that only PD was released from Ch-SP in aqueous media of neutral pH. These results supported that Ch-SP was formed via an amide bond between the amino groups of Ch and the carboxy groups of SP. The present Ch-SP showed a drug (PD) content of approximately 8% (w/w). The binding efficiency, given by (observed drug content)/(theoretical drug content), was fairly high, approximately 40%.

Conjugate microspheres (Ch-SP-MS) were prepared by emulsification and solvent evaporation using Ch-SP aqueous solution. Since Ch-SP-MS exhibited no burst release unless they were treated with a strong alkaline aqueous solution, Ch-SP-MS were considered to scarcely contain free PD. The particle size and drug content of the microparticles are described in Table 1. Ch-SP-MS had an almost spherical shape, and Ch-SP-MS/EuL and Ch-SP-MS/EuS showed a spherical or ellipsoidal shape with a rough surface. The mean particle size of Ch-SP-MS was 1.3 μm, and the size was distributed in the range of 0.7 – 2.9 μm. This size was smaller than that of previous microspheres [11,12], which was considered to be mainly due to sonication. The present Ch-SP-MS was considered more suitable for mucosal retention and penetration because of the reduction of the particle size [7]. Ch-SP-MS/EuL showed approximately 24 times the mean size of Ch-SP-MS, and Ch-SP-MS/EuS were approximately 22 times the mean size of Ch-SP-MS. Eudragit-coated Ch-SP-MS, the size of which ranged from 10 to 60 μm, were bigger than Ch-SP-MS (less than 3 μm), suggesting that the coating was well completed under the present conditions.

The content of Ch-SP-MS was 4.6% (w/w), indicating that PD was lost in the preparation of microspheres. As Ch-SP-MS were prepared at high temperature, the release and decomposition of the drug might occur in the process; however, this drug content was considered acceptable because a high dose is not required for PD treatment. The drug content was higher than that reported previously

Table 1
Particle characteristics of Ch-SP-MS, Ch-SP-MS/EuL and Ch-SP-MS/EuS

Microparticulate formulation	Particle diameter[a] (μm)	Drug content[b] (%, w/w)
Ch-SP-MS	1.30 ± 0.41	4.60 ± 0.74
Ch-SP-MS/EuL	31.78 ± 11.11	3.22 ± 0.68
Ch-SP-MS/EuS	28.39 ± 9.86	3.09 ± 0.60

[a] Results are expressed as means ± SD ($n = 160$).
[b] Results are expressed as means ± SD ($n = 3$).

[11,12], though the drug content has to be determined for each lot because it varies to some extent. As to Eudragit-coated Ch-SP-MS, the drug content was reduced due to the addition of Eudragit. However, the drug content (approximately 3%) was much greater than that reported previously, and considered acceptable due to the same reason as in Ch-SP-MS.

3.2. Morphological features and size change in different pH media

For morphological analysis of the microparticles incubated in different pH media, SEM was applied for washed samples. As stated below, the particle features observed by SEM agreed with those observed by CLSM, which supported that SEM analysis was available for the present morphological change studies. Moreover, although the particle size and size distribution of Ch-SP-MS in JP14 2nd fluid were analyzed by dynamic light scattering, particle aggregation was observed (data not shown), which was made clear by CLSM. This was another reason that SEM was used for morphological analyses.

SEM photomicrographs of the microparticles after incubation for 15 min and 4 h are shown in Fig. 1. In JP14 1st fluid (pH 1.2), Ch-SP-MS swelled quickly, and their aggregation and dissolution increased gradually. In JP14 2nd fluid (pH 6.8) and PBS (pH 7.4), Ch-SP-MS swelled to some extent, but the spherical shape was almost maintained. Both Ch-SP-MS/EuL and Ch-SP-MS/EuS almost kept their original shape and size at pH 1.2, though more pores and cracks were found as compared with their initial states. At pH 6.8 and 7.4, the size of Eudragit-coated Ch-SP-MS decreased fast to several μm due to dissolution of the Eudragit.

Time courses of the size of microparticles in different pH media are shown in Fig. 2. At pH 1.2, the size of Ch-SP-MS increased gradually because of the aggregation of swelled particles. The mean particle size became 49 μm at 4 h, and size distribution was very large. The status of the particles was made clearer from the observation of FITC-Ch-MS by CLSM, which gave the intact status of the microspheres because suspended microspheres were observed directly with no treatment. FITC-Ch-MS before incubation showed morphological features similar to Ch-SP-MS (Fig. 3a). FITC-Ch-MS swelled and aggregated extensively after the incubation at pH 1.2 for 4 h (Fig. 3b), but several micrometers were maintained at pH 6.8 and 7.4 (Fig. 3c and d). These results were consistent with the results in Figs. 1 and 2. Eudragit-coated Ch-SP-MS retained their particle size at pH 1.2. This was confirmed by CLSM of Eudragit-coated FITC-Ch-MS (Fig. 4a and b). Ch-SP-MS/EuL and Ch-SP-MS/EuS decreased in size with time at pH 6.8 and 7.4 (Figs. 2b, c and 4c, d). The mean sizes of Ch-SP-MS/EuL and Ch-SP-MS/EuS were 7 and 5 μm at 15 min, respectively, and their mean sizes became a few μm at 4 h.

Thus, the size and shape of Ch-SP-MS changed markedly at pH 1.2, which was considered due to the dissolution properties of Ch [13], suggesting that Ch-SP-MS is vulnerable to morphological change in the stomach. Therefore, enteric coating was needed to maintain the morphology of Ch-SP-MS at gastric pH. Ch-SP-MS/EuL and Ch-SP-MS/EuS kept their initial morphology well at pH 1.2, while

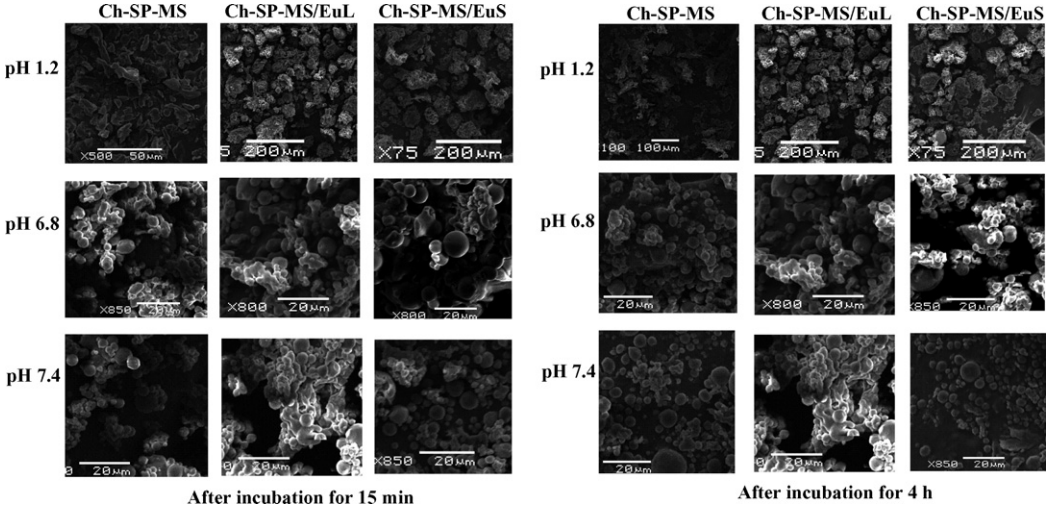

Fig. 1. SEM micrographs of Ch-SP-MS, Ch-SP-MS/EuL and Ch-SP-MS/EuS after incubation in JP14 1st fluid (pH 1.2), JP14 2nd fluid (pH 6.8) and PBS (pH 7.4) at 37 °C for 15 min and 4 h. Scale is shown with a white bar in each micrograph.

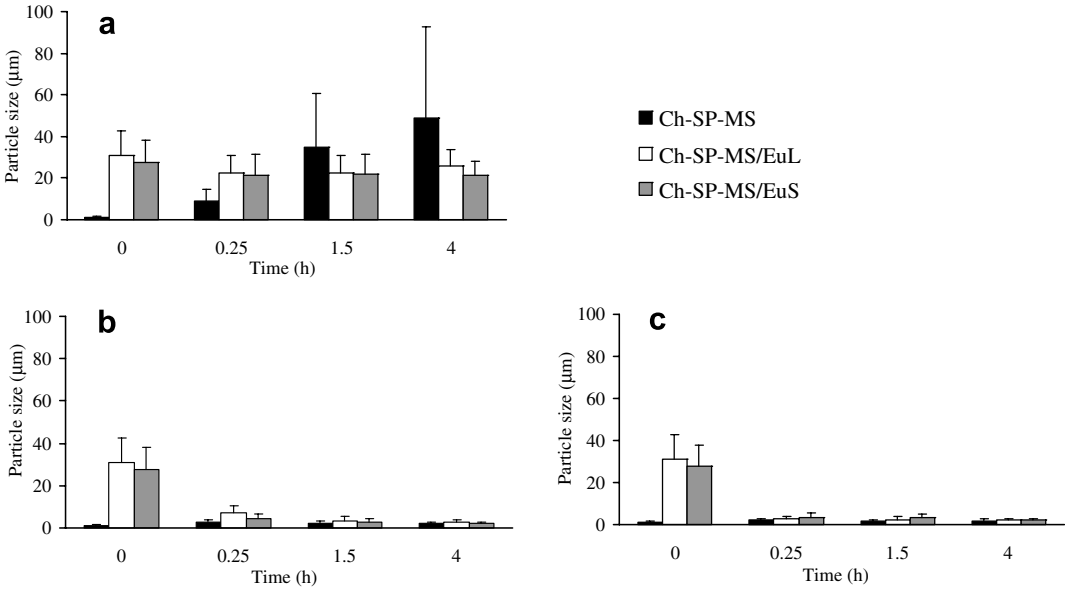

Fig. 2. Change in particle size of Ch-SP-MS, Ch-SP-MS/EuL and Ch-SP-MS/EuS during incubation in different pH media at 37 °C. (a) JP14 1st fluid (pH 1.2); (b) JP14 2nd fluid (pH 6.8); (c) PBS (pH 7.4). The results are expressed as means ± SD ($n = 160$).

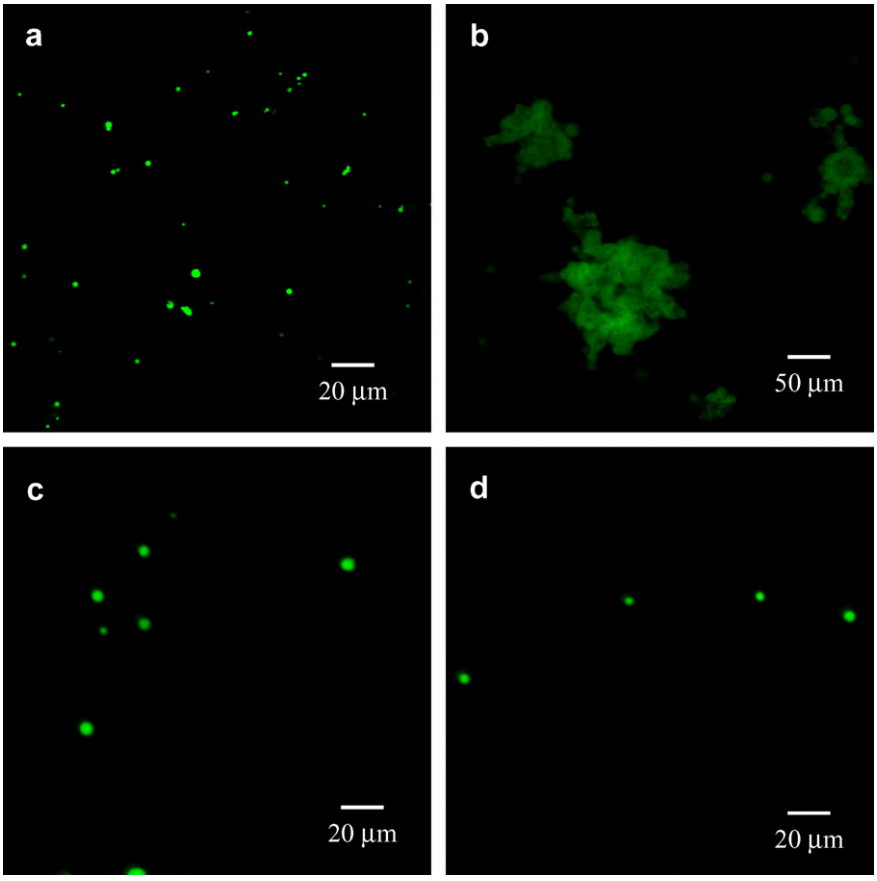

Fig. 3. CLSM micrographs of FITC-Ch-MS after incubation in different pH media at 37 °C for 4 h. (a) Before incubation; (b) JP14 1st fluid (pH 1.2); (c) JP14 2nd fluid (pH 6.8); (d) PBS (pH 7.4). Scale is shown with a white bar in each micrograph.

their particle size decreased quickly to several μm at pH 6.8 and 7.4. Although Eudragit L100 and S100 are said to be dissolved around pH 6 and upwards, and around pH 7 and upwards, respectively [14], Ch-SP-MS/EuS were similar in dissolution behavior to Ch-SP-MS/EuL at pH 6.8 and 7.4. One reason was probably due to the thin coating

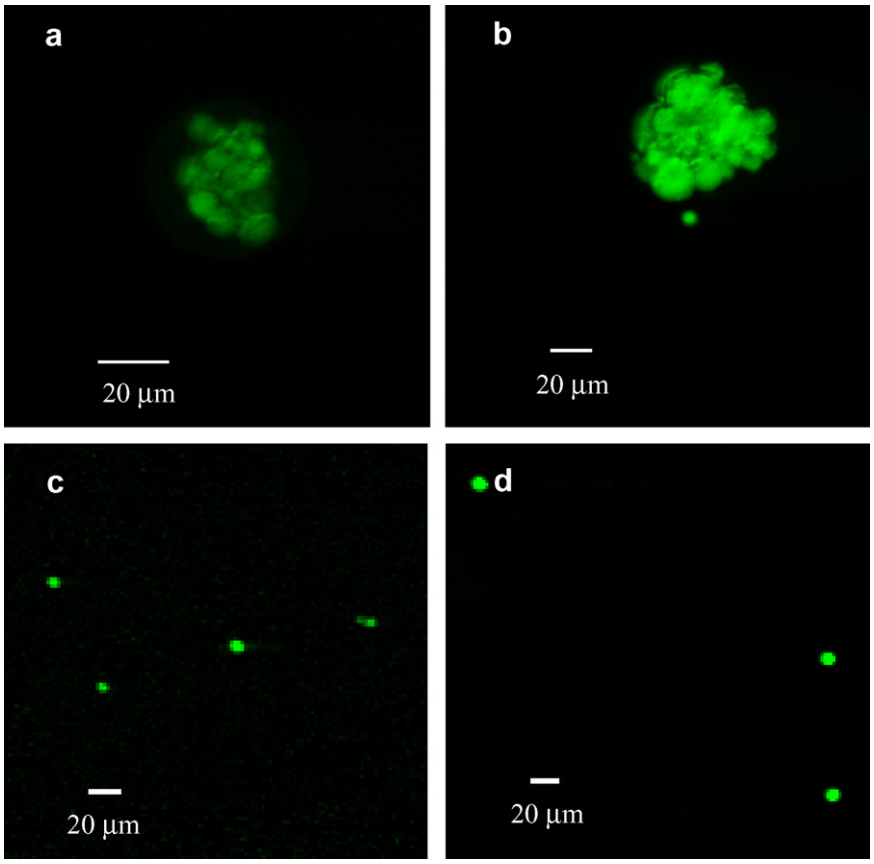

Fig. 4. CLSM micrographs of Eudragit-coated FITC-Ch-MS after incubation in different pH media at 37 °C for 4 h. (a) FITC-Ch-MS/EuL in JP14 1st fluid (pH 1.2); (b) FITC-Ch-MS/EuS in JP14 1st fluid (pH 1.2); (c) FITC-Ch-MS/EuL in JP14 2nd fluid (pH 6.8); (d) FITC-Ch-MS/EuS in PBS (pH 7.4). Scale is shown with a white bar in each micrograph.

layer. Actually, as reported previously [12], Eudragit L100-coated Ch-SP-MS with a greater content of Eudragit L100 displayed slower dissolution of the coating layer. In addition, as Ch is a polymer with many amino groups, it may influence the dissolution behavior of the surrounding coating polymer. Thus, both Ch-SP-MS/EuL and Ch-SP-MS/EuS exhibited similar morphological changes at both gastric and intestinal pH, suggesting they should be available to efficiently deliver Ch-SP-MS with a few μm to the intestine.

3.3. In vitro release

The drug release profiles are shown in Fig. 5. At pH 1.2, PD was released very slowly for all types of microparticles. At 6 h, Ch-SP-MS, Ch-SP-MS/EuL and Ch-SP-MS/EuS exhibited PD release of 3.8%, 1.4% and 1.0%, respectively. At pH 6.8, Ch-SP-MS displayed a gradual and almost linear drug release, and approximately 30% of the drug was released at 6 h; however, Ch-SP-MS/EuL and Ch-SP-MS/EuS displayed the suppressed drug release during the initial 3 h. At 3 h, the cumulative release was approximately one-third that of Ch-SP-MS. After the initial 3 h, the release rate increased up to that of Ch-SP-MS. Ch-SP-MS/EuL and Ch-SP-MS/EuS showed PD release of nearly 20% at 6 h. Both types of Eudragit-coated Ch-SP-MS exhibited similar drug release profiles.

Controlled drug release is important to deliver drugs efficiently to the diseased area. As reported previously [11,12], hydrolysis of the ester bond of the SP moiety appeared to be a major rate-limiting process in drug release, because Ch-SP-MS showed similar drug release behavior to Ch-SP. Namely, as Ch-SP-MS swelled quickly in aqueous media, the ester was considered to be rapidly exposed to media and to quickly respond to the medium conditions. The release profiles from Eudragit-coated Ch-SP-MS were favorable for efficient delivery of PD to the lower intestine. At pH 1.2, the coating layer appeared to prevent diffusion of the solvent and contained molecules. At pH 6.8, the coating layer appeared to suppress solvent diffusion and drug release until it was dissolved to a considerable extent. The release profiles from both types of Eudragit-coated Ch-SP-MS were considered suitable for the specific delivery of PD to IBD sites because the release was expected to be suppressed in the stomach and upper intestine, and promoted in the lower intestine.

In conclusion, the present Ch-SP-MS and Eudragit-coated Ch-SP-MS showed the particle characteristics available for in vivo or practical application. Namely, Eudragit

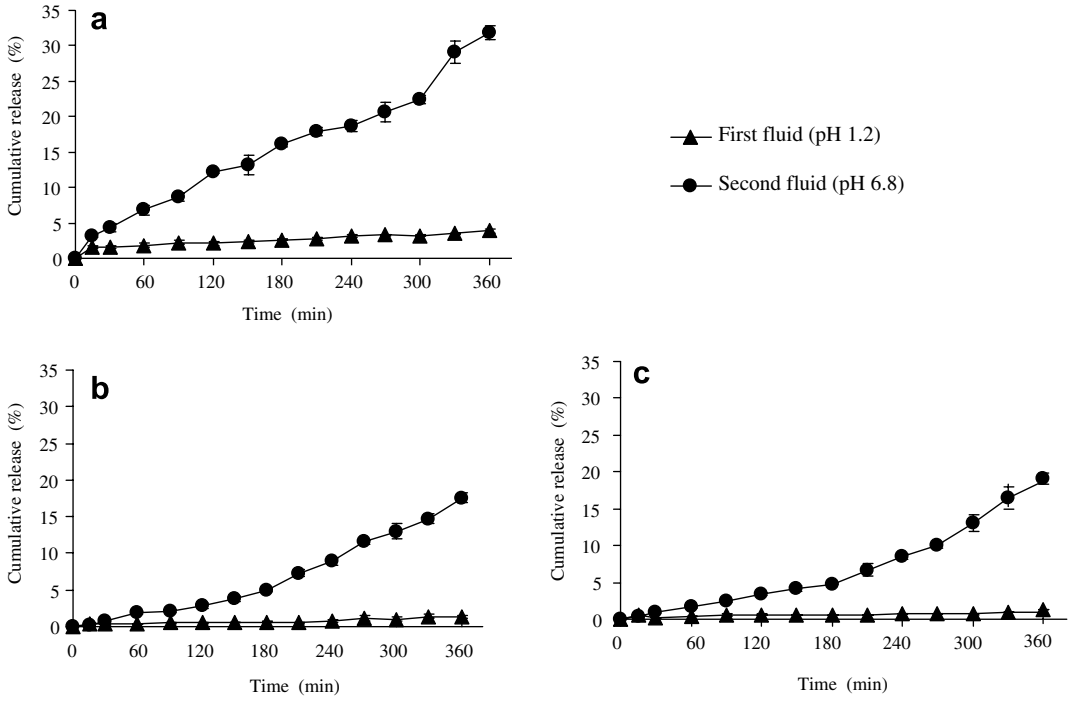

Fig. 5. Release of PD from microparticles during incubation in JP14 1st fluid (pH 1.2) and JP14 2nd fluid (pH 6.8) at 37 °C. (a) Ch-SP-MS; (b) Ch-SP-MS/EuL; (c) Ch-SP-MS/EuS. The results are expressed as means ± SD ($n = 3$).

coating could protect Ch-SP-MS morphology at gastric pH of 1.2 and allow almost complete regeneration of Ch-SP-MS at intestinal pH of 6.8 a few h after exposure at this pH. Drug release was also suppressed at gastric pH and raised at intestinal pH. The present Eudragit-coated Ch-SP-MS were suggested to be potentially useful as a specific delivery system of PD to IBD sites.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2007.06.016.

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