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Preparation and In Vitro Evaluation of Chitosan Microspheres Containing Prednisolone: Comparison of Simple and Conjugate Microspheres

Hiraku Onishi, Tomoko Oosegi, and Yoshiharu Machida
Department of Drug Delivery Research, Hoshi University, Shinagawa-ku, Tokyo, Japan

James W. McGinity
Division of Pharmaceutics, College of Pharmacy, University of Texas at Austin, Austin, Texas, USA

ABSTRACT The purpose of the present study was to obtain a novel microparticulate formulation of prednisolone, which was adequate for the treatment of inflammatory bowel disease (IBD). The formulations prepared were evaluated in vitro. Two types of chitosan microspheres containing prednisolone, named Ch-Pred and Ch-SP-MS, were prepared by an emulsification-solvent evaporation method using a chitosan-prednisolone mixture and a chitosan-succinyl-prednisolone conjugate (Ch-SP), respectively. Ch-Pred and Ch-SP-MS were obtained in almost spherical shape. Ch-Pred showed a relatively high drug content of 13.2% (w/w), but the particle size was distributed from 10 to 45 μm and a large initial burst release of approximately 60% was observed. On the other hand, although Ch-SP-MS exhibited a fairly low drug content of 3.5% (w/w), their particle size ranged from several hundred nanometers to 20 μm, with the mean diameter of 5 μm, and a gradual drug release profile was achieved. These characteristics on particle size and in vitro release suggested that Ch-SP-MS should have good potential as a microparticulate system for the treatment of IBD.

KEYWORDS Chitosan microsphere, Conjugate, Prednisolone, Particle characteristics, Drug release

INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is often severe and chronic. Its etiology has not been clearly elucidated yet. The major cause of IBD is considered to include autoimmune disease and an imbalance of microorganisms (Kleessen et al., 2002; Pallone et al., 2003). Anti-inflammatory drugs such as 5-aminosalicylic acid (5-ASA) and steroidal and nonsteroidal anti-inflammatory drugs are frequently used for the

Address correspondence to Hiraku Onishi, Department of Drug Delivery Research, Hoshi University, 2-4-41, Ebara, Shinagawa-ku, Tokyo 142-8501, Japan; Fax: 0081-3-3787-0036; E-mail: onishi@hoshi.ac.jp

treatment of IBD (Brzezinski et al., 1995; Ekstrom, 1998). However, when these drugs are administered in simple conventional oral dosage forms, they are absorbed systemically to a large extent and not delivered efficiently to the diseased site in the gastrointestinal tract. The nonselectivity of the drugs means that a high dose is required, and consequently, drug distribution in the entire body often results in toxic side effects.

Therefore, various approaches have been examined in an attempt to achieve the efficient delivery of such drugs to the target site (Kinget et al., 1998; Watts & Illum, 1997). Small molecular and high molecular weight prodrugs have been developed in order to achieve colon targeting (Erdmann & Uhrich, 2000; Galvez et al., 2000; Klotz, 1985; Yano et al., 2002). For example, salazosulfapyridine, a prodrug used clinically, releases the active compound 5-ASA in the colon, which is caused by the colonic bacteria (Klotz, 1985). However, as sulfapyridine is regenerated from salazosulfapyridine, salazosulfapyridine can cause toxic side effects due to the intestinal absorption of sulfapyridine (Klotz, 1985). Other novel dosage forms such as controlled-release formulations, bacteria-degradable capsules, and biodegradable matrix systems have been investigated to achieve a more effective delivery to the colonic area (Larouche et al., 1995; Tozaki et al., 2002). A commercial tablet, Pentasa, is a delayed-release system and releases only 5-ASA around the colonic region, resulting in less toxicity than salazosulfapyridine (Larouche et al., 1995; Tozaki et al., 2002).

Recently, other approaches have been investigated to obtain more effective systems for the treatment of IBD. In particular, microparticulate systems containing biodegradable polymers have been examined actively. Microparticles with a diameter of less than 200 μm remain longer at the site of colitis due to a thicker mucous layer, and are required for the avoidance of elimination by diarrhea (Lamprecht et al., 2000a, 2000b, 2001a, 2001b, 2001c; Watts et al., 1992). Further, microparticles of several hundred nanometers to several micrometers are effectively taken up by macrophages appearing in large numbers (Nakase et al., 2000; Tabata & Ikada, 1988; Tabata et al., 1996). Biocompatible and biodegradable polymers are useful to deliver the drugs in the inflamed area, and may accelerate the drug release due to the enzymes of bacteria and macrophages. For example, chitosan, a polymer derived from a natural polymer, chitin, is highly safe when taken orally, and effectively undergoes biodegradation by bacteria (Tozaki et al., 2002). Actually, chitosan capsules are reported to be useful for delivery to diseased parts of the colon. Therefore, chitosan microparticles containing anti-inflammatory drugs are attractive as a new system for the treatment of IBD. Thus, in the present study, simple chitosan microspheres containing prednisolone were prepared by emulsification-solvent evaporation of a solution of chitosan-prednisolone mixture. Furthermore, chitosan microspheres loaded with prednisolone were also prepared by emulsification-solvent evaporation of a solution of a chitosan-succinyl-prednisolone conjugate. These two microspheres were compared and evaluated in vitro.

MATERIALS AND METHODS

Chemicals

Chitosan (Ch) (medium molecular weight grade: Brookfield viscosity=200,000 cps) and prednisolone 21-hemisuccinate (SP) sodium salt were purchased from Sigma-Aldrich Co. (St. Louis, MO). Prednisolone (Pred) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) were supplied by Spectrum Chemical Mfg. Co. (Gardena, CA). All other chemicals used were of reagent grade.

Preparation of Chitosan Microspheres

Simple Microspheres

Simple chitosan microspheres were prepared by an emulsification-solvent evaporation method, as follows. Ch (200 mg) was dissolved in a 2% (v/v) acetic acid aqueous solution (10 mL), then Pred (50 mg) was added to the solution. The mixture was stirred to give a uniform suspension. The mixture was put in liquid paraffin (150 mL) (80°C) containing 1% (w/v) Span 80, which was stirred at 500 rpm with a three-blade propeller stirrer. The emulsion was stirred at 80°C at 500 rpm for 1 h. Further, the emulsion was stirred at 95°C at 500 rpm for another 1 h to completely evaporate the aqueous phase. The microspheres

formed were washed three times with n-hexane and dried in air, and named Ch-Pred.

Conjugate Microspheres

Microspheres were produced using a chitosan-succinyl-prednisolone conjugate (Ch-SP), the proposed structure of which is shown in Fig. 1. First, Ch-SP was prepared as follows: Ch (125 mg) was dissolved in a hydrochloric acid aqueous solution by adjusting the pH to 5.5 (40 mL). SP sodium salt (40 mg) was dissolved and the final pH was adjusted to 5.5 by the addition of dilute hydrochloric acid, when the final volume was adjusted to 50 mL. EDCI (160 mg) was added to the mixture and vigorously stirred with a three-blade propeller stirrer under ice-cooling conditions. This mixture was stirred for 1.5 h with ice cooling and subsequently for 1 h at room temperature. Then, EDCI (160 mg) was added again, and stirring of the mixture at 500 rpm was continued at room temperature for 21.5 h. The product was precipitated by the addition of acetone (200 mL). The precipitate was washed with an 80% (v/v) acetone aqueous solution, and lyophilized after being suspended in water to obtain the Ch-SP powder. Next, Ch-SP microspheres (Ch-SP-MS) were prepared using Ch-SP in the following manner: Ch-SP (32 mg) was dissolved in 6 mL of a 1% (v/v) acetic acid aqueous solution. This solution was emulsified in liquid paraffin (100 mL) containing 1% (w/v) sorbitan sesquioleate (SO-15), which was stirred with a three-blade propeller at 95°C at 900 rpm. The stirring was continued under the same conditions for 1 h to evaporate the aqueous solvent. The microspheres were obtained by washing with n-hexane and drying in air.

Yields and Drug Content

The yield was determined as the weight of the final product. To determine the drug content of Ch-Pred, Ch-Pred was vigorously shaken in a 0.1 N hydrochloric acid aqueous solution at 0.2 mg Ch-Pred/mL. The mixture was incubated by circumvolution at 175 rpm at 37°C for 24 h. After the specified incubation periods, aliquots were taken and centrifuged to obtain a clear solution, and the supernatant was analyzed by UV absorption at 246 nm. Immediately after the measurement of each sample, the supernatant and precipitate were returned to the incubation medium. When the drug concentration in the supernatant reached a plateau, the drug content was calculated from that plateau level.

The drug content in conjugate Ch-SP was determined by alkaline hydrolysis and subsequent measurement of the regenerated Pred. That is, Ch-SP was put in a 0.1 N sodium hydroxide aqueous solution at 0.3 mg Ch-SP/mL. The suspension was kept at 43°C, and shaken gently every 5 min. After the specified incubation periods, aliquots were taken and centrifuged, and the supernatant was measured spectrophotometrically at 246 nm. Immediately after the measurement, the supernatant and precipitate were put back in the incubation medium. The plateau level of the regenerated Pred was calculated as the drug content. As to the

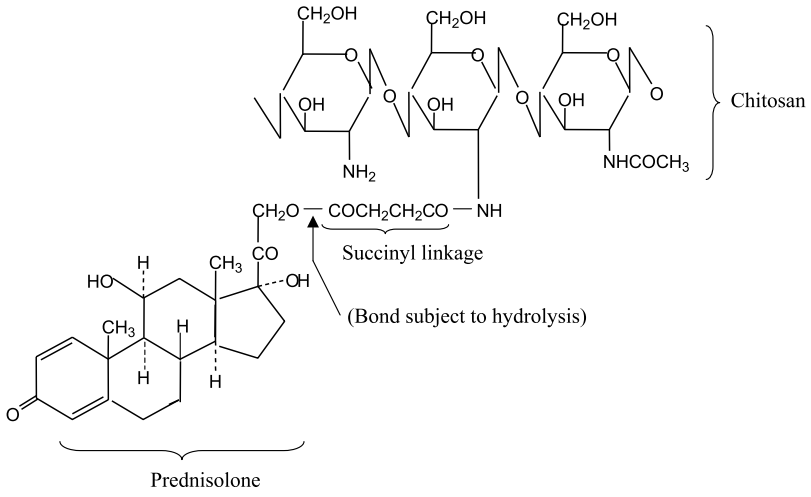

FIGURE 1 **Proposed Chemical Structure of Ch-SP.**

microspheres Ch-SP-MS, the Pred content was determined in the same manner as in Ch-SP.

Size, Shape, and Surface Structure of Microspheres

The particle diameter and shape of Ch-Pred and Ch-SP-MS were examined based on scanning electron microscopy (SEM) for each powder prepared. The powder samples were coated thinly with Pd-Au, and observed using a Hitachi S-4500 field emission scanning electron microscope. The particle size and its distribution were examined by measuring the Green diameters of 150 particles chosen at random. The particle shape and surface were analyzed using the same SEM micrographs. The size, shape, and surface features of the microspheres were also investigated following the dissolution studies. That is, the microspheres were precipitated by centrifugation after the drug release tests, and the supernatant was discarded. The microspheres precipitated were washed with water and dried in air before the SEM analysis. SEM was performed in the same manner as for the intact microspheres.

In Vitro Drug Release Studies

Ch-Pred and Ch-SP-MS were put in a 0.05 M phosphate buffered solution, pH 7.4, at 0.2 mg Ch-Pred/mL and 0.3 mg Ch-SP-MS/mL, respectively, and incubated by circumvolution at 175 rpm at 37°C. At appropriate time points, samples were taken and centrifuged, and the supernatant was analyzed spectrophotometrically at 246 nm. Immediately after each measurement, the supernatant and precipitate were put back in the incubated mixture. The percentage of drug released was calculated as a ratio to the mean drug content described above.

HPLC Assay

HPLC studies were performed to confirm what kinds of compounds were included in the samples and if the determination by the spectrophotometric method was valid. A Waters (Milford, MA) HPLC system connected to a Model 996 photodiode array detector was used under isocratic conditions with a 16% (w/v) 2-propanol aqueous solution containing 0.1% (w/v) trifluoroacetic acid as the mobile phase. A Capcell Pak C18 column (3 mm in inner diameter × 10 cm in length) (Shiseido Co., Ltd., Japan) was used as an analytical column, the temperature of which was kept at 30°C by the column oven. The mobile phase was eluted at 0.3 mL/min, and the sample was detected spectrophotometrically at 246 nm. The determination was performed by the absolute calibration curve method.

RESULTS AND DISCUSSION

Drug Content and Yields

Most of the Ch-Pred could be dissolved in a 0.1 N hydrochloric acid aqueous solution, but a small amount remained in the form of a semi-solid. However, the amount of drug recovered in the solution was the same for the samples incubated for 1.5 h and for 24 h (Fig. 2a). At 22 d after the sample had been kept at room temperature under acidic conditions, the solution was analyzed based on UV absorption and HPLC. Both the methods showed almost the same drug concentration, which was similar with methods, and similar to that calculated from the UV absorption for the sample incubated for 24 h (data

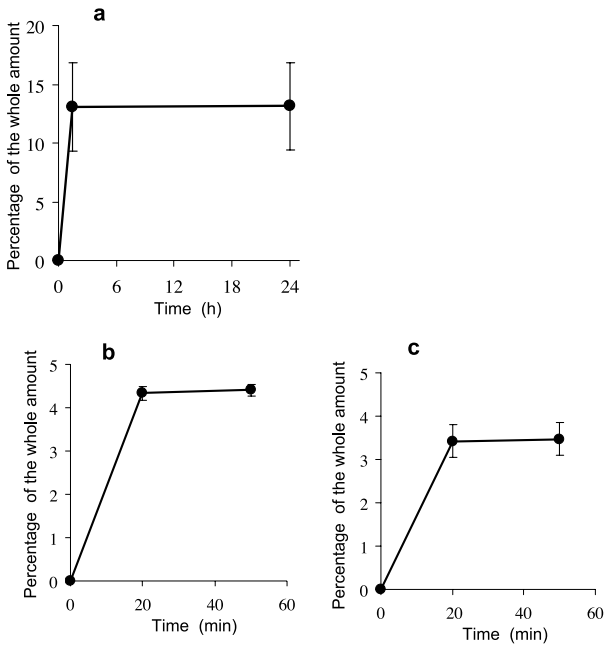

FIGURE 2 **Release of Pred from Ch-Pred in 0.1 N Hydrochloric Acid Aqueous Solution at 37°C (a), and Release of Pred from Ch-SP (b) and Ch-SP-MS (c) in 0.1 N Sodium Hydroxide Aqueous Solution at 43°C. Each Point Represents the Mean ± S.D. (n = 3).**

TABLE 1 Yields and Drug Contents of Ch-Pred, Ch-SP, and Ch-SP-MS

Product	Total yield[a] (%, w/w)	Drug content[b] (%, w/w)	Pred yield[c] (%, w/w)	Polymer yield[c] (%, w/w)	Incorporation efficiency (%)[d]
Ch-Pred	55	13.2±3.7	36	60	66
Ch-SP	67	4.4±0.1	16	83	—
Ch-SP-MS	63	3.5±0.4	50	64	80

[a]Obtained amount/total amount used.
[b]The results are expressed as the mean±S.D. (n=3).
[c]Calculated based on the mean drug content.
[d]Calculated as the ratio of mean observed content to mean ideal content.

not shown). These results indicate that Pred was stable under acidic conditions and the drug content in Ch-Pred could be determined from the UV absorption for the sample incubated for 24 h.

Ch-SP was obtained as a fluffy powder. The proposed chemical structure described in Fig. 1 is based on the reaction pathway by EDCI. Ch-SP was not dissolved completely in the acidic aqueous solution, and a small amount remained in a semi-solid state. Therefore, the drug content was determined by cleaving the ester bond with an alkaline solution. The NMR spectrum of the soluble part of the conjugate in the D_2O/D_3PO_4 solution, measured using VARIAN INOVA 500, showed Ch-SP to be a conjugate composed of chitosan and SP: Chitosan was found to have a deacetylation degree of approximately 80% (mol/mol), and the integrated intensity of 2′-H (3.1 ppm) of glucosamine units of chitosan was approximately 13 times greater than that of each of 1-H (7.5 ppm), 2-H (6.3 ppm), and 4-H (6.1 ppm) of prednisolone. A 0.1 N sodium hydroxide solution well permeated the powder. The drug regeneration profile is shown in Fig. 2b. The drug content was estimated from the plateau level of the UV absorption. The UV absorption at 246 nm of Pred in a 0.1 N sodium hydroxide solution changed little during incubation for 90 min, though Pred underwent degradation or deformation in these alkaline conditions (data not shown). Since only Pred was released from Ch-SP-MS in the release study at pH 7.4 as described below, the UV method could be used for the determination of drug content. Thus, the drug content was calculated from the plateau level of the drug regeneration profile obtained by UV absorption. The drug content of Ch-SP, calculated based on UV absorption, paralleled the value given by the NMR spectrum stated above. The drug content in Ch-SP-MS was determined in the same manner. The regeneration profile from Ch-SP-MS is described in Fig. 2c, and the drug content was calculated from the plateau level.

The total yield was calculated as a ratio of the obtained total (drug plus chitosan) amount to the initial total amount. The yields of polymer and drug were calculated based on the drug content and the subsequently calculated amount of drug contained. The incorporation efficiency was calculated as a ratio of the observed drug content to the theoretical drug content (Table 1).

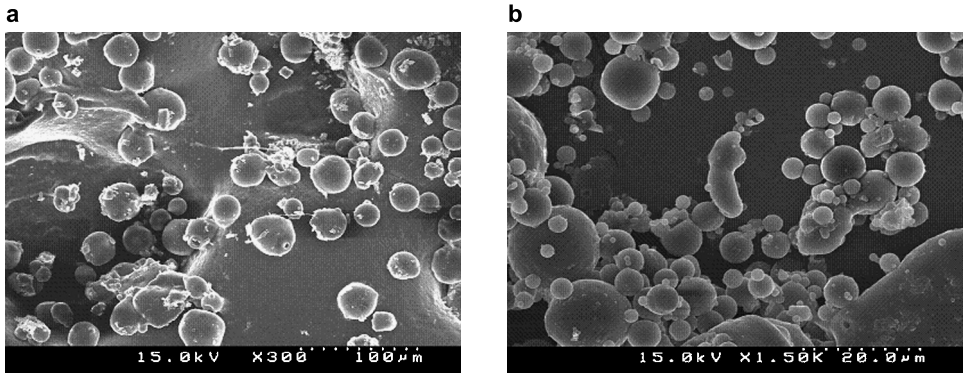

FIGURE 3 Scanning Electron Micrographs of Intact Ch-Pred (a) and Ch-SP-MS (b).

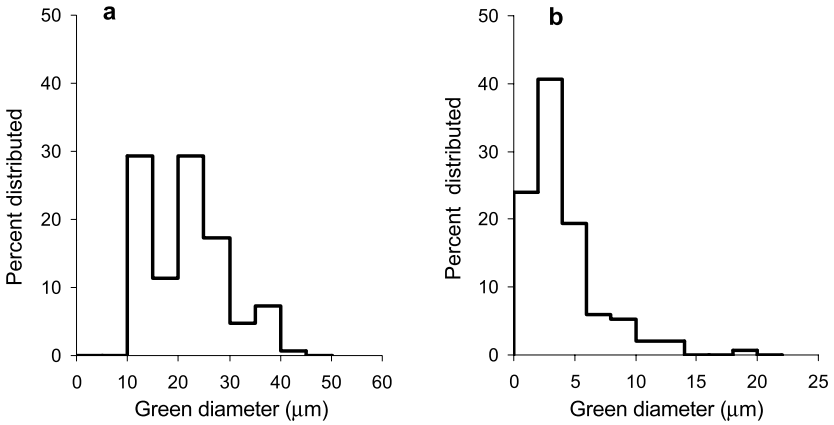

FIGURE 4 **Particle Size Distribution of Ch-Pred (a) and Ch-SP-MS (b): the Green Diameters of 150 Particles Chosen at Random were Measured for Both Microspheres.**

Particle Size and Shape

The intact Ch-Pred and Ch-SP-MS were analyzed by SEM. Their particle shape and surface structure are shown in Fig. 3. Ch-Pred showed an almost spherical shape, and the small crystal-like particles were observed on the surface of many particles (Fig. 3a). The mean particle size of Ch-Pred was 23 μm and the Green diameters were distributed from 10 to 45 μm (Fig. 4a). Ch-SP-MS also exhibited a spherical shape, but possessed a smooth surface (Fig. 3b). The mean particle diameter of Ch-SP-MS was 5 μm, and particle size was distributed from several hundred nanometers to 20 μm, with many particles having a diameter of less than 5 μm (Fig. 4b).

Microparticles of several hundred nanometers to several micrometers were reported to be taken up more easily by macrophages in IBD (Nakase et al., 2000; Tabata & Ikada, 1988; Tabata et al., 1996), indicating that Ch-SP-MS would be an appropriate delivery system for the treatment of IBD. Thus, Ch-SP-MS was suggested to be better than Ch-Pred from the viewpoint of particle size. SO-15 might be superior to Span 80 as a surfactant in the production of microspheres with respect to a reduction of particle size.

In Vitro Drug Release

The drug release was examined at pH 7.4 and 37°C. The results are shown in Fig. 5. Ch-Pred exhibited a large initial burst of approximately 60%, then released the drug slowly. On the other hand, Ch-SP-MS showed a gradual drug release, with 43% released after incubation for 24 h. When the compound released from Ch-SP-MS was checked by HPLC, it was identified as Pred, containing no SP. Namely, the amount of Pred released as determined by HPLC was similar to that determined by UV absorption (Fig. 6). Dextran-succinyl-methylprednisolone, having been examined as a soluble conjugate (McLeod et al., 1993, 1994; Mehvar et al., 2000),

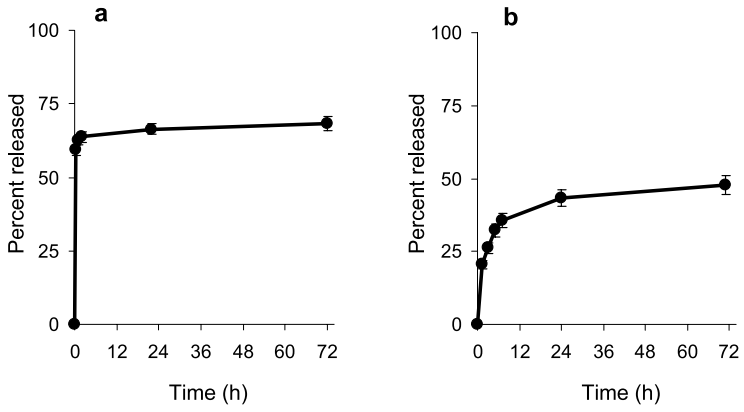

FIGURE 5 **Drug Release Profiles for Ch-Pred (a) and Ch-SP-MS (b) in 0.05 M Phosphate Buffer, pH 7.4, at 37°C: Each Point Represents the Mean ± S.D. (n = 3).**

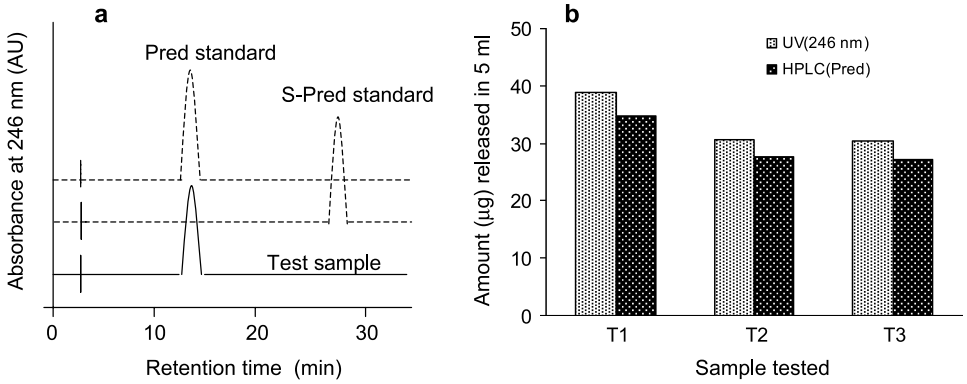

FIGURE 6 **HPLC Profile of the Final Sample in the Drug Release Test (a) and Amount of Pred Released Determined Based on UV Absorption and HPLC (b). The Final Sample was Derived from the Drug Release Experiment Shown in Fig. 5(b).**

releases methylprednisolone and succinyl-prednisolone simultaneously, because succinyl-methylprednisolone is combined with dextran via an ester bond. On the other hand, since SP was combined with Ch in Ch-SP-MS via an amide bond, the binding of the chitosan and succinyl linker was considered to very stable, resulting in the release of Pred alone. The release rate from 24 h after the start of the release test was considerably slow. This reason is unclear, but some reasons are proposed. For example, methylprednisolone-21-hemisuccinate, having a structure similar to that of SP, is subject to acyl migration between 17- and 21-hydroxyl groups, and the release of methylprednisolone from methylprednisolone-17-hemisuccinate is much slower than that from methylprednisolone-21-hemisuccinate (Anderson & Taphouse, 1981). If such acyl migration happens to some extent in the preparation of SP and Ch-SP-MS, it may cause slow release later in the release test. Hydrolysis of the ester bond or diffusion of the drug released seems to be suppressed in the latter period of the release test. Further detailed analysis will be needed to explain clearly the drug release profiles from Ch-SP-MS.

Figure 7 shows the particle shape and surface structure after the release tests. For both Ch-Pred and Ch-SP-MS, the spherical shape was mostly preserved, but the surface was eroded to a slight extent. The small crystal-like particles on the surface of Ch-Pred disappeared, suggesting their dissolution might be associated with the rapid initial release. Adhesion of the particles was observed, particularly in Ch-SP-MS, suggesting that the surface polymers should be swollen and entangled.

Thus, Ch-Pred was considered inadequate for concentration and sustained supply of Pred in the diseased intestinal sites, because it had a particle diameter of more than several micrometers and exhibited large initial release. On the other hand, Ch-SP-MS had a mean diameter of 5 μm, and its particle size ranged from several hundred nanometers to 20 μm, which was appropriate for the treatment of

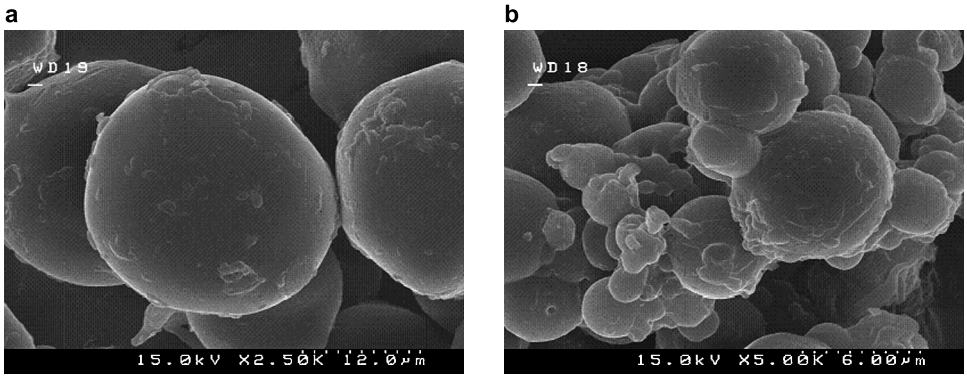

FIGURE 7 **Particle Shape and Surface Structure of Ch-Pred (a) and Ch-SP-MS (b) After the Release Experiment at pH 7.4 and 37°C. The Samples were Derived from the Drug Release Experiment shown in Fig. 5.**

IBD. Particles with such diameters are considered to be distributed more in the inflamed area and to be subject to uptake by macrophages appearing around the IBD region (Lamprecht et al., 2000a, 2000b, 2001a, 2001b, 2001c; Nakase et al., 2000; Tabata & Ikada, 1988; Tabata et al., 1996; Watts et al., 1992). Such small microspheres are thought to remain for a long time at the IBD site; reportedly, small microspheres were retained in the diseased area for at least a few days (Lamprecht et al., 2000b). Further, the gradual drug release from Ch-SP-MS was also suggested to be appropriate for a sustained supply of Pred at the diseased site. The increase in drug content was considered to be achieved by raising the substitution degree of SP in the conjugate Ch-SP. However, the substitution degree of SP in Ch-SP appears to affect the solubility of Ch-SP because Pred is less water-soluble. In fact, Ch-SP obtained in the present study included to some extent a portion that was less soluble in acid aqueous solution. This point will have to be examined in more detail. In addition, since chitosan microparticles are mucoadhesive (Shimoda et al., 2001; Takishima et al., 2002), such particles have to be protected from mucosal membrane until they reach the diseased part. The protection is considered to be achieved by coating or encapsulation with enteric polymers; actually, we have already found such protection can be achieved in vitro (data not shown). By these techniques, the delivery system for the treatment of IBD using Ch-SP-MS will be completed as an orally available dosage form.

CONCLUSION

Two types of prednisolone-loaded chitosan microspheres, Ch-Pred and Ch-SP-MS, were obtained by emulsification-solvent evaporation using a simple chitosan-prednisolone mixture and a chitosan-succinyl-prednisolone conjugate (Ch-SP), respectively. Ch-Pred showed a relatively high drug content, but the particle size was distributed from 10 to 45 μm, and a large initial burst release of approximately 60% was observed. Therefore, they were considered inadequate for prolonged retention and sustained drug release in the diseased intestinal sites. Chitosan-succinyl-prednisolone conjugate (Ch-SP) with a moderate drug content was obtained by water-soluble carbodiimide condensation. Ch-SP microspheres (Ch-SP-MS), prepared by w/o emulsification and subsequent evaporation of the aqueous phase, had a moderate drug content and particle size appropriate for prolonged retention in the diseased sites (mean size=5 μm). Further, Ch-SP-MS showed sustained drug release. Thus, it is suggested that Ch-SP-MS should be a useful delivery system for the treatment of IBD.

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