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Research paper

pH-Sensitive nanospheres for colon-specific drug delivery in experimentally induced colitis rat model

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ABSTRACT

Novel pH-sensitive nanospheres designed for colon-specific delivery were prepared using polymeric mixtures of poly (lactic-co-glycolic) acid (PLGA) and a pH-sensitive methacrylate copolymer. Budesonide (BSD), a topically active corticosteroid, was entrapped as a model drug. The therapeutic efficacy of the prepared nanospheres was assessed using the trinitrobenzenesulfonic acid (TNBS) colitis rat model, in comparison with conventional enteric microparticles. In addition, the colon targeting properties, systemic bioavailability, and specific uptake by the inflamed colon mucosa were evaluated using coumarin-6 (C-6)-loaded nanospheres. The prepared nanospheres showed strongly pH-dependent drug release properties in acidic and neutral pH values followed by a sustained release phase at pH 7.4. Animal experiments revealed the superior therapeutic efficiency of BSD-loaded nanospheres in alleviating the conditions of TNBS-induced colitis model. The in vivo studies using C-6-loaded nanospheres displayed higher colon levels and lower systemic availability of the fluorescent marker when compared with simple enteric coating. Moreover, quantitative analysis of the fluorescent marker and confocal laser scanning studies showed strong and specific adhesion of the nanospheres to the ulcerated and inflamed mucosal tissue of the rat colon. In conclusion, the proposed nanosphere system combined the properties of pH-sensitivity, controlled release, and particulate targeting that could be useful for colon-specific delivery in inflammatory bowel disease.

1. Introduction

Inflammatory bowel disease (IBD) is an idiopathic inflammatory disorder involving the mucosa and sub-mucosa of the colon. In humans, the majority of IBD occurs in a variety of forms, the most common being Crohn's disease and ulcerative colitis. The two forms of the disease are clinically related and histologically distinct chronic inflammation of the bowel that is characterized by intermittent courses of acute attacks [1,2]. Steroidal and nonsteroidal anti-inflammatory drugs in addition to immunosuppressive agents are commonly used to control acute attacks of the disease and to prevent further attacks during remission [3,4]. To achieve an optimum therapeutic efficacy, the delivery system must transport the drug in adequate concentration and without significant loss before reaching the proximal part of the colon. Unfortunately, most of the currently available therapeutic strategies lack this selectivity [5].

Colon-specific drug delivery has been widely investigated for the treatment of local colonic diseases or for the systemic administration of drugs that are adversely affected by the upper gastrointestinal tract. The use of pH-dependent systems represents the leading formulation approach for the site-specific colon delivery of drugs in the oral treatment of IBD. The currently existing colon delivery devices for this disease include enteric coated tablets, pellets, and granules. However, the residence time of these delivery systems is usually decreased by diarrhea, a common symptom of IBD [6,7]. Diarrhea associated with IBD has a reported frequency as high as 66–92% [8], which may limit the efficacy of single unit and large particulate delivery systems. Early drug release in the small intestine as a result of inter- and intra-individual variability of gut pH, with subsequent decrease of therapeutic efficacy, is another problem of pH-dependent systems [9]. Therefore, a pressing need still exists for the development of a drug delivery system that is able to target selectively the inflamed tissue of the colon. Such a system could maximize the therapeutic efficacy and reduce the systemic side-effects associated with the anti-inflammatory drugs.

Recently, a new therapeutic approach targeting the immune-regulating cells in IBD using micro-particulate drug delivery system has been proposed by Nakase et al. [10]. It has been proven that small size particulate drug delivery systems can be efficiently taken up by macrophages and M cells available at the site of inflammation. Moreover, the disruption of the intestinal barrier function could allow for the accumulation of the particulate delivery system at the site of inflammation [11]. Lamprecht et al. have

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observed that this accumulation is particle size-dependent with an increasing effect for smaller particle size and highest efficiency for the nanoparticles of around 100 nm [12].

On the basis of the above-mentioned considerations, small particulate drug delivery systems that combine pH-sensitivity, controlled-release properties, and particulate targeting to the inflamed mucosal tissue could be promising for colon drug targeting in IBD. The previously reported systems depended mainly on entrapping the drug-loaded polymeric micro- or nanoparticles into enteric micro-particulate carriers [3,13,14]. However, these systems usually comprise two preparation steps of emulsification and solvent evaporation, which render the preparation procedures more complicated and time consuming. In addition, it is difficult to ensure the regeneration of the dispersible state of the sub-micron sized particles in the GI tract after oral administration.

In our current study, matrix type nanospheres were prepared using a combination of the biodegradable PLGA copolymer and a pH-sensitive methacrylate copolymer. The objectives of this combination were to minimize early drug release in the proximal small intestine, to allow for a controlled-release phase in the distal part of the GI tract, and to target the site of colonic inflammation by using the drug-loaded nanoparticles. BSD, a corticosteroid with high topical efficacy and minimal systemic activity [15], was selected as a model anti-inflammatory drug to be entrapped in the pH-sensitive nanospheres. The in vivo therapeutic efficacy of the new drug delivery system was evaluated in comparison with conventional drug-loaded enteric microparticles in a TNBS-induced colitis rat model. In addition, the potentials of colon targeting and specific uptake by the ulcerative mucosal tissue were evaluated using fluorescent marker-loaded systems.

2. Materials and methods

2.1. Materials

Poly(DL-lactic-co-glycolic) acid (PLGA 75/25, molecular weight 20 kDa), 2,4,6-trinitrobenzenesulfonic acid (TNBS), hexadecyltrimethylammonium bromide (HTAB) were purchased from Wako Pure Chemicals (Osaka, Japan). The pH-sensitive poly(methacrylic acid-methyl methacrylate, 1:2) copolymer (Eudragit® S100) was received as a gift from Röhm GmbH (Darmstadt, Germany). Budesonide (BSD) was obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Coumarin-6 (Laser grade) and *O*-dianisidine dihydrochloride were purchased from MP Biomedicals (Illkirch, France). Polyvinyl alcohol (PVA-403) was kindly supplied by Kuraray Co., Ltd. (Osaka, Japan). Water used in this study was purified and deionized using a Milli-Q system (Millipore, France). All other chemicals were obtained from Nacalai Tesque Inc. (kyoto, Japan), and were of analytical grade.

2.2. Preparation of pH-sensitive nanospheres

The pH-sensitive nanospheres were prepared by an adaption of the modified spontaneous emulsification solvent diffusion (SESD) method previously described by our laboratory [16]. Polymeric mixtures of the biodegradable PLGA polymer and the pH-sensitive polymer (Eudragit® S100) were used in 2:1 or 1:1 weight ratios based on a preliminary investigation. Fixed amounts of the polymeric mixtures (90 mg) and BSD (10 mg) were co-dissolved in a solvent system consisting of acetone (2 ml) and methanol (1 ml). The obtained polymeric drug solution was added to 20 ml of aqueous PVA solution (0.5% w/w) using a peristaltic pump at a flow rate of 2.0 ml/min while continuously stirring at 400 rpm with a propeller mixer. The entire dispersion was immediately centrifuged (45,000*g*, 4 °C for 30 min, Kubota Co., Japan), and the residue was redispersed in Milli-Q water to remove excess PVA and unentrapped drug. This process was repeated twice, and the final dispersion was subjected to freeze drying at −120 °C for 72 h (FD-81TS, Tokyo Rikakikai Co. Ltd., Japan).

2.3. Preparation of enteric microparticles

BSD-loaded enteric microparticles were prepared by using the spray-drying method. Typically, an ethanolic solution (100 ml) containing 100 mg BSD and 900 mg Eudragit® S100 was spray-dried using a nitrogen circulating spray-dryer (GS-31 Yamato Labotech., Tokyo, Japan). The process conditions were as follows: the inlet temperature was 120 °C, the outlet temperature was 70 ± 5 °C, the spray pressure was 0.13 MPa, and the feeding rate of solution was 10 ml/min.

2.4. Physicochemical characterization

The particle size of the freeze-dried nanospheres was determined by photon correlation spectroscopy (PCS) using Zetasizer 3000 (Malvern Instruments, UK). The measured parameters by PCS were the average particle diameter (ZAve) and the polydispersity index (PI). Particle size of the spray-dried microparticles was measured by a laser-based time-of-transition system (Cis-1, Galai Inc., Israel). All determinations were carried out in triplicates, and results were expressed as mean ± SD.

The drug content within the nanospheres and microparticles was determined by a previously reported reversed-phase HPLC procedures [17]. Typically, samples (10 mg) were dissolved in a solvent mixture of acetonitrile/methanol (1:1 v/v) and filtered through 0.2 m filter. The drug concentration in the filtrate was measured, and the entrapment efficiency was calculated as the percentage of actual drug loading to the theoretical drug loading.

The surface morphology of the nanospheres and microparticles was evaluated by using scanning electron microscopy (SEM, JSM-330A, Nihon Denshi, Japan).

The in vitro release characteristics of BSD from the prepared nanospheres and microparticles were evaluated in gradually pH-changing buffers (pH 1.2, 6.8, and 7.4). These buffer systems were selected based on the normal variations of the pH along the gastrointestinal (GI) tract from the stomach (pH ∼1.5) and the proximal small intestine (pH 6.0–6.8) to the ileocecal region (pH ∼7.3) [18]. Samples (10 mg) were suspended in 50 ml of the release medium and incubated into a shaking water bath (60 rpm, 37 °C). After predetermined time intervals, 1 ml samples were collected and centrifuged at 45,000*g* for 20 min. The drug concentration in the supernatant was directly analyzed by the HPLC procedures mentioned before.

2.5. In vivo anti-inflammatory effects

All animal experiments in the present study were performed in compliance with the regulations of Gifu Pharmaceutical University (Gifu, Japan) in line with the Japanese legislation on animal studies. Male Wistar rats weighing 230–250 g, 10 weeks old, were obtained from Japan SLC Inc. (Shizuoka, Japan). The TNBS-induced colitis rat model has been established as a useful method to test new therapeutic strategies for human IBD [19–21]. For the induction of colitis model, the rats were fasted for 24 h, narcotized with ether, and catheterized 8 cm intrarectally. The colitis control group and the treatment groups received 500 l TNBS solution in 50% ethanol by rectal instillation (dose 120 mg/Kg), while the healthy control group received only 50% ethanol solution. The rats were monitored for three days without treatment to allow for the development of IBD model. The treatment groups received orally 0.5 ml suspension of BSD pH-sensitive nanospheres or enteric microparticles once daily for five consecutive days (BSD dose: 0.2 mg/Kg). The colitis

control group received 0.5 ml normal saline instead of the drug-containing particles.

2.6. Assessment of colonic inflammation

2.6.1. Clinical activity score

The activity of the induced colitis model was quantified with a clinical score assessing weight loss, stool consistency, and rectal bleeding as previously reported [1,4,22].

2.6.2. Colon/bodyweight ratio

The rats were sacrificed either 24 h or 7 days after the last drug administration and the distal colon specimens (6 cm length) were resected, opened longitudinally, and rinsed with iced phosphate buffer. The ratio of the wet weight of the colon specimen to the bodyweight was calculated for each rat as an index of colonic inflammation [23].

2.6.3. Myeloperoxidase activity (MPO)

The activity of MPO, caused by the infiltration of activated neutrophils, is a reliable index to quantify the degree of inflammatory colitis. Measurements were performed according to the standard procedures [24]. Typically, the distal colon specimen (200 mg) was minced in 1 ml of HTAB buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0) on ice and homogenized (Polytron homogenizer, Kinematica, Switzerland) three times for 30 s on ice. The homogenate was sonicated 10 s, freeze thawed three times, and centrifuged at 10,000 rpm for 3 min. MPO activity in the supernatant was measured spectrophotometrically. Supernatant (0.1 ml) was added to 0.167 mg/ml of *o*.dianisidine hydrochloride and 0.0005% hydrogen peroxide, and the change in absorbance at 460 nm was measured. One unit of MPO activity was defined as the amount that degraded 1 mol peroxide per min at 25 °C.

2.7. In vivo studies using coumarin-6 (C-6)

Budesonide was replaced by the hydrophobic fluorescent marker (C-6) to facilitate the in vivo analytical detection and confocal imaging after oral administration of the different particulate formulations. C-6 was loaded in a weight ratio of 1% into PLGA/Eudragit® S100 (1:1 w/w) nanospheres and Eudragit® S100 microparticles using the same aforementioned preparation procedures. The entrapment efficiency of C-6 (λ_{Ex} = 460 nm, λ_{Em} = 505 nm) was determined after dissolving specific weight of the particles in methanol/chloroform mixture (1:1 v/v) using a fluorescence spectrophotometer (F-3010, Hitachi Ltd., Japan).

2.7.1. Disposition of C-6-loaded particles in the rat GIT

The rats were fasted for 48 h prior to oral administration of C-6-loaded nanospheres and microparticles (C-6 dose of 50 g/rat). The rats were sacrificed 3, 8, or 12 h after oral administration, and the whole GI tract was resected and divided into four segments (stomach, small intestine, caecum, and colon). The tissue specimens (including the luminal fluid) were homogenized with normal saline, extracted with a methanol/chloroform mixture (1:1 v/v), and assayed for C-6 content by fluorophotometry.

2.7.2. Bioavailability of C-6 after oral administration

The plasma concentration of C-6 after oral administration of C-6-loaded nanospheres and microparticles (C-6 dose of 50 g/rat) was determined for the evaluation of the systemic drug bioavailability from the different formulations. Blood samples (0.5 ml) were collected from the jugular vein, and plasma was separated by centrifugation (10 min, 10.000 rpm). C-6 was extracted from plasma with a methanol/chloroform mixture for quantification.

2.7.3. Adhesion and uptake of C-6-loaded particles by the inflamed rat colon

The inflammatory-mediated adhesion and uptake of the fluorescent dye containing particles was evaluated by fluorophotometry and Confocal Laser Scanning Microscopy (CLSM). Inflammatory colitis was induced in rats using TNBS as previously described. Three days later, the rats were orally administered either C-6-loaded nanospheres or microparticles (C-6 dose of 50 g/rat). The rats were sacrificed 24 h after oral administration, and the colon tissue was resected. The inflamed portion of the colon, identified by marked swelling and hyperemia, was separated from the non-inflamed parts. The different colon specimens were opened longitudinally, rinsed in cold saline to remove luminal contents, and homogenized. The homogenate was extracted with a methanol/chloroform mixture and assayed for C-6 content by fluorophotometry.

For CLSM, the freshly excised colon tissues were cryofixed in Tissue-Tek® Compound, sectioned (10 m thickness) using a cryomicrotome (Leica CM, Germany), and imaged by a Zeiss LSM 510 Confocal Laser Scanning System (Carl Zeiss Inc., Germany) equipped with a laser operating at 488 nm for fluorescence excitation.

2.8. Statistical analysis

All the results were expressed as mean values ±S.D. The student's *t* test was applied to study the significance of difference between two treatment groups (One-way analysis of variance [ANOVA] followed by Tukey–Kramer test in case of multiple comparison). However, if normality and/or equal variance was not achieved, the nonparametric methods (Mann-Whitney *U* test for two treatment groups and the Kruskal–Wallis test for multiple comparisons) were applied. All statistical calculations were performed using the StatsDirect software (StatsDirect Ltd.). In all cases, an associated *P* value of less than 0.05 was considered to be significant.

3. Results

3.1. Preparation and characterization of BSD-loaded nanospheres and microparticles

PLGA/Eudragit® nanospheres were prepared by an adoption of the SESD method. The nanospheres had a uniform size distribution with average diameters in the range of 260–290 nm and low polydispersity values (Table 1). All nanosphere formulations showed high production yields and high entrapment efficiency of the model drug (89.8–92.7%). On the other hand, BSD enteric microparticles were prepared by spray-drying method using Eudragit® S100. The average particle size of BSD-loaded microparticles was 1.97 ± 0.78 m with a production yield of about 42% and a drug recovery of 40%.

The morphology of BSD-loaded nanospheres and microparticles was evaluated by SEM (Fig. 1). Nanospheres prepared with PLGA/Eudragit® S100 co-polymers displayed a well-defined spherical shape with a smooth surface and narrow size distribution, while spray-dried Eudragit® S100 microparticles exhibited an elliptical shape with shriveled surfaces.

The in vitro release profiles of BSD from the prepared particulate systems were studied in gradually pH-changing buffers (Fig. 2). Nanospheres prepared in different weight ratios of PLGA to Eudragit® S100 exhibited differential pH-dependent release properties. PLGA/Eudragit® S100 nanospheres (in 2:1 w/w ratio) released about 32 and 55% of the initial drug load at pH 1.2 and 6.8, respectively, indicating insufficient control of drug release in

Table 1
Physicochemical characteristics of BSD-loaded nanospheres and microparticles.

Formulation	Average particle diameter (nm)	Polydispersity index	Yield (%)	Entrapment efficiency (%)
PLGA/ES100 (2:1 w/w) NS	261.7 ± 19.7	0.134 ± 0.04	89.2 ± 1.0%	89.78 ± 0.31%
PLGA/ES100 (1:1 w/w) NS	282.8 ± 23.4	0.197 ± 0.08	93.4 ± 1.2%	92.68 ± 0.53%
ES100 MP	1.97 ± 0.78	–	41.9%	40.01 ± 0.35%

Data are presented as mean ± SD (n = 3).

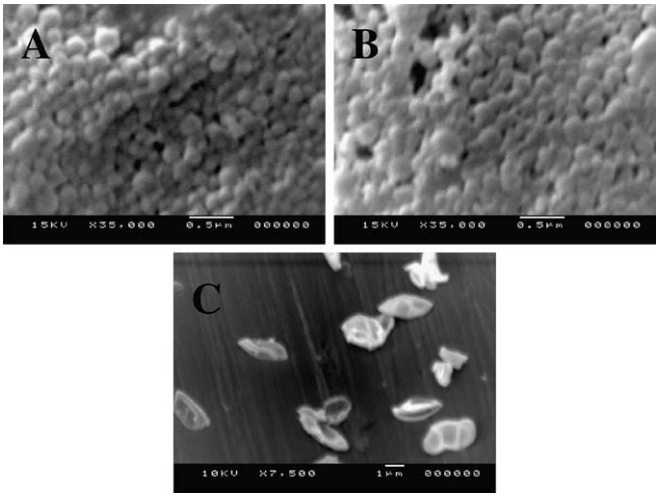

Fig. 1. Scanning electron micrographs of BSD-loaded particles; (A) PLGA/Eudragit® S100 (2:1 w/w) nanospheres, (B) PLGA/Eudragit® S100 (1:1 w/w) nanospheres, and (C) Eudragit® S100 microparticles.

the desired part of the GI tract. Increasing the ratio of Eudragit® S100 in the nanospheres (1:1 w/w of PLGA/Eudragit® S100) resulted in a considerable decrease in BSD burst release, only about 20% and 28% at pH 1.2 and 6.8, respectively. Once the enteric polymer had dissolved when the pH was changed to 7.4, which corresponds to the pH of the ileocecal region of the GI tract, the remaining drug was released from the PLGA matrix by slow diffusion in a sustained manner. The release pattern of BSD from the conventional Eudragit® S100 microparticles was evaluated using the same buffer systems. As expected, BSD was almost retained in the microparticles when tested at pH 1.2 and 6.8, and was immediately released when the pH was shifted to 7.4.

3.2. In vivo anti-inflammatory effects

For the induction of colitis rat model, all animals received an intrarectal instillation of TNBS (dose = 120 mg/kg) on the third day of the experiment, except for the healthy control group. The activity of the induced colitis was evaluated using a clinical activity score system taking in consideration bodyweight loss, stool consistency, and rectal bleeding [1,4]. After induction of experimental colitis, all the animals suffered from severe diarrhea and rectal bleeding. The clinical activity score system increased rapidly and consistently in all the groups that received TNBS, while the healthy control group did not show any significant increase (Fig. 3). Starting from day 6, rats received orally either BSD pH-sensitive nanospheres (1:1 w/w of PLGA/Eudragit® S100) or enteric microparticles (Eudragit® S100) once daily for five consecutive days, while the colitis group received saline instead. During the treatment period, the signs of inflammation started to decrease in severity for both the microparticle and nanosphere-treated groups. The nanosphere-treated group showed statistically different results from the colitis group (days 8–11, $P < 0.05$), while there was no statistical difference between the microparticle-treated group and the colitis group. During the next 7 days of monitoring without drug treatment, the nanosphere-treated rats continued to improve markedly in comparison with the microparticle-treated animals.

The rats were sacrificed either 24 h or 7 days after the last drug administration, and the distal colon specimens (6 cm length) were obtained. All the animals that received TNBS developed areas of localized inflammation, thickening, and hyperemia of the bowel wall when examined macroscopically (data not shown). These inflammatory signs were significantly reduced in the nanosphere-treated group when compared with the colitis and microparticle groups. The colon/bodyweight ratio was determined for quantitative evaluation of the inflammatory colitis of the different groups. As shown in Fig. 4A, the colon/bodyweight ratio, deter-

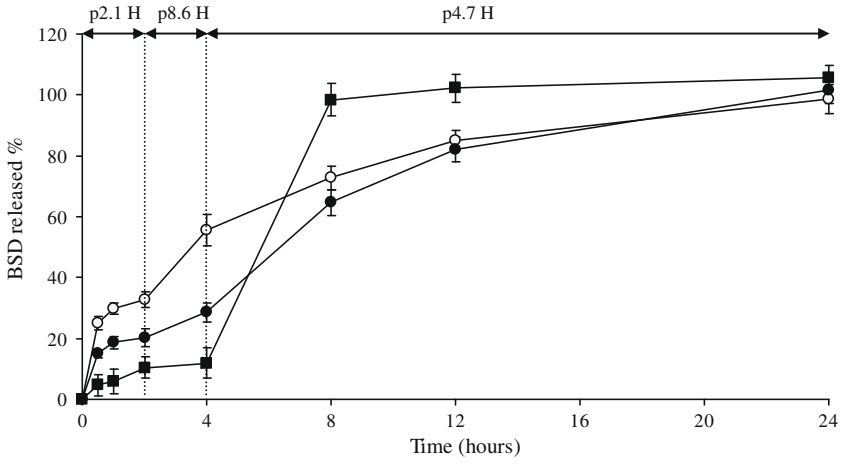

Fig. 2. Release profiles of BSD from the prepared nanospheres and microparticles in gradually pH-changing buffers; (○) PLGA/Eudragit® S100 (2:1 w/w) nanospheres, (●) PLGA/Eudragit® S100 (1:1 w/w) nanospheres, and (■) Eudragit® S100 microparticles.

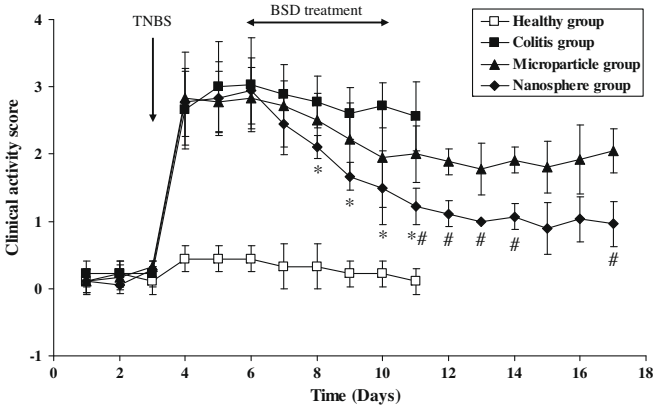

Fig. 3. Clinical activity score system of the different animal groups. Data are presented as mean ± SD (n = 3–6 animals/group, $^{*}p < 0.05$ compared with colitis control group, $^{\#}p < 0.05$ compared with microparticle-treated group).

mined 24 h after the last drug administration, decreased significantly ($P < 0.05$) in the case of nanosphere-treated group when compared with the colitis control group. In addition, the nanosphere-treated group showed a statistical difference from the microparticle-treated group when the colon/bodyweight ratio was determined 7 days after the last drug administration.

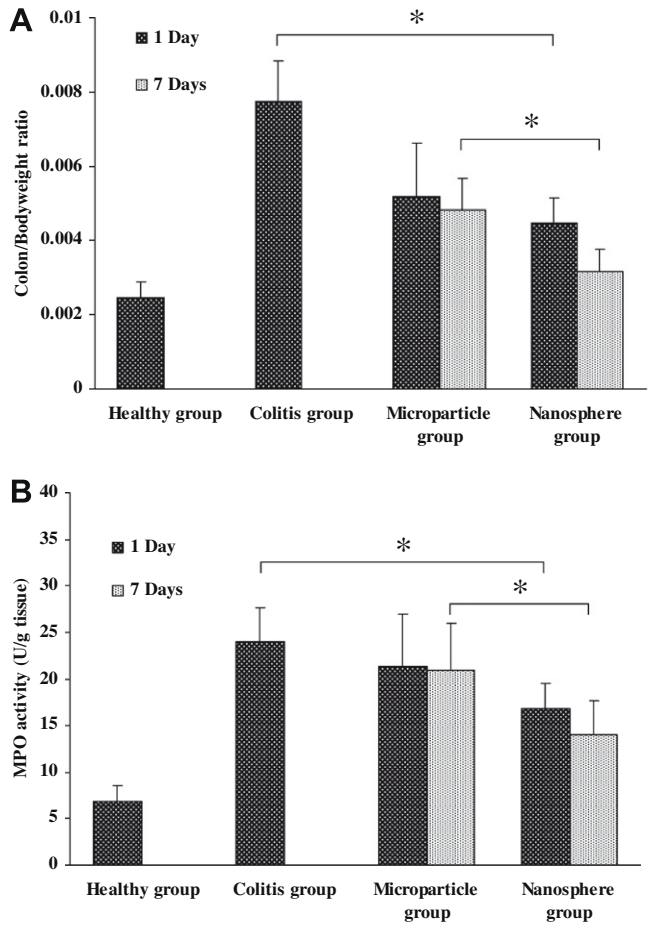

Fig. 4. Colon/bodyweight ratio (A) and MPO activity (B) determined 1 day or 7 days after final drug administration. Data are presented as mean ± SD (n = 3 animals/group, $^{*}p < 0.05$).

The activity of MPO enzyme is usually associated with the presence of neutrophils in the mucosa and sub-mucosa of the inflamed tissues. Therefore, measurement of the MPO activity can be used as a reliable index to quantify the degree of inflammatory colitis. The MPO activity after the intrarectal administration of TNBS to the colitis group was markedly increased as compared with the intrarectal administration of 50% ethanol in the healthy control group (Fig. 4B). The patterns of MPO activity in the isolated colon tissues of the treatment groups were quite similar to the results of the colon/bodyweight determinations.

3.3. In vivo studies using C-6

Coumarin-6 was loaded in the pH-sensitive nanospheres and enteric microparticles for evaluation of the GI disposition and possible colon targeting characteristics of the prepared particulate systems. C-6-loaded PLGA/Eudragit® S100 nanospheres and Eudragit® S100 microparticles had average particle diameters of 240 nm and 2.3 μm and entrapment efficiencies of 85.9 ± 1.05% and 40.3 ± 1.39%, respectively.

Fig. 5 represents the time course of C-6 dose percent in the different regions of the GIT after oral administration of pH-sensitive nanospheres and enteric microparticles. The pH-sensitive nanospheres achieved significantly higher levels of C-6 in the colon in comparison with simple enteric microparticles. The colon C-6 levels in the case of pH-sensitive nanospheres were 3.7, 9.2, and 15.4 folds higher than that of enteric microparticles after 3, 8, and 12 h, respectively.

The systemic bioavailability was assessed by measuring the plasma C-6 concentrations after oral administration of the two formulations (Fig. 6). The oral administration of Eudragit® S100 microparticles resulted in a relatively rapid increase in plasma C-6 concentrations with a peak value at about 2–3 h. In contrast, after oral administration of pH-sensitive nanospheres, the peak value was delayed to 6 h and the systemic availability was reduced by about 50%.

The specific adhesion and uptake of the fluorescent pH-sensitive nanospheres to the inflamed mucosa of the colitis rat model

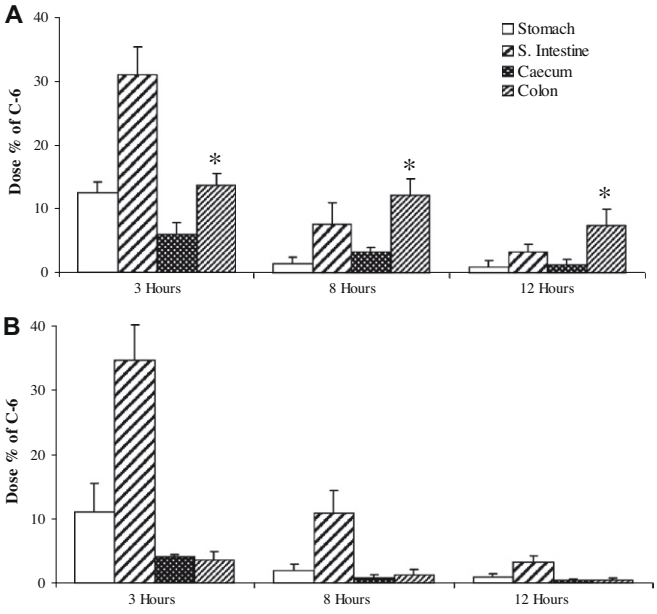

Fig. 5. Dose % of C-6 in the different regions of the GI tract after oral administration of pH-sensitive nanospheres (A) and enteric microparticles (B). Data are presented as mean ± SD (n = 3 animals for each time point, $^{*}p < 0.05$ compared with enteric microparticles at the corresponding time point).

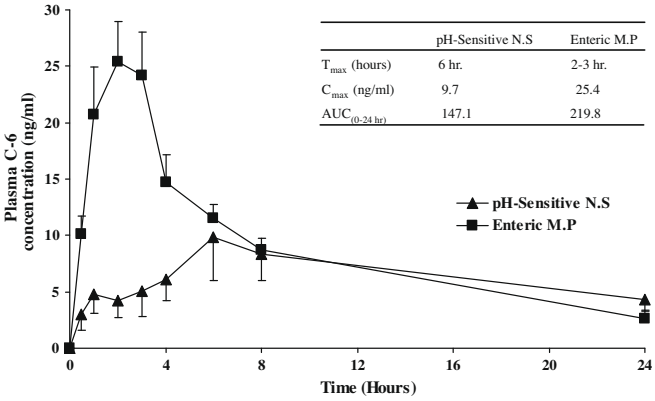

Fig. 6. Plasma concentration of C-6 after oral administration of pH-sensitive nanospheres and enteric microparticles. Data are presented as mean ± SD ($n = 3$ animals/group).

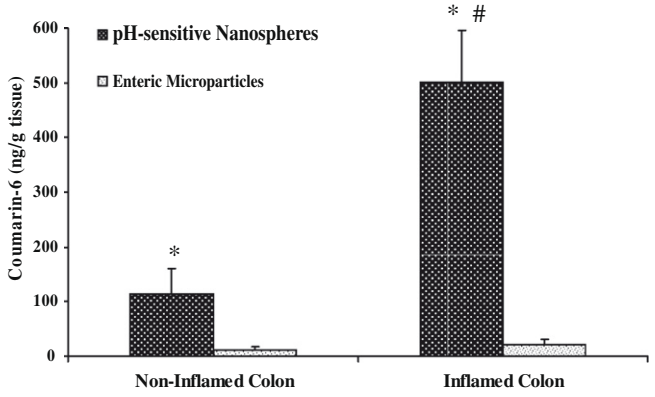

Fig. 7. Quantitative determination of C-6 in the mucosal tissue of the non-inflamed and inflamed colon specimens 24 h after oral administration of pH-sensitive nanospheres and enteric microparticles. Data are presented as mean ± S.D. ($n = 4$ animals/group, $^{*}p < 0.05$ compared with the corresponding microparticle group, $^{\#}p < 0.05$ compared with the non-inflamed colon of the nanosphere group).

was evaluated 24 h after oral administration. Fig. 7 shows the C-6 levels in the mucosal tissues of inflamed and non-inflamed colon specimens after oral administration of pH-sensitive nanospheres and enteric microparticles. The pH-sensitive nanospheres achieved a highly significant difference from the enteric microparticles both in the inflamed and in the non-inflamed colon parts. Moreover, the C-6 adhesion and penetration into the inflamed colon tissue was about 5-folds higher than that of the non-inflamed part after oral administration of pH-sensitive nanospheres. These results were further confirmed by CLSM. The confocal images of the non-inflamed and inflamed colon tissues after oral administration of enteric microparticles displayed negligible fluorescence intensity (data not shown). On the other hand, pH-sensitive nanospheres resulted in a noticeable degree of green fluorescence in the non-inflamed part and strong fluorescence in the areas of active inflammation, especially on the mucus layer and ulcerated mucosal tissue (Fig. 8).

4. Discussion

The modified SESD method has been developed by Murakami et al. [16] for the preparation of PLA and PLGA nanoparticles. This technique offers many advantages; for instance, avoidance of toxic solvents such as dichloromethane which require long evaporation and purification time, uniform nanoparticles formation even under moderate stirring, and high nanoparticle yield that is acceptable for industrial application. In this study, the SESD method proved to be efficient in the preparation of BSD-loaded PLGA/Eudragit® nanoparticles with optimum physicochemical properties.

Colon delivery systems based on the combination of pH-dependent and controlled-release properties have been previously reported [3,13,14]. The clear objectives of such a combination are to protect the drug from the early release in the proximal part of the GI tract by using pH-sensitive polymers and, at the same time, to prevent the complete drug release before reaching the colon by using a controlled-release polymer. We have tried to achieve these objectives by formulating the two types of polymers in a single matrix nanosphere delivery system. The achieved drug release profile from PLGA/Eudragit® nanospheres (1:1 w/w) could decrease the early drug loss before reaching the site of action, a problem commonly encountered with pH-dependent systems [9,18]. In addition, the initial burst release associated with PLGA nanoparticles could be minimized by incorporation of Eudragit® S100 in the matrix system. Accordingly, the proposed drug delivery system combined the advantages of pH-sensitive delivery of methacrylate co-polymers together with the sustaining and particulate targeting properties of biodegradable polymers.

The therapeutic efficacy of BSD pH-sensitive nanospheres was evaluated using the TNBS-induced colitis rat model in comparison with BSD enteric microparticles. The TNBS colitis model shares many of the histopathological and clinical features of the human

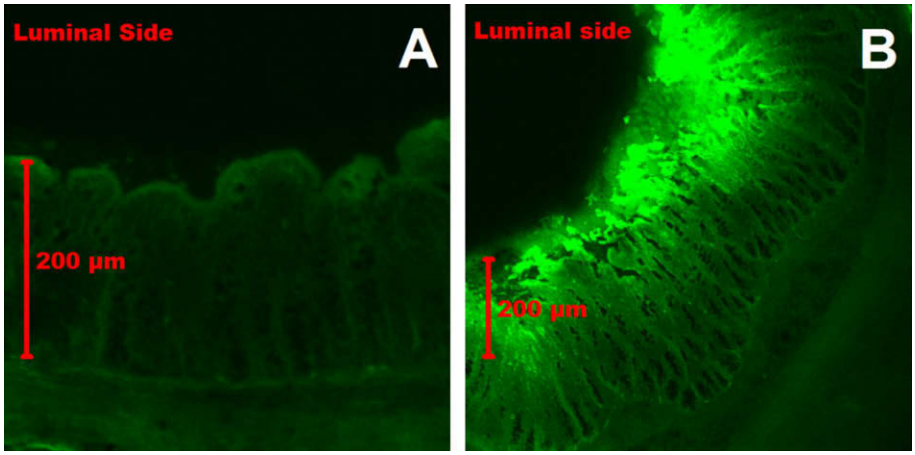

Fig. 8. CLSM images of colon cross-sections prepared 24 h after oral administration of C-6-loaded pH-sensitive nanospheres; (A) non-inflamed colon mucosa, and (B) inflamed colon mucosa.

IBD [25]. In addition, it has been established as a useful method to test new therapeutic strategies for human IBD [19–21]. Morris et al. [19] reported that the TNBS-induced ulceration and marked thickening of the bowel wall persisted for at least 8 weeks following a TNBS dose of 30 mg/rat. After the induction of experimental colitis, the clinical activity score, colon/bodyweight ratio, and MPO activity markedly increased in the colitis group. Oral treatment using BSD-loaded pH-sensitive nanospheres significantly lowered these parameters in comparison with the colitis group, while BSD enteric microparticles could not achieve such a difference. Moreover, comparing these parameters one week after final drug administration revealed a significant difference between the two treatment groups. These results may suggest a higher colon targeting potential and drug accumulation after treatment with pH-sensitive nanospheres in comparison with simple enteric coating of the drug.

For further elucidation of the observed pharmacological responses, we have loaded the different systems with C-6 for quantitative evaluation of the colon targeting properties. The fluorescent marker C-6 is a stable lipophilic compound with a small molecular weight of about 350 Da, which makes it suitable for uptake and penetration studies [26]. The pH-sensitive nanospheres achieved considerably higher levels of C-6 in the colon and lower systemic bioavailability in comparison with simple enteric microparticles. These results may be attributed to the release pattern of each system. While Eudragit® S100 microparticles were expected to release all the C-6 content in the ileum or the ileocecal region, higher drug concentration was available for the systemic absorption from the small intestine before reaching the colon. However, in the case of pH-sensitive nanospheres, the slow and incomplete release of C-6 in the terminal ileum resulted in lower plasma concentrations and lower systemic bioavailability of the fluorescent marker with subsequent achievement of higher levels in the colon. Moreover, the slow and incomplete absorption of drugs from the colon, compared to that of small intestine, may attribute for the delay of the peak value of C-6 after administration of pH-sensitive nanospheres. Despite the fact that translocation of the drug-loaded nanoparticles through the intestinal mucosa can result in increased bioavailability and systemic side effects of anti-inflammatory drugs, many studies refer that not more than 5% of the ingested sub-micron particles can reach systemic circulation [27,28].

Previous reports have pointed out the size-dependent adhesion and uptake of micro- and nanoparticles into the intact mucosal tissues of the GI tract. Desai et al. [29] have shown that the highest uptake was observed with 100 nm particles, and that the degree of uptake depends on the type of GI tissue. However, in the case of mucosal inflammation (e.g., ulcerative colitis), a higher degree of particles deposition can be expected. It has been reported that some intestinal inflammatory diseases, such as the Crohn disease, could modify epithelium permeability [30,31], and thus nanoparticles could be more easily taken up by the inflamed mucosal tissues. Lamprecht et al. [12] have investigated the adhesion of fluorescent polystyrene particles of different particle sizes to the inflamed colonic mucosa. They have observed that mucoadhesion is particle size-dependent with an increasing effect for smaller particle size and highest efficiency for the nanoparticles of around 100 nm. It has been concluded that small particles can better attach to the mucus layers due to easier penetration into the layer and their relatively small mass. In our current study, we have observed strong mucoadhesion and penetration of the fluorescent nanospheres in the ulcerated mucosal tissue of the colitis rat model. This deposition behavior was observed 24 h after oral administration of the particles, which may intensify the ability of pH-sensitive nanospheres to accumulate at the site of inflammation. In addition, this behavior may account for the extended therapeutic efficacy of BDS nanospheres in the treatment of induced colitis. According to the previously published data, there are several factors that contribute to such a specific deposition. Nanoparticles can penetrate through the disrupted and ulcerated mucosal barrier at the site of inflammatory colitis [11]. Another reason is the increased mucus production at the site of inflammation, which allows for a higher degree of particles adhesion [12]. In addition, small particles can be easily taken up by macrophages and other immune-regulating cells available in the colonic mucosal tissue of ulcerative colitis [21]. In fact, all these factors can work together to increase the accumulation of the particulate carrier at the site of inflammation.

In summary, a nanosphere system for colon-specific delivery in ulcerative colitis has been proposed in this study. The delivery system combined the properties of pH-sensitive delivery of pH-dependent polymers with the controlled release and particulate targeting properties of biodegradable polymers. The nanospheres loaded with BSD showed significant anti-inflammatory effects in the TNBS-induced colitis rat model. This therapeutic response could be related, first, to the higher drug levels that reached to the colon, and second, to the specific adhesion and uptake of the nanospheres at the site of inflammation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2008.12.013.

References

[1] G. Hartmann, C. Bidlingmaier, B. Siegmund, S. Albrich, J. Schulze, K. Tschoep, A. Elgler, H.A. Lehr, S. Endres, Specific type IV phosphordiesterase inhibitor rolipram mitigates experimental colitis in mice, J. Pharmacol. Exp. Ther. 292 (2000) 22–30.
[2] S. Klein, J. Stein, J. Dressman, Site-specific delivery of anti-inflammatory drugs in the gastrointestinal tract: an in-vitro release model, J. Pharm. Pharmacol. 57 (2005) 709–719.
[3] M. Rodrigeuz, J.A. Antunez, C. Taboada, B. Seijo, D. Torres, Colon-specific delivery of budesonide from microencapsulated cellulosic cores: evaluation of the efficacy against colonic inflammation in rats, J. Pharm. Pharmacol. 53 (2001) 1207–1215.
[4] A. Lamprecht, H. Yamamoto, H. Takeuchi, Y. Kawashima, Nanoparticles enhance therapeutic efficiency by selectively increased local drug dose in experimental colitis in rats, J. Pharmacol. Exp. Ther. 315 (2005) 196–202.
[5] Lamprecht, Multiparticulate systems in the treatment of inflammatory bowel disease, Curr. Drug Targets Inflamm. Allergy 2 (2003) 137–144.
[6] P.J. Watts, L. Barrow, K.P. Steed, C.G. Wilson, R.C. Spiller, C.D. Melia, M.C. Davies, The transit rate of different-sized model dosage forms through the human colon and the effects of a lactulose-induced catharsis, Int. J. pharm. 87 (1992) 215–221.
[7] Y. Meissner, Y. Pellequer, A. Lamprecht, Nanoparticles in inflammatory bowel disease: particle targeting versus pH-sensitive delivery, Int. J. Pharm. 316 (2006) 138–143.
[8] S. Urayama, E.B. Chang, Mechanism and treatment of diarrhea in inflammatory bowel diseases, Inflamm. Bowel Dis. 3 (1997) 114–131.
[9] L. Yang, J.S. Chu, J.A. Fix, Colon-specific drug delivery: new approaches and in vitro/in vivo evaluation, Int. J. Pharm. 235 (2002) 1–15.
[10] H. Nakase, K. Okazaki, Y. Tabata, S. Uose, M. Ohana, K. Uchida, Y. Matsushima, C. Kawanami, C. Oshima, Y. Ikada, T. Chiba, Development of an oral drug delivery system targeting immune-regulating cells in experimental inflammatory bowel disease: a new therapeutic strategy, J. Pharmacol. Exp. Ther. 292 (2000) 15–21.

[11] J. Stein, J. Ries, K.E. Barret, Disruption of intestinal barrier function associated with experimental colitis: possible role of mast cells, Am. J. Physiol. 274 (1998) 203–209.
[12] A. Lamprecht, U. Schafer, C.M. Lehr, Size-dependent bioadhesion of micro- and nanoparticulate carriers to the inflamed colonic mucosa, Pharm. Res. 18 (2001) 788–793.
[13] M. Rodriguez, J.L. Vila-Jato, D. Torres, Design of a new multiparticulate system for potential site-specific and controlled drug delivery to the colonic region, J. Control. Release 55 (1998) 67–77.
[14] A. Lamprecht, H. Yamamoto, H. Takeuchi, Y. Kawashima, A pH-sensitive microshere system for the colon delivery of tacrolimus containing nanoparticles, J. Control. Release 104 (2005) 337–346.
[15] A. Chopra, D.S. Pardi, E.V. Loftus, W.J. Tremaine, L.J. Egan, W.A. Faubion, K.A. Hanson, T.A. Johnson, W.J. Sandborn, Budesonide in the treatment of inflammatory bowel disease: the first year of experience in clinical practice, Inflam. Bowel Dis. 12 (2006) 29–32.
[16] H. Murakami, M. Kobayashi, H. Takeuchi, Y. Kawashima, Preparation of poly (DL-lactide-co-glycolide) nanoparticles by modified spontaneous emulsification solvent diffusion method, Int. J. Pharm. 187 (1999) 143–152.
[17] M. Gupta, H. Bhargava, Development and validation of a high-performance liquid chromatographic method for the analysis of budesonide, J. Pharmaceut. Biomed. 40 (2006) 423–428.
[18] T.F. Vandamme, A. Lenourry, C. Charrueau, J.-C. Chaumeil, The use of polysac charides to target drugs to the colon, Carbohyd. Polym. 48 (2002) 219–231.
[19] G.P. Morris, P.L. Beck, M.S. Herridge, W.T. Depew, M.R. Szewczuk, J.L. Wallace, Hapten-induced model of colonic inflammation and ulceration in the rat colon, Gastroenterology 96 (1989) 795–803.
[20] J. Fuss, T. Marth, M.F. Neurath, G.R. Perlstein, A. Jain, W. Strober, Anti-interleukin12 treatment regulates apoptosis of Th1 cells in experimental colitis in mice, Gastroenterology 117 (1999) 1078–1088.
[21] H. Nakase, K. Okazaki, Y. Tabata, S. Uose, M. Ohana, K. Uchida, T. Nishi, D. Debreceni, T. Itoh, C. Kawanami, M. Iwano, Y. Ikada, T. Chiba, An oral drug delivery system targeting immune-regulating cells ameliorates mucosal injury in trinitrobenzene sulfonic acid-induced colitis, J. Pharmacol. Exp. Ther. 297 (2001) 1122–1128.
[22] A. Lamprecht, N. Ubrich, H. Yamamoto, U. Schafer, H. Takeuchi, P. Maincent, Y. Kawashima, C.M. Lehr, Biodegradable nanoparticles for targeted drug delivery in treatment of inflammatory bowel disease, J. Pharmacol. Exp. Ther. 299 (2001) 775–781.
[23] G. Yue, F.F. Sun, C. Dunn, K. Yin, P.Y.K. Wong, The 21-aminosteroid tirilazad mesylate can ameliorate inflammatory bowel disease in rats, J. Pharmacol. Exp. Ther. 276 (1996) 265–270.
[24] E. Krawisz, P. Sharon, W.F. Stenson, Quantitative assay of acute intestinal inflammation based on myeloperoxidase activity, Gastroenterology 87 (1984) 1344–1350.
[25] Y. Yamada, S. Marshall, R.D. Specian, M.B. Grisham, A comparative analysis of two models of colitis in rats, Gastroenterology 102 (1992) 1524–1534.
[26] C. Lombry, C. Bosquillon, V. Préat, R. Vanbever, Confocal imaging of rat lungs following intratracheal delivery of dry powders or solutions of fluorescent probes, J. Control. Release 83 (2002) 331–341.
[27] A.T. Florence, The oral absorption of micro- and nanoparticulates: neither exceptional nor unusual, Pharm. Res. 14 (1997) 256–259.
[28] E. Allemann, J.C. Leroux, R. Gurny, Polymeric nano- and microparticles for the oral delivery of peptides and peptidomimetics, Adv. Drug Deliv. Rev. 34 (1998) 171–189.
[29] M.P. Desai, V. Labhasetwar, G.L. Amidon, R.J. Levy, Gastrointestinal uptake of biodegradable microparticles: effect of particle size, Pharm. Res. 13 (1996) 1838–1845.
[30] P. Hofman, Pathological interactions of bacteria and toxins with the gastrointestinal epithelial tight junctions and/or the zonula adherens: an update, Cell. Mol. Biol. 49 (2003) 65–75.
[31] C.A. Cuvelier, J. Quatacker, H. Mielants, V.M. De, E. Veys, H. Roels, M cells are damaged and increased in number in inflamed human ileal mucosa, Eur. J. Morphol. 31 (1993) 87–91.