Size-Dependent Bioadhesion of Micro- and Nanoparticulate Carriers to the Inflamed Colonic Mucosa

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Purpose. The size-dependent deposition of microparticles and nanoparticles after oral administration to rats using an experimental model colitis was examined. Local delivery of an entrapped drug could reduce side effects and would be a distinct improvement compared with existing colon delivery devices.

Methods. Ulcerative colitis was induced in Lewis rats with trinitrobenzenesulfonic acid. Fluorescent polystyrene particles with a size of 0.1, 1, or 10 μm were administered for 3 days. The animals then were sacrificed and their guts resected. Particle distribution in the colon was imaged by confocal laser scanning microscopy and quantified by fluorescence spectrophotometry.

Results. In the inflamed tissue, an increased adherence of particles was observed at the thicker mucus layer and in the ulcerated regions. A size dependency of the deposition was found, and an increased number of attached particles to the colon was determined compared with the control group. For 10-μm particles, only fair deposition was observed (control group: 1.4 ± 0.6%; colitis: 5.2 ± 3.8% of administered particle mass). One-micrometer particles showed higher binding (control group: 2.0 ± 0.8%; colitis: 9.1 ± 4.2%). Highest binding was found for 0.1-μm particles (control group: 2.2 ± 1.6%; colitis: 14.5 ± 6.3%). The ratio of colitis/control deposition increased with smaller particle sizes.

Conclusions. The use of submicron-sized carriers holds promise for the targeted delivery of drugs to the inflamed colonic mucosal areas in inflammatory bowel disease.

KEY WORDS: microspheres; nanospheres; drug targeting; inflammatory bowel disease; ulcerative colitis.

INTRODUCTION

The conventional treatment of inflammatory bowel disease (IBD) requires the daily intake of anti-inflammatory drugs. Chemical compounds requiring frequent intake at high doses by oral route often lead to absorption in the small intestine, thereby causing possible strong adverse effects. Therefore, several strategies have been followed for an oral drug delivery, which include the development of prodrugs delivering conventional drugs specifically in the large bowel after cleavage of the active part from the carrier (1–4) and solid-dosage forms that release the drug in the colon when activated by specific enzymes only present in the colon (5–7). The prodrug approach typically requires a relatively complex chemical process to couple drug to the hydrophilic polymeric carrier. Its feasibility depends on the structure of drug. Although prodrugs lead to reduced adverse effects, a more comfortable dosage frequency cannot be achieved. Sustained drug release devices, e.g. pellets, capsules, or tablets, have been developed to deliver the drug specifically in the colon for a longer time period. However, their efficiencies are decreased in many cases due to the symptoms of IBD, i.e., diarrhea (8,9). The administration of drugs by the rectal route is also currently used. However, it is not effective when the inflamed tissues are located in the upper parts of the colon.

Beside tablets and pellets, smaller drug-carrier systems ≥200 μm are subjected to the diarrhea symptoms. This results in a decreased gastrointestinal transit time and therefore to a distinct decrease in efficiency (8,9). However, systems smaller than 200 μm show a more prolonged passage time, so some efforts have been made to develop microparticles to achieve controlled drug release for IBD (10,11).

Both of these therapeutic pathways, e.g., prodrug or controlled-release device, also risk causing adverse effects resulting from the systemic drug absorption after their nonselective drug delivery all over the colon. Thus, a carrier system that delivers the drug specifically and exclusively to the inflamed tissues would be more desirable. Such a system could reduce side effects significantly in the case of conventional chemical anti-inflammatory compounds. In the case of colitis, several symptoms are known to activate the cellular immune response, i.e., in general, cell extravasion with increased presence of neutrophils, natural killer cells, mast cells, and regulatory T cells in the inflamed area. Several studies have indicated that involvement of macrophages, dentritic cells and T cells have an important role of pathophysiology of IBD (12–14). Moreover, it has been reported that microspheres and nanoparticles can be efficiently taken up by macrophages (15), mostly by way of phagocytosis (16). Thus, it may be expected that particle uptake into those immune-related cells or the disruption of the intestinal barrier function (17) could allow the accumulation of the particulate carrier system in the desired area. A subsequent increase in residence time, which would be postulated for smaller particles compared with existing drug delivery systems, allows for a dose reduction. One parameter of major importance for a particulate drug carrier system in this case is the particle size.

This project evaluates the use of micro- and nanoparticles for their targeted deposition in inflamed tissue. We examined the deposition behavior of polymeric carrier systems in the inflamed tissue of IBD and the dependence of particle diameter on this deposition. The main objective was to visualize and to quantify potential size-dependent particle accumulations and the resulting distribution of the particles in the gut.

MATERIALS AND METHODS

Materials

Trinitrobenzenesulfonic acid (TNBS) and *o*-dianisidine hydrochloride were obtained from Sigma (Deisenhofen, Germany), hexadecyltrimethylammonium bromide (HTAB) was obtained from Fluka (Deisenhofen, Germany). Astra blue, eosine G, and nuclear fast red for the histological staining were purchased from Merck AG (Darmstadt, Germany). All other compounds were obtained from Merck AG (Darmstadt, Germany) and were of analytical grade purity.

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Microspheres and Nanospheres

Polystyrene fluorescent micro- and nanoparticles with sizes of 0.1, 1, and 10 μm were purchased from Polysciences Ltd. (Eppenheim, Germany). Particle sizes and zeta potential were confirmed using a Zetasizer II® (Table I). All particles were washed twice with distilled water to remove residual surfactants or antibacterial agents, separated by centrifugation, and dispersed in distilled water or phosphate buffer (pH 6.8).

Animals

Experiments were performed in compliance with the regulations of the responsible committee of the City of Saarbrücken in line with the German legislation on animal experiments. Female Lewis rats (average weight 180–200 g; 12–15 weeks) were used in all experiments. Each group of treated animals contained at least six rats and was housed in a metabolic cage. The colitis group was treated by the following procedure to effectuate an inflammation: After light narcotizing with ether, the rats were catheterized 8 cm intrarectally, and 500 μl of TNBS in ethanol was applied (dose: 80 mg/ml TNBS in ethanol 50% solution). The rats were housed for 3 days without treatment to maintain the development of a full IBD model. The control group contained the same number of healthy rats. On day 4 and for 2 subsequent days, a 0.5-ml particle suspension (12.5 mg particles/kg body weight) was administered orally with a blunted, curved needle to all rats. Thereafter, no particles were administered, to wash out unabsorbed particles from the colon. Seventy-two hours after the last particle application, the animals were sacrificed with carbon dioxide, and the stomach, small intestine, caecum, and colon were resected.

To determine the particle residence time in the inflamed colon, the wash-out phase of the standard procedure was prolonged for an additional 1-, 3-, or 5-day period before the rats were sacrificed. These experiments were performed using 0.1-μm particles only with groups of six animals for each prolonged time interval.

Assessment of the Colon Wet Weight/Body Weight Ratio

The colon was opened by longitudinal incision and rinsed with phosphate buffer. An 8-cm segment of distal colon, including the major gross pathologic changes, was weighed. The colon/body weight ratio was calculated as an index of tissue edema (18).

Assessment of the Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was measured to ensure the full development of the colitis. MPO activity is a reliable index of inflammation caused by infiltration of activated neutrophils into the inflamed tissue. Activities were analyzed according to Krawisz *et al.* (19). Briefly, distal colon specimens were minced in 1 ml of HTAB buffer (0.5% in 50 mM phosphate buffer) on ice and homogenized. The homogenate was sonicated for 10 s, freeze-thawed three times, and centrifuged at 10,000 rpm for 3 min. MPO activity in the supernatant was measured spectrophotometrically. 0.1 ml of supernatant was added to 0.167 mg/ml *o*-dianisidine hydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured, with one unit of MPO activity being defined as the amount which degraded 1 μmol of peroxidase per minute at 25°C. All values of MPO activity were normalized to wet colon tissue weight.

Table I. Size and Zeta Potential of Particles Used for the *In Vivo* Study

Theoretical size	Measured size	Zeta potential (mV)
100 nm	126.7 ± 6.1 nm	–24.5 ± 1.8
1 μm	1.08 ± 0.08 μm	–48.3 ± 2.6
10 μm	9.89 ± 0.05 μm	n.d.

Confocal Laser Scanning Microscopy (CLSM) for the Qualitative Localization of Particles

A Biorad MRC 1024 Laser Scanning Confocal Imaging System (Hemel Hempstead, UK), equipped with an argon ion laser (American Laser Corp., Salt Lake City, UT, USA) and a Zeiss Axiovert 100 microscope (Carl Zeiss, Oberkochen, Germany), was used to qualitatively detect the fluorescent particles in the tissue sections. The laser was adjusted in the green fluorescence mode, which yielded an excitation wavelength at 488 nm.

Quantitative Determination of Particle Deposition in the Gut

After resection of the gut, longitudinal cuts of the intestine sections were made, and the tissue was rinsed carefully to remove food residues. Stomach, small intestine, and caecum were cut into small segments. Colon was processed in the same way, except the inflamed areas, which were identified by the macroscopic damages of the mucosal tissue, were separated from the noninflamed tissue before segmentation. All tissue samples were lyophilized in the dark to avoid bleaching of the fluorescence marker. Samples were incubated with 10 ml of chloroform at 30°C in a shaking water bath for 24 h in the dark. The extraction procedure was repeated two times to dissolve the polystyrene completely. The polymer solutions were diluted with chloroform to a total volume of 100 ml and were analyzed for their fluorescence by fluorescence spectrophotoscopy (F-2000 Fluorescence Spectrophotoscope, Hitachi Lt., Tokyo, Japan). Blank lyophilized particles were used as references. Untreated gut samples with and without ground chow were used for background correction.

To determine the amount of particles either attached to the mucus or taken up into the tissue mucus was removed from the mucosal tissue by repeated intensive washing steps with phosphate buffer pH 6.8 followed by their fluorescence quantification with the fluorescence spectrophotoscope in the washing medium and in the complementary tissue.

Statistical Analysis

The results were expressed as mean values ± SD. The Mann–Whitney–Wilcoxon U-test was used to investigate differences statistically because the number of animals in each group was relatively low. However, in all statistical analysis normality and equal variance was passed. Therefore, the Student's *t* test was also applied to examine significance of differences. In all cases, $P < 0.05$ was considered to be signifi-

cant, and the marked significant differences are valid for both statistical test systems.

RESULTS AND DISCUSSION

The behavior of the proposed drug delivery system was examined with respect to particle deposition and accumulation after oral administration. Because of its reproducibility, the TNBS colitis model was selected. Furthermore, TNBS colitis might be a relevant model because it involves the use of a hapten and develops a chronic inflammation rather than an acute mucosal injury (20). The experimental colitis model in rat after the intrarectal administration of TNBS (21) should allow an *in vivo* characterization of the particulate carrier system under the influence of chronic inflammation symptoms.

After inducing the colitis, histologic sections were performed to get visual evidence of the inflammation and to characterize the differences to healthy tissue. MPO activity was determined to ensure and quantify the inflammation in the colonic area (21). The average MPO activity in the areas with macroscopic damages was 0.59 ± 0.11 units/mg tissue, while in the healthy control the activity was as low as 0.09 ± 0.03 units/mg tissue. These results were comparable to values reported from earlier work (18,21). Additionally, it was observed that the colon wet weight/body ratio increased by a factor of 2.9 compared with the healthy control group, which has been reported as an indication for inflammation as well (18,21). The inflamed tissue showed an extreme increase in mucus production in the area of distal colon (Fig. 1b) compared with the histology of healthy gut sections from the control group (Fig. 1a). Moreover, strong damages of the intestinal tissue, e.g., ulcerations, were observed. In these areas, a highly increased amount and infiltration activity of immune related cells was found (Fig. 1c).

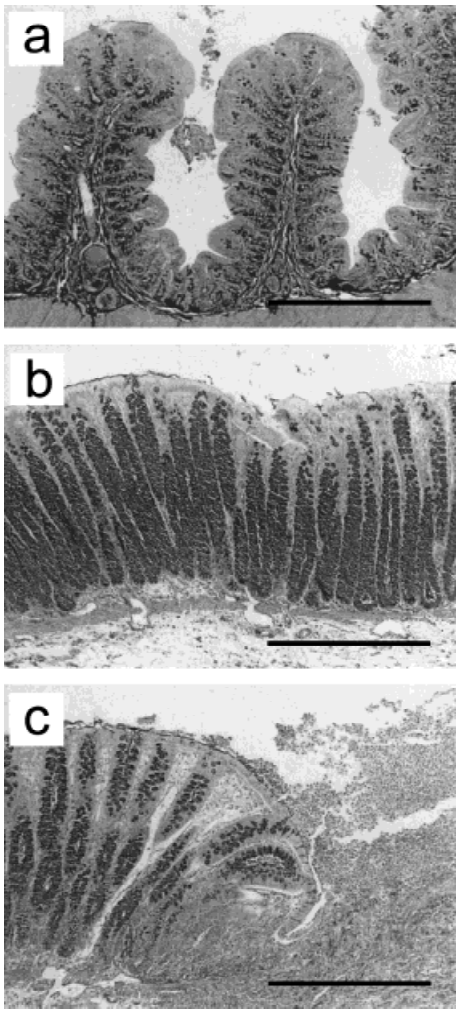

Fig. 1. Microscopical images of a colon section through a tissue sample of the healthy control group (a) and of the colitis group (b) showing the strong increased mucus layer on the mucosa (dark areas represent the astra blue-stained mucus) and a typical ulceration in the colon (c; left, mucosal tissue; right, ulceration with cell extravasion; increased presence of neutrophils, natural killer cells, mast cells, and regulatory T cells). Scale bars represent 50 μm.

A size-dependent particle deposition in the gastrointestinal tract of healthy rats was reported in earlier research work (22–24) and has been combined with bioadhesion depending on the particle surface properties (25). The authors reported an increased adherence for smaller particles in the whole gut. We observed the same phenomenon in the healthy control animals, but the trend was less pronounced in our study. Qualitatively, an increased adherence of all three sizes of particles was obtained in the inflamed tissue. Particles showed a higher deposition as observed by imaging with CLSM in the histological tissue cross-sections, especially in the areas of active inflammation. A strong increase of particle deposition compared with the healthy control model was observed in the inflamed tissue with macroscopic visible edema, whereas a dramatically higher particle accumulation was found inside ulcerated tissue sections (Fig. 2).

Tissue samples of both the control group and colitis group were analyzed quantitatively for their fluorescence to obtain data directly from the targeted area. A size dependency, as already mentioned for the healthy control group, also was observed in the inflamed area, but with a highly increased amount of deposition. To allow an easier direct comparison of the different groups a targeting index was defined as the ratio between particle deposition in colitis ($P_{colitis}$) and control group ($P_{control}$).

The decrease in diameter of the particles led to a distinct increase of accumulation in both the healthy gut and the inflamed tissue. Furthermore, a higher targeting index was observed with decreasing particle diameter; the targeting indices are shown in Figure 3. One-micrometer particles showed a nearly 5-fold higher percentage of particle binding in TNBS colitis compared to the healthy control. The highest deposition in inflamed tissue was observed for the 0.1-μm particles, showing a 6.5-fold increase in percentage of particle binding. It is remarkable that the comparison of the different colon regions, which is between ulcerated tissue and areas without macroscopic damages, showed an enhanced accumulation in the ulcerated regions for all particle sizes (Fig. 4).

To examine their overall distribution in the different gut regions after oral administration the particle deposition in the whole gut was quantified by fluorescence spectrophotoscopy (Fig. 5). Only small differences between the healthy and colitis groups were observed for the recovered particles from stomach, small intestine, and caecum. However, significant differences were determined between healthy and damaged

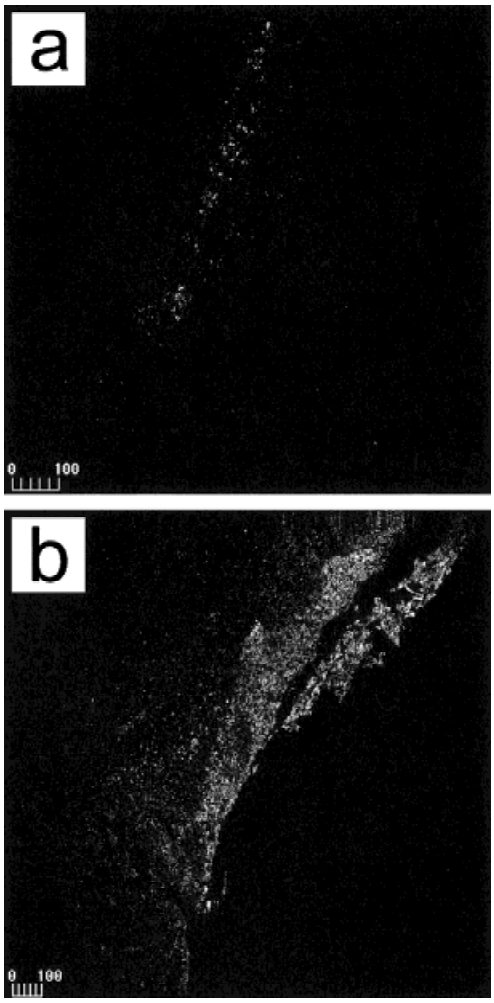

Fig. 2. Confocal laser scanning microscopy images of colon cross-sections from the healthy control group after administration of 100-nm particles (a) and the colitis group after administration of 100-nm particles (b). Scale bars represent 100 μm.

regions of the colon, which should allow a selective bioadhesion to the inflamed tissue.

To differentiate between particles located in the mucus and those attached or internalized by any tissue structure, the mucus was separated from the tissue by several washing steps and particles were quantified separately. Removing the mucus from the inflamed tissue decreased the total fluorescence signal in the tissue down to 38.6 ± 5.3%. This percentage was further decreased with increased particle size (1 μm: 31.1 ± 4.5%; 10 μm: 13.4 ± 6.9%). This decrease of the fluorescence signal from the tissue after the washing steps showed that a high amount of the particles are attached to the thicker mucus layer rather than taken up by macrophages. Moreover, according to the observations of Tirosh and Rubinstein (26), it might be speculated that there are further amounts of particles that were not taken up into the inflamed mucosal tissue

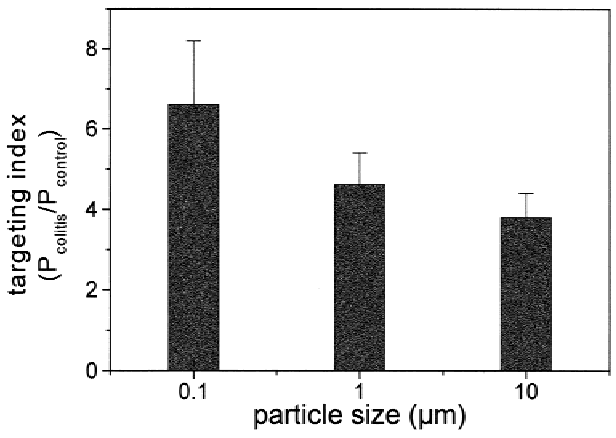

Fig. 3. Targeting indeces for the different particle diameters after quantitative determination of fluorescence signals from the resected colonic tissues.

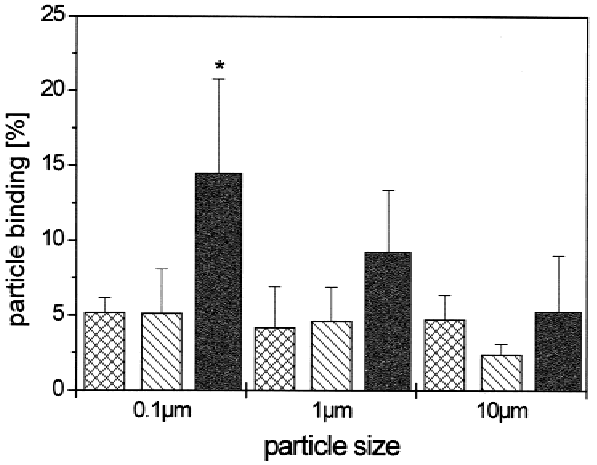

Fig. 4. Quantitative determination of particle deposition in the colitis group comparing different colon regions on day 9 after colitis induction. Small intestine (cross-hatched bars), colon without macroscopic damages (diagonal-marked bars), and inflamed colonic area (filled bars). Results are shown in percent of administered particle mass as mean values ± SD. *P <0.05 compared with colon deposition in colitis rats given 10-μm particles.

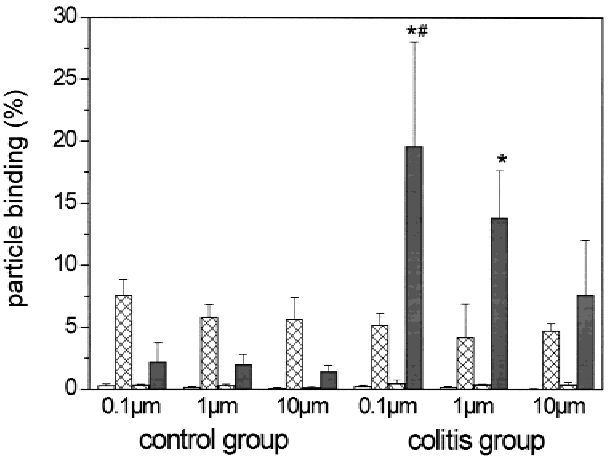

Fig. 5. Quantitative determination of particle deposition throughout the whole intestine comparing healthy control group with the colitis group on day 9 after colitis induction. Stomach (unfilled bars), small intestine (cross-hatched bars), caecum (diagonal-marked bars), and colon (filled bars). Results are shown in percent of administered particle mass as mean values ± SD. #P <0.05 compared with colon deposition in colitis rats given 10-μm particles *P <0.05 compared with colon deposition in healthy rats given particles of equivalent diameter.

but rather attached to the insoluble mucus. Two major reasons can be stated for the more distinct size-dependent deposition in the case of colitis. Smaller particles are taken up more easily by macrophages in the area of active inflammation. Nevertheless, particles with a size bigger than 4 μm, which have been reported to be taken up by macrophages less effectively (27), also showed a remarkable deposition in the inflamed tissue in our experiments. Therefore, it might be concluded that carrier systems in that size are able to attach to the mucus layer and accumulate in the inflamed region without the need for macrophage uptake. The strong increase in mucus production leads to a thicker mucus layer in the inflamed areas and results in a higher quantity of particle attachment. In addition, smaller particles can better attach to mucus layers due to easier penetration into the layer and their relatively small mass. Moreover, it was reported that in the healthy colon where the mucus level is relatively low, alterations in mucus amount and turnover have greater impact on particles adherence. Thus, the higher mucus production in IBD may affect the bioadhesion significantly (26).

In the case of IBD, an increased presence of neutrophils, natural killer cells, mast cells, and regulatory T cells in the inflamed tissue can be observed (28). Thus, an enhanced uptake of administered particles by these cell types should be expected. This results in an accumulation of the carrier system in the inflamed area. But on the other hand, there is an increased risk that the carrier system might be degraded after internalization (27,29), followed by a loss of activity of the carrier system. From this point of view, it seems to be advantageous that particles tend to be attached to the mucus layer rather than to be taken up by macrophages.

Based on the findings of a high amount of mucus attachment, it was of interest to determine the particle residence time in the inflamed colon in order to evaluate the efficiency of such a model carrier system (performed with 0.1-μm particles). The wash-out phase of the standard procedure was prolonged for an additional 1-, 3-, or 5-day period. With this procedure, the recovered particle amount further decreased with time. After one additional day of the wash out phase, 9.1 ± 2.8% of total administered particles were still found in the colon and a further decrease was observed after 3 and 5 days (3.4 ± 2.2% and 1.9 ± 1.1%, respectively).

Charge interactions are reported to further enhance binding to the inflamed tissue, especially in the stomach (30). Negatively charged particles may adhere more readily to inflamed tissue (Table I) because it has been reported that ulcerated tissues contain high concentrations of positively charged proteins that increase the affinity to negatively charged substances. Thus, this might be another reason for the enhanced attachment to the inflamed mucus areas. The influences of ionic interactions between particles and inflamed tissue were beyond the scope of this study and still have to be examined.

An optimal particle size for the design of a particulate carrier system must be chosen based on two major influencing factors. It should be kept in mind that by increasing the particle size, a higher drug loading capacity can be reached that allows the transport of higher drug amounts with less polymer. On the contrary, we observed a higher deposition rate and a better inflammation index for smaller particles. The results of this study show distinct advantages of smaller particles for this approach.

CONCLUSIONS

Polymeric particulate carrier systems proved in an *in vivo* model to be a promising approach to the bioadhesion to an inflamed area of gut in IBD. The particle deposition showed a significant increase in rats suffering from colitis compared to the equivalent tissue areas of the healthy control group. This enhanced particle accumulation in the targeted tissue suggests that a new concept of treatment using micro- or nanoparticles is possible. The size-dependent deposition of particles in the inflamed tissue should be given particular consideration in the design of new carrier systems for the treatment of inflammatory bowel disease. Moreover, these results suggest the need for further development of nanoparticulate carriers for IBD.

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