



Enzyme/pH dual sensitive polymeric nanoparticles for targeted drug delivery to the inflamed colon



Muhammad Naem, Wooseong Kim, Jiafu Cao, Yunjin Jung, Jin-Wook Yoo*

College of Pharmacy, Pusan National University, Busan 609-735, South Korea

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ABSTRACT

Novel nanoparticles whose drug release profiles are controlled by both enzyme and pH were prepared for the colon-specific drug delivery using a polymeric mixture of enzyme-sensitive azo-polyurethane and pH-sensitive Eudragit S100 (ES-Azo.pu). The enzyme/pH dual sensitive nanoparticles were designed to release a drug based on a two-fold approach which specifically aimed to target drug delivery to the inflamed colon while preventing the burst release of drugs in the stomach and small intestine. Single pH-sensitive (ES) and dual sensitive (ES-Azo.pu) nanoparticles were prepared using an oil-in-water emulsion solvent evaporation method and coumarin-6 (C-6) was used as a model drug. The successful formation of ES and ES-azo.pu nanoparticles that have 214 nm and 244 nm in mean particle size, respectively, was confirmed by scanning electron microscopy and qNano. ES nanoparticles showed almost 100% of burst drug release at pH 7.4, whereas ES-Azo.pu nanoparticles prevented the burst drug release at pH 7.4, followed by a sustained release phase thereafter. Furthermore, ES-Azo.pu nanoparticles exhibited enzyme-triggered drug release in the presence of rat cecal contents obtained from a rat model of colitis. An in vivo localization study in rat gastrointestinal tract demonstrated that ES-Azo.pu nanoparticles were selectively distributed in the inflamed colon, showing 5.5-fold higher C-6 than ES nanoparticles. In conclusion, the enzyme/pH dual sensitive nanoparticles presented in this study can serve as a promising strategy for colon-specific drug delivery against inflammatory bowel disease and other colon disorders.

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1. Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn's disease [1], is characterized by relapsing and remitting episodes of active inflammation and chronic mucosal injury [2]. The etiology of IBD remains unclear, but many studies have shown several genetic and environmental factors contribute to its pathogenesis [3,4]. The most common symptoms of IBD are diarrhea, bloody stools, weight loss, abdominal pain, fatigue, and fever [5]. The basic aims of IBD treatment are the induction and maintenance of remission, which facilitates mucosal healing [6,7].

Colon-specific drug delivery is a desirable approach in the topical treatment of IBD due to increased drug availability to the inflamed colon [8,9]. Various approaches to colon-targeted drug delivery such as prodrugs, pH-sensitive polymer coatings, time-dependent release systems, and enzyme-dependent release systems have been developed using conventional dosage forms including capsules, tablets, and pellets [10]. However, the use of

conventional dosage forms are subjected to accelerated elimination due to IBD-associated high frequency diarrhea, which diminishes their ability to deliver a drug to the colon [11–13]. Therefore, strategies to deliver a drug specifically and sufficiently to the inflamed colon for a prolonged period are required for successful treatment of IBD.

The majority of commercialized systems for local drug delivery to the colon are based on the pH changes during gastrointestinal tract (GIT) passage [14]. pH-sensitive polymers such as methacrylate copolymer (Eudragit S100 (ES)) are most widely used for the systems owing to their characteristic dissolution at pH values above 7.0 [15]. Considering the normal pH in the ileum which is above pH 7.0, however, the prematurely released drug from a pH-dependent system in the ileum can be systemically absorbed, resulting in drug loss before reaching the colon as well as unwanted systemic side effects [16]. In this regard, a pH-dependent system per se is not suitable for efficient colon-targeted drug delivery.

Another strategy for colon-targeted drug delivery is to utilize enzymatic reduction of azo-containing polymers by colonic microflora that triggers drug release [17]. Among various azo-containing polymers, Yamaoka et al., synthesized a linear type azo-polyurethane (Azo.pu) by reacting isophorone diisocyanate

* Corresponding author. Tel.: +82 51 510 2807.

E-mail address: jinwook@pusan.ac.kr (J.-W. Yoo).

with a mixture of *m,m'*-di(hydroxymethyl)azobenzene (azo-aromatic segment), polyethylene glycol (hydrophilic segment), and 1,2-propanediol (hydrophobic segment) [18]. By reduction of the azo group, the pellets coated with polyurethane film became fragile and started releasing the drug, indicating that Azo.pu would have potential applicability to colon-specific drug delivery [19].

Recently, nanoparticle-based systems have emerged as a new strategy for targeting IBD due to their distinctive ability to accumulate in inflamed tissues in the colon [20,21]. In human patients and in animal models, the nanoparticle were taken up more readily by inflamed mucosa than larger sized carriers [22,23]. Therefore, a colon-specific drug delivery strategy combined with nanotechnology would offer a promising approach to the targeting of inflamed areas in colon.

In the present study, dual-sensitive nanoparticles (ES-Azo.pu nanoparticles) were prepared using a combination of the pH-sensitive ES and the enzyme degradable Azo.pu. This combination was chosen to avoid burst drug release in the stomach and the small intestine [25] and to facilitate drug release in the diseased colon by the enzymatic degradation of the ES-Azo.pu nanoparticles. We also prepared single pH-sensitive nanoparticles (ES nanoparticles) for comparison of their colon-targeting ability with ES-Azo.pu nanoparticles. Coumarin-6 (C-6), which is widely used as a hydrophobic model drug in various drug delivery systems, was selected as a model drug and loaded into nanoparticles because many of drugs that are used for the treatment of IBD, such as glucocorticoids and immunosuppressants, are hydrophobic in nature [24]. The nanoparticles were characterized for size, shape and drug loading capability. The drug release profiles from the nanoparticles were evaluated in different pH environments resembling those of the GIT and in the presence and absence of rat cecal contents containing azo-reductase. In vivo localization of the nanoparticles in the GIT was also evaluated in a rat model of colitis.

2. Materials and methods

2.1. Materials

Eudragit S100 (ES) was generously donated by Evonik Korea Ltd (Seoul). The fluorescent dye coumarin-6 (C-6) (98%), polyvinyl alcohol (PVA, mol wt 30,000–70,000), isophorone diisocyanate (IPDI), 1,2-propanediol (PD), poly (ethylene glycol) (PEG Mn = 2000) and tin octanoate were purchased from Sigma-Aldrich (St. Louis, MO, USA). *m,m'*-Di (hydroxymethyl) azobenzene (DAB) was prepared as described in the literature [26]. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents and solvents were of the highest analytical grade commercially available.

2.2. Synthesis and characterization of azo-containing polyurethane

Azo-polyurethane (Azo.pu) was synthesized using a previously reported method [18]. Briefly, 5.85 g (24.2 mmol) of DAB and 15.7 g (7.8 mmol) of PEG were placed in 300 mL round bottom flask equipped with a mechanical stirrer and a dropping funnel. The flask was evacuated using a vacuum pump for a few hours and flushed with dry nitrogen to dry the contents. Then, 6.7 g (88 mmol) of PD and 0.11 g of tin octanoate were added, and the flask was heated to 120 °C with stirring under nitrogen flow. IPDI 6.7 g (88 mmol) was then added drop wise using a dropping funnel over 4 h. Stirring was maintained after the addition until the viscosity of the contents increased to the required degree. A few grams of ethanol were then added to stop the polymerization, and the product was dissolved in 80 mL of ethanol and poured into 1000 mL of diethyl

ether for precipitation. After filtration, the precipitate was dried in a vacuum oven. The prepared Azo.pu, which had a brown glassy appearance, was characterized by ¹H nuclear magnetic resonance (NMR) spectroscopy (Varian 400 MHz). The UV spectrum of the polymer was measured after dissolving it in chloroform using a UV/VIS spectrophotometer (Optizen 2120, Mecasys, Korea).

2.3. Nanoparticles preparation

ES nanoparticles and ES-Azo.pu nanoparticles were prepared by an oil-in-water emulsion/solvent evaporation method with some modifications [27]. Briefly, 100 mg of ES or ES-Azo.pu (1:1, w/w) was dissolved with C-6 (2 mg) in a 10 mL of acetone and ethanol (7:3, v/v). This solution was slowly injected using a syringe pump at a flow rate of 0.33 mL/min into 40 mL of citrate buffer (pH 5.0) containing 0.1% (w/v) PVA solution with stirring. After evaporating residual solvent under a fume hood, the nanoparticles were collected by centrifugation at 20,000 × g for 30 min and washed with deionized water three times. The obtained nanoparticles were immediately used for the following experiments.

2.4. Characterization of nanoparticles

2.4.1. Scanning electron microscopy (SEM)

The morphology of nanoparticles was analyzed by SEM. Nanoparticles suspended in water were dropped on a carbon tape and air dried at room temperature in a fume hood or desiccator. Samples were then coated with platinum for 2 min in a vacuum and viewed by field emission scanning electron microscopy (FE-SEM, S4800, Hitachi, Japan) at an acceleration voltage of 1–5 kV.

2.4.2. Particle size analysis

A qNano size analyzer (Izon Sciences, Christchurch, New Zealand) coupled with an air based variable pressure module (VPM) was used for nanoparticles size determinations using 200 nanopore and 200 nm calibration particles. Nanoparticles and calibration particles (5 μL) were suspended separately in 1000 μL of Izon Tris buffer electrolyte and sonicated for at least 30 min prior to use. First, the nanopore and cells were cleaned with electrolyte and a baseline current (70–140 nA) was developed. Diluted nanoparticles or calibration particles (40 μL) were loaded in the upper fluid cell and the lower fluid cell was filled with 80 μL of electrolyte. All samples were run under the same applied voltage (0.5 V), average current (90 ± 3 nA), stretch (47 mm), and pressure gauge was at 7. Each recorded measurement was based on at least 500 particles. Particle sizes were determined using Izon control suite 2.2 software.

2.4.3. Drug loading and entrapment efficiency

The entrapped drug in nanoparticles was determined using a fluorescence multi-well plate reader (Tristar LB941, Berthold). A specific amount of ES nanoparticles or ES-Azo.pu nanoparticles were dissolved in ethanol/dimethyl sulfoxide (DMSO) mixture (1:1, v/v). After suitable dilutions, each sample (200 μL) was transferred to a 96-well plate and the C-6 fluorescence intensity ($\lambda_{Ex} = 460$ nm, $\lambda_{Em} = 505$ nm) was measured immediately. Samples were protected from light throughout the sample preparation. Samples were prepared in triplicate and encapsulation efficiency (%) of the drug was calculated using the following equation.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Amount of C-6 in nanoparticles}}{\text{Amount of C-6 initially added}} \times 100$$

2.4.4. Differential scanning calorimetry (DSC)

The physical status of the entrapped drug in nanoparticles was analyzed by DSC (N-650, SCINCO, Seoul, Korea). Samples (6 mg) of

C-6, ES, Azo.pu, and the nanoparticles were accurately weighted into aluminum pans and then hermetically sealed with aluminum lids. The DSC thermograms of samples were obtained by heating from 25 to 300 °C at a scanning rate of 10 °C/min under dry nitrogen. Empty pans were used as reference.

2.5. *In vitro* drug release in different pH

In vitro drug release from ES nanoparticles and ES-Azo.pu nanoparticles was evaluated in gradually pH changing buffers at pH values of 1.2, 4.0, and 7.4 [28]. These pH values reflect the increase in pH along the GIT and correspond to the stomach, upper small intestine, and colon, respectively [29]. Nanoparticles (10 mg) were added to 50 mL of the release medium and incubated in a shaking water bath (60 rpm, 37 °C). Tween-80 (5%, w/v) was added to the release medium to facilitate the solubilization of C-6 released from nanoparticles. At predetermined time intervals, 150 μ L aliquots were sampled and volumes were made up with fresh buffer solution. The aliquots were centrifuged at 17,000 \times g for 30 min and supernatants containing C-6 released from the nanoparticles were analyzed using a fluorescence plate reader as described above.

2.6. Induction of colitis

All animal experiments were performed in accordance with the regulations of Pusan National University and Korean

legislation on animal studies. Male Sprague-Dawley (SD) rats (290–320 g, 9 weeks old) were purchased from Samtako Bio Korea (Osan, Korea) and housed in the university animal facility at a 25 \pm 3 °C under a 12 h light/dark cycle controlled rooms. Colitis was induced by the previously reported method [30]. Briefly, before induction of colitis, rats were starved for 24 h but had free access to water. The rats were lightly anesthetized with ether and a rubber cannula (o.d., 2 mm) was inserted rectally into the colon such that the tip was 8 cm proximal to the anus, approximately at the splenic flexure. TNBS dissolved in 50% (v/v) aqueous ethanol was instilled into the colon via the rubber cannula (15 mg/0.3 mL/rat).

2.7. *In vitro* drug release in the presence of rat cecal contents

To assess the degradation of Azo.pu by colonic microbial enzymes, drug release from ES-Azo.pu nanoparticles was studied in the presence of rat cecal contents obtained from a rat model of colitis. Prior to the release study, blank ES-Azo.pu nanoparticles containing no drug were administered directly in rat stomachs by oral gavage for five days to induce the expressions of enzymes capable of reducing the azo-bond of Azo.pu. On the day of experiment, rats were sacrificed and cecal contents were collected inside nitrogen chamber. Cecal contents (5%) were dispersed in PBS (pH 5.5) [31–33] and used for drug release study from ES-Azo.pu nanoparticles in a nitrogen chamber (60 rpm, 37 °C). The release study

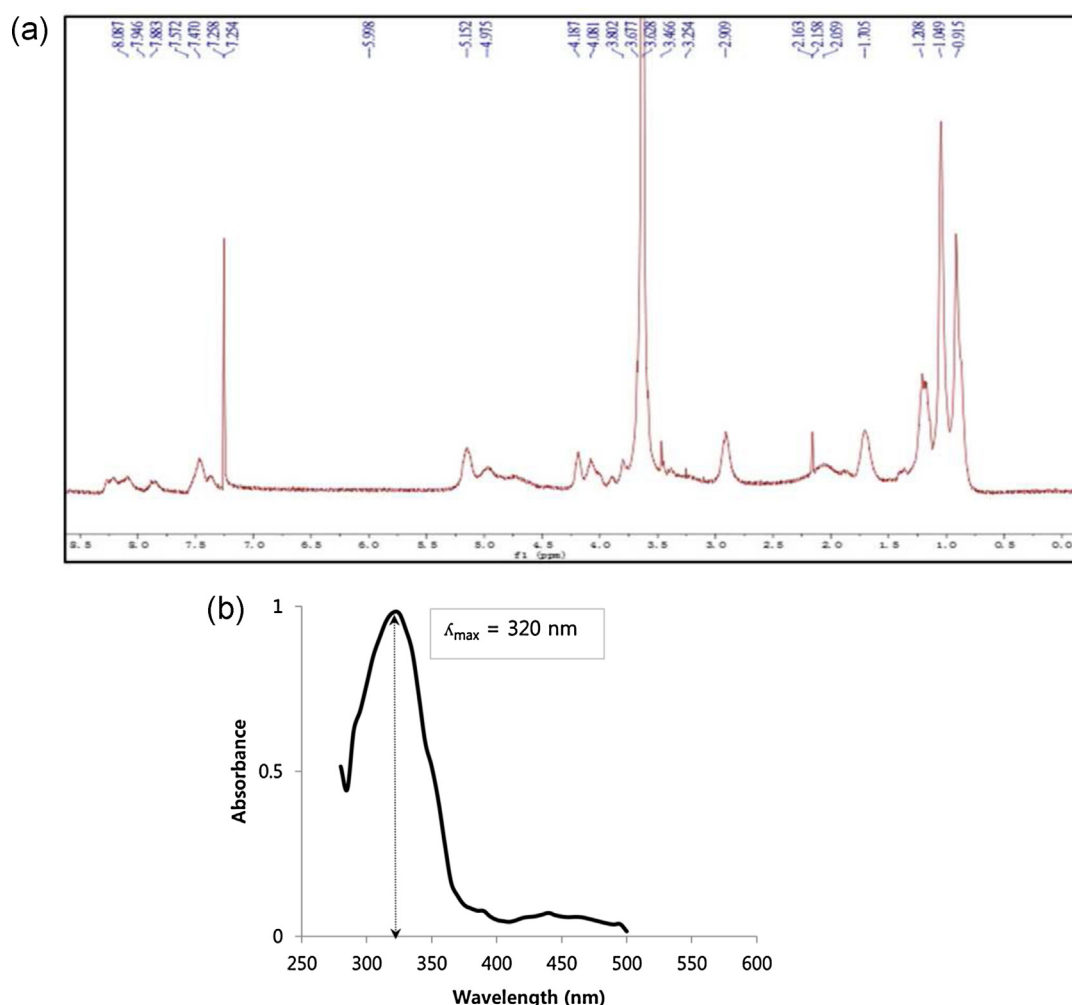


Fig. 1. Characterization of azo-pu. (A) ¹H NMR spectrum and (B) UV-vis spectrum.

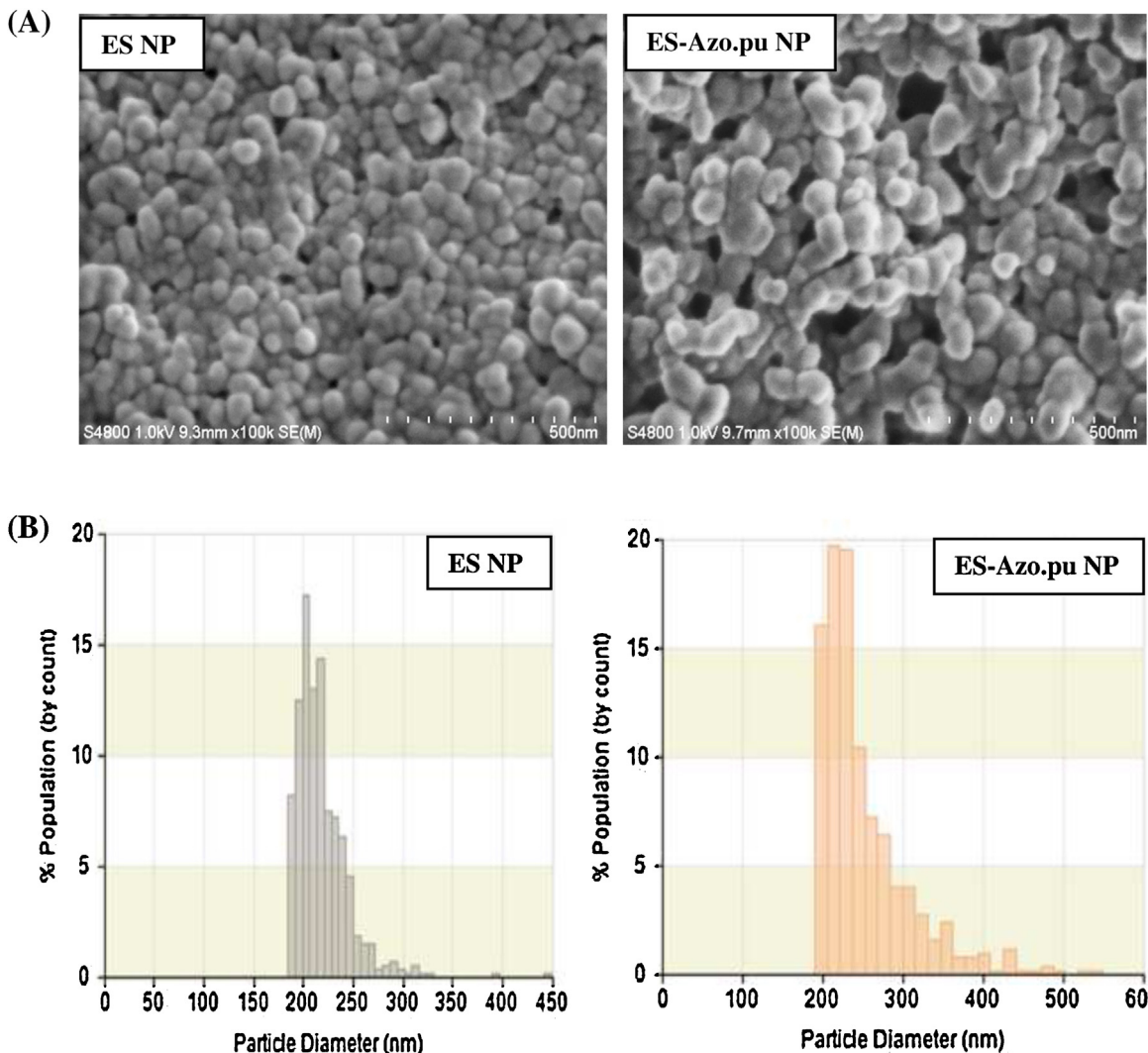


Fig. 2. Characterization of ES nanoparticles and ES-Azo.pu nanoparticles. (A) SEM images and (B) size histogram by qNano.

was carried out in a nitrogen chamber because colonic microflora requires an anaerobic environment. Sample aliquots were withdrawn at specific time intervals. After centrifugation, amounts of C-6 released were determined using a fluorescence plate reader as described above.

2.8. *In vivo* localization in GIT

In vivo localization of ES nanoparticles and ES-Azo.pu nanoparticles in GIT was evaluated using a colitis rat model to mimic IBD environments. Food was withheld from all rats 24 h prior to the administration. The rats were divided randomly into three groups ($n=3$). The first group was given C-6 solution in 10% ethanol containing 0.1% Tween-80. The second and third groups were given ES nanoparticles and ES-Azo.pu nanoparticles suspended in PBS (pH 5.0), respectively. The C-6 solution and the nanoparticles at a C-6 dose of 0.15 mg/kg were orally administered by oral gavage under mild ether anesthesia and the rats were sacrificed 8 h after administration. The all GIT segments including luminal contents were collected and divided into four sections (stomach, small intestine, cecum, and colon). The tissue samples were homogenized in PBS and then subsequently extracted with ethanol/DMSO mixture (1:1 v/v). C-6 contents in samples were assayed using a fluorescence plate reader as described above.

3. Results and discussion

3.1. Characterization of Azo.polyurethane

Azo.pu was characterized by ^1H NMR spectroscopy as shown in Fig. 1A. Deuterated chloroform (CDCl_3) was used as an internal standard. ^1H NMR (in CDCl_3): $\delta=0.915$ (s, CH_3 for IPDI unit), 1.049 (s, CH_3 for IPDI unit), 1.208 (m, CH_3 for PD unit), 1.705 (broad m, CH_2 for PD unit), 2.909 (broad m, CH_2 for IPDI unit), 3.582 (broad m, CH for IPDI unit), 3.628 (s, CH_2 for PEG unit), 4.081 (broad m, CH_2 for IPDI unit), 4.905 (broad m, CH for PD unit), 5.151 (broad m, CH_2 for DAB unit), 7.304–8.208 ppm (m, C_6H_4 for DAB unit). These values are identical to those from the previously reported ^1H NMR spectrum of Azo.pu [18]. We also characterized the prepared Azo.pu by UV-spectroscopy. Fig. 1B shows the UV spectrum of the polymer dissolved in the chloroform. The maximum absorption band was observed at a wavelength of 320 nm, which is attributed to the characteristic absorption of the azo aromatic chromospheres. The ^1H NMR and UV spectra results confirmed the successful synthesis of Azo.pu.

3.2. Preparation and characterization of nanoparticles

ES nanoparticles and ES-Azo.pu nanoparticles were prepared using the oil-in-water emulsion/solvent evaporation method [27].

To find the optimal fabrication method for ES-Azo.pu nanoparticles, various solvent systems and polymer compositions were tested. A single solvent system such as acetone and ethanol has been used for ES nanoparticles, but severe aggregations were occurred during fabrication of ES-Azo.pu nanoparticles due to the solubility difference between ES and Azo.pu. To increase the solubility of the two polymers, we developed a co-solvent system of acetone and ethanol (7:3, v/v), where a cloudy suspension of ES-Azo.pu nanoparticles was produced without aggregations. Different compositions of the two polymers were also examined for further optimization. ES-Azo.pu at a composition of 1:2 (w/w) resulted in sticky aggregates, while fine emulsions were produced at 1:1 (w/w). This can be explained by the sticky nature of Azo.pu unlike ES which is a powdered polymer. In some batches, minor polymer aggregates were removed by slow centrifugation speed ($100 \times g$ for 5 min) first and then nanoparticles were separated by high speed centrifugation ($20,000 \times g$ for 30 min). The pH of the aqueous phase was maintained at pH 5.0 to prevent solubilization of ES during the fabrication. Finally, spherical nanoparticles with high yields, uniformed sizes and high encapsulation efficiency were obtained as shown in Table 1 and Fig. 2A.

Size is considered an important factor in the development of colon-specific drug delivery strategies for the treatment of IBD because carrier size impacts their accumulation in the inflamed colon [22]. Particle size was determined using qNano size analyzer (Izon Sciences) (Table 1). The mean sizes of ES nanoparticles and ES-Azo.pu nanoparticles were 214 ± 27 nm and

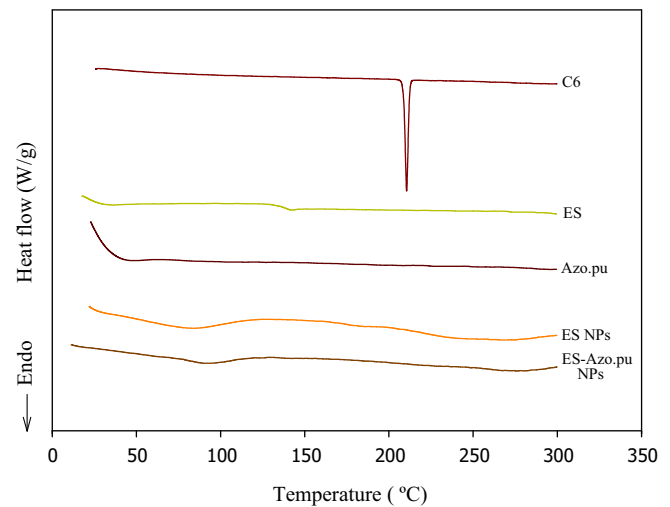


Fig. 3. DSC thermogram of C-6, polymers and the nanoparticles.

244 ± 38 nm, respectively. ES and ES-Azo.pu nanoparticle size histograms showed that the most nanoparticles fell in the size range 200–250 nm (Fig. 2B). All measurements were obtained under the same conditions of applied voltage, stretch, and pressure.

The physical statuses of entrapped drug in the nanoparticles were evaluated by DSC as shown in Fig. 3. The endothermic peak

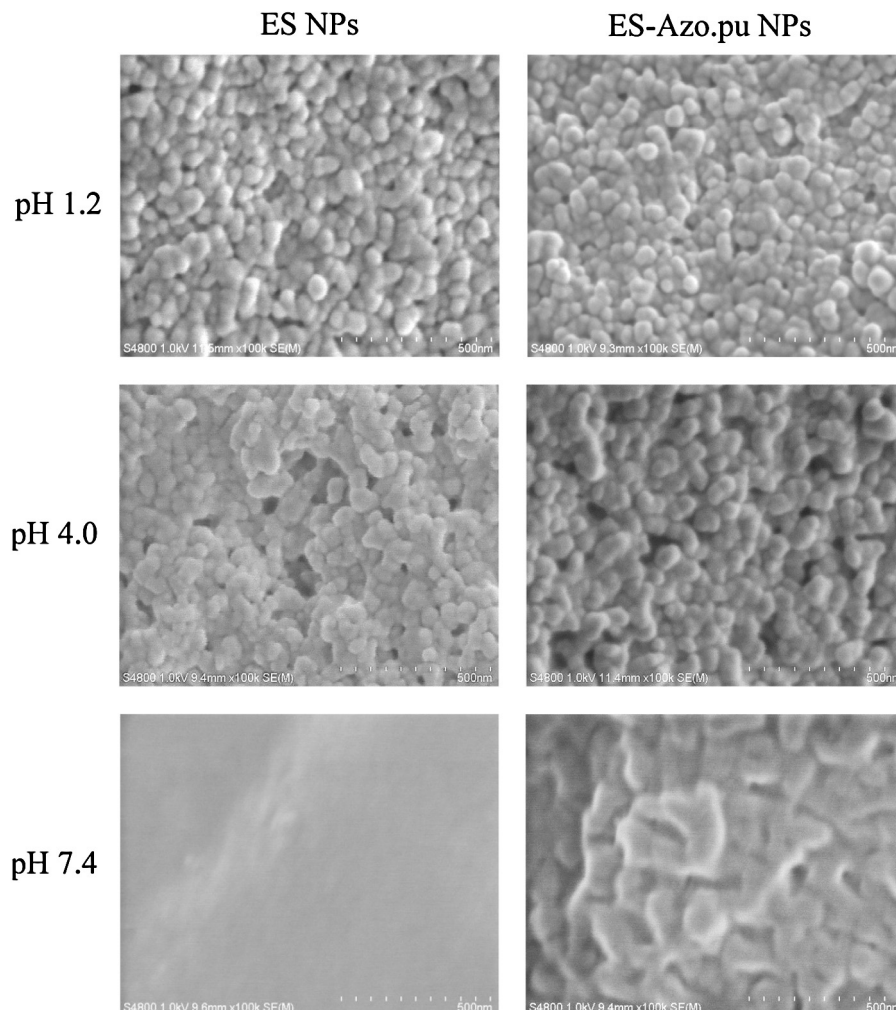


Fig. 4. Morphological changes of ES nanoparticles and ES-Azo.pu nanoparticles in different pHs. The nanoparticles were incubated for 2 h at pH 1.2 and 4 h for pH 4.0 and 7.4.

Table 1
Physicochemical characteristics of ES nanoparticles and ES-Azo.pu nanoparticles.

Formulation	ES:Azo.pu (mg)	Cosolvent ^a (mL)	Yield ^b (%)	Encapsulation efficiency ^b (%)	Particle size ^b (nm)
ES NPs	100:0	10	84 ± 4	52 ± 2.4	214 ± 27
ES-Azo.pu NPs	50:50	10	71 ± 6.1	58 ± 3	244 ± 38

^a Acetone:ethanol (7:3).

^b Results are expressed as mean ± S.D. (n = 3).

(i.e. melting point) of C-6 in DSC curves was observed at 210 °C whereas no C-6 peaks were found in the nanoparticles, indicating that C-6 was present in molecularly dispersed state and well encapsulated in nanoparticles.

3.3. pH-dependent morphological changes

To analyze the morphological stability and degradation behavior of the nanoparticles in GIT, the nanoparticles were suspended in buffers of different pH values (pH 1.2, 4.0, or 7.4) and incubated in a shaking water bath (60 rpm, 37 °C) for specific times. These conditions were chosen to mimic GIT pHs during the passage from the stomach to the colon. First, ES and ES-Azo.pu nanoparticles were incubated in pH 1.2 buffers for 2 h to mimic stomach pH and passage time. Similarly, particles were incubated in pH 4.0 buffer for 4 h to mimic the upper small intestine pH and passage time. Nanoparticles were also incubated at pH 7.4, representing ileum and colonic pH for 4 h. Changes in morphology of the nanoparticles were observed by SEM (Fig. 4). SEM images showed no significant

morphological change in ES nanoparticles at pH 1.2 and 4.0. This result was predictable because ES is a pH-sensitive polymer which does not dissolve at pH 4.0, thus water cannot enter the particle and there was no swelling. Interestingly, the morphology of ES-Azo.pu nanoparticles was maintained at pH 1.2 or 4.0 without swelling or degradation, implying that Azo.pu is also stable in acidic conditions. At pH 7.4, ES nanoparticles quickly dissolved because of their pH-dependent solubility. However ES-Azo.pu nanoparticles exhibited swelling behavior at pH 7.4 while maintaining their nanoparticulate morphology, implying that they can reach the colon in a particulate form. The swelling behavior of ES-Azo.pu nanoparticles can be attributed to the hydrophilic property of ES at pH 7.4 and the hydrophilic PEG segment in Azo.pu [18].

3.4. pH-dependent drug release

Following the morphological study, we investigated the effects of pH on drug release from the nanoparticles. The pH dependent drug release from ES nanoparticles and ES-Azo.pu nanoparticles were evaluated at different pHs (1.2, 4.0, and 7.4). ES-Azo.pu nanoparticles exhibited different pH release profiles from ES nanoparticles (Fig. 5A). At pH 1.2 and 4.0 (i.e. pH of the stomach and the upper part of small intestine, respectively), there was no significant difference in the drug release profiles between both nanoparticles, showing that less than 20% of the drug were released during the first 6 h possibly due to the slow diffusion or loosely attached drug on the surface of nanoparticles. At pH 7.4 (i.e. pH of the ileum), however, ES nanoparticles and ES-Azo.pu nanoparticles showed markedly different release profiles. ES nanoparticles showed a sudden burst release (nearly 100%) of the drug due to their complete dissolution at pH 7.4 as shown in Fig. 4, confirming that the pH-dependent system would not be suitable for colon-specific delivery due to the burst drug release in the ileum. The premature drug release in the ileum can result in the systemic

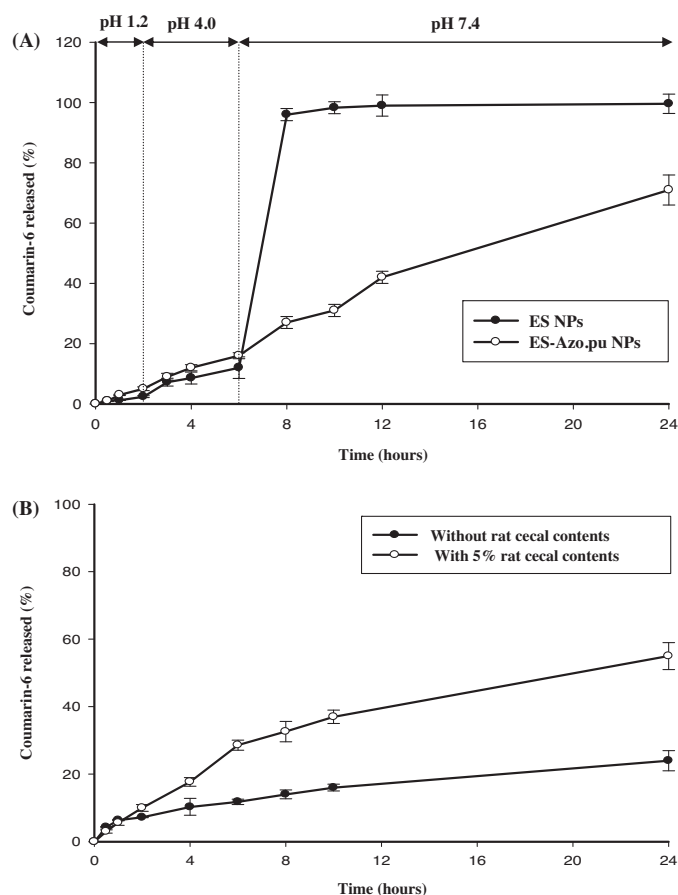


Fig. 5. In vitro drug release from (A) ES nanoparticles and ES-Azo.pu nanoparticles in different pHs and (B) ES-Azo.pu nanoparticles in the presence and absence of rat cecal contents obtained from a rat model of colitis. Results are presented as mean ± S.D. (n = 3).

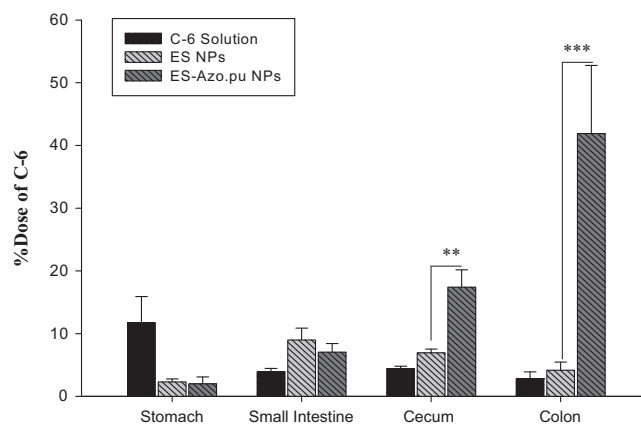


Fig. 6. In vivo localization of ES nanoparticles and ES-Azo.pu nanoparticles. The % dose of C-6 in GIT segment (stomach, small intestine, cecum, and colon) in a rat model of colitis was calculated 8 h after the oral administration of C-6 solution, ES nanoparticles and ES-Azo.pu nanoparticles (**p < 0.01, ***p < 0.001). Statistical analysis was performed using the paired *t* test in Sigma Plot 10.0 (SYSTAT, Inc., Chicago, IL, USA) on the data set of ES-Azo.pu vs. ES nanoparticles in the cecum and colon. Results are presented as mean ± S.D. (3 rats per group).

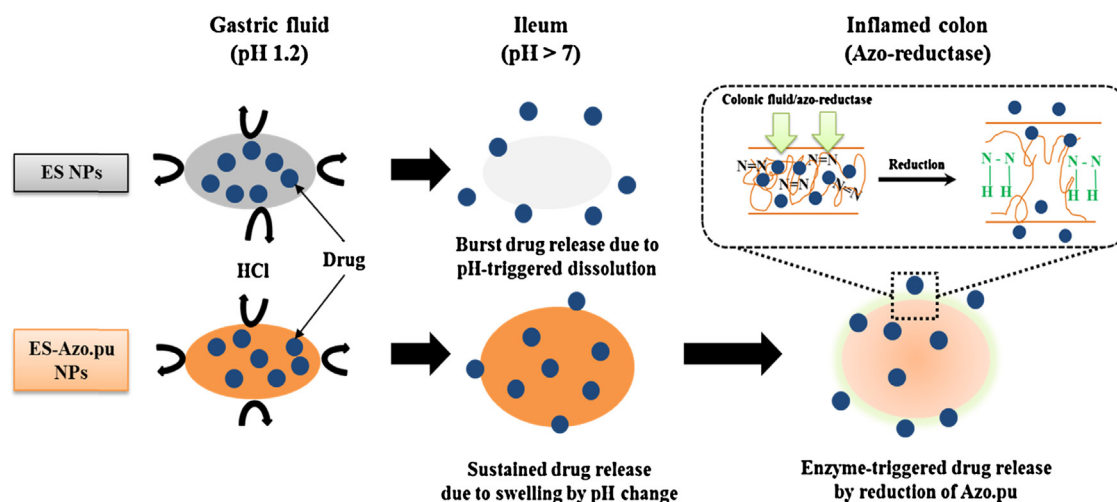


Fig. 7. Proposed mechanism of drug release from the colon-targeted nanoparticles under GIT-mimicking conditions.

absorption, leading to unwanted side effects as well as insufficient drug delivery to the colon. On the other hand, ES-Azo.pu nanoparticles exhibited no drastic change in drug release at pH 7.4 but slightly faster drug release than at acidic pHs. The increased release rate can be explained by the swelling of ES-Azo.pu nanoparticles at pH 7.4 as discussed and shown in Fig. 4. These results demonstrated that unlike ES nanoparticles, ES-Azo.pu nanoparticles can efficiently retain the entrapped drug from the stomach until reaching the colon to increase the drug availability in the colon, which is a desirable approach for colon-targeted delivery.

3.5. Enzyme-triggered drug release

After the drug release study in GIT-mimicking pHs, the next question was whether ES-Azo.pu nanoparticles has an ability to sufficiently release a drug in the diseased colon. The drug release from ES-Azo.pu nanoparticles was examined in the presence and absence of rat cecal contents obtained from a rat model of colitis, which contain azo-reductase produced by the colonic microflora which is responsible for reduction of the azo group to hydrazo group in Azo.pu. The drug release study was performed at pH 5.5 to mimic the colonic fluid environment of IBD patients [34]. In the absence of rat cecal contents, ES-Azo.pu nanoparticles showed the slow drug release profile (Fig. 5B), which is consistent to drug release in acidic pHs (Fig. 5A). In the presence of rat cecal contents, the drug release profile during the first hour was similar to that in their absence because it takes time for ES-Azo.pu nanoparticles to absorb water and swells in order for microbial flora to have an access to the azo groups in the nanoparticle for the enzymatic reduction. In other words, azo reductase first reduces the surface azo groups to hydrazo groups for making the particle surface loose and facilitating the penetration of colonic fluid into the nanoparticles [18]. Following the first hour, significantly increased drug release from ES-Azo.pu nanoparticles were observed in the presence of rat cecal contents as compared to their absence, indicating that the reduction of the azo group to hydrazo group by azo-reductase makes ES-Azo.pu nanoparticles fragile and quickly release the entrapped drug.

3.6. In vivo localization in the rat GIT

The nanoparticles were orally administered to rats with colitis to evaluate the localization and their colon-specific delivery. The % dose of C-6 in each GIT segment (stomach, small intestine,

cecum, and colon) was assayed 8 h after the oral administration of C-6 solution, ES nanoparticles and ES-Azo.pu nanoparticles (Fig. 6). C-6 solution exhibited relatively high C-6 level in the stomach but negligible amounts in other GIT segments including the cecum and the colon. The total % dose of C-6 solution in GIT was around 20%, implying that most of C-6 was absorbed in GIT. For ES nanoparticles, very low levels of C-6 were found in the cecum (7%) and the colon (4%), confirming that a pH-sensitive system per se is not suitable for colon-specific drug delivery. The total % dose of ES nanoparticles in GIT was around 32%. Considering the nearly 100% drug release from ES nanoparticles at the ileum pH as shown in Fig. 5A, it can be assumed that most of the C-6 is released and absorbed in the ileum and only a few remaining C-6 was transferred to the cecum and the colon. ES-Azo.pu nanoparticles also showed low C-6 levels in stomach and small intestines. However, % dose of C-6 with ES-Azo.pu nanoparticles was 2.5- and 5.5-fold higher than that of ES nanoparticles in the cecum (17%) and colon (42%), respectively, demonstrating that the dual sensitive nanoparticles enables the avoidance of premature drug release in the stomach and small intestine and deliver the entrapped drug specifically to the colon.

All of the above results suggest that the ES-Azo.pu nanoparticles have the ability to deliver a sufficient amount of drug specifically to the inflamed colon. The proposed drug release mechanisms of the novel enzyme/pH sensitive nanoparticles in different GIT segments are summarized in Fig. 7. These autonomous and complementary release mechanisms incorporated into the devised polymer system should overcome the limitation associated with the single-triggered release approach and improve site-specificity.

4. Conclusions

Novel enzyme/pH dual sensitive ES-Azo.pu nanoparticles were successfully prepared by the modified oil-in-water emulsion/solvent evaporation method. In vitro release profiles showed that the entrapped drug was retained by ES-Azo.pu nanoparticles at acidic conditions, but released in a sustained manner at a physiological pH and in the presence of rat cecal contents. This result was further supported by in vivo drug localization study in rat GITs, which showed that ES-Azo.pu nanoparticles achieved significantly high levels of C-6 in the inflamed colon than ES nanoparticles. Accordingly, we conclude that the dual sensitive approach combined with the use of nanoparticles provides a promising strategy for colon-specific drug delivery. Further studies in experimentally

induced colitis animal models are required to evaluate the efficacy of these carriers in the treatment of IBD.

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