Pharmaceutical nanotechnology

pH-sensitive nanoparticles for colonic delivery of curcumin in inflammatory bowel disease

Ana Beloqui a,1, Régis Coco a,1, Patrick B. Memvanga a,b, Bernard Ucakar a, Anne des Rieux a, Véronique Préat a,*

a Université catholique de Louvain, Louvain Drug Research Institute, Advanced Drug Delivery and Biomaterials, 1200 Brussels, Belgium
b University of Kinshasa, Faculty of Pharmaceutical Sciences, Laboratoire de Pharmacie galénique, BP 212 Kinshasa, Democratic Republic of the Congo

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A B S T R A C T
Nano-scaled particles have been found to preferentially accumulate in inflamed regions. Local delivery of anti-inflammatory drugs loaded in nanoparticles to the inflamed colonic site is of great interest for inflammatory bowel disease (IBD) treatment. Curcumin (CC) is an anti-inflammatory local agent, which presents poor ADME properties. Hence, we evaluated, both in vitro and in vivo, the local delivery of CC using pH-sensitive polymeric nanoparticles (NPs) combining both poly(lactide-co-glycolide) acid (PLGA) and a polymethacrylate polymer (Eudragit® S100). CC-NPs significantly enhanced CC permeation across Caco-2 cell monolayers when compared to CC in suspension. CC-NPs significantly reduced TNF-α secretion by LPS-activated macrophages (J774 cells). In vivo, CC-NPs significantly decreased neutrophil infiltration and TNF-α secretion while maintaining the colonic structure similar to the control group in a murine DSS-induced colitis model. Our results support the use of nanoparticles made of PLGA and Eudragit® S100 combination for CC delivery in IBD treatment.

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1. Introduction

Ulcereative colitis (UC) and Crohn’s disease (CD) are the two major forms of inflammatory bowel disease (IBD) (Alhauyek et al., 2011). IBD can be described as chronic but relapsing inflammatory disorders of the gastrointestinal tract. Whilst UC is characterized by inflammation that is limited to the colon, CD involves any part of the gastrointestinal tract, most commonly the terminal ileum or the perianal region (Khor et al., 2011). Abdominal pain, diarrhea, rectal bleeding and weight loss are frequent symptoms in patients suffering from IBD. For over four decades, the conventional treatment of IBD consisted of anti-inflammatory medications, such as corticosteroids, aminosalicylates, immunosuppressants and biologic agents (i.e., anti-TNF-α monoclonal antibodies) (Beloqui et al., 2013a; Talaei et al., 2013). However, these drugs present several side effects due to their unspecific efficacy upon administration (Rogler, 2010; Stallmach et al., 2010; Yang and Lichtenstein, 2002). A promising strategy toward IBD therapy would be to selectively target the inflamed colonic tissue in order to ameliorate drug related side effects and, thus, to increase its therapeutic efficacy (Grimpen and Pavli, 2010).

Among the existing drug delivery systems, nano-sized drug delivery systems represent a promising alternative for IBD treatment, mostly due to their preferential accumulation in the inflamed regions of the gut (Collnot et al., 2012; Ulbrich and Lamprecht, 2010). Increased mucus production, mucosal surface alterations, crypt distortions and ulcers, are associated with a disrupted intestinal barrier and infiltration of immune-related cells (i.e., macrophages, lymphocytes or dendritic cells). These pathophysiological changes lead to a higher accumulation of nanoparticles in the inflamed colonic regions compared to healthy tissues (Lamprecht et al., 2001). Poly(lactide-co-glycolide) acid (PLGA) is the most investigated, biocompatible and biodegradable polymer, which has been formulated as nanoparticles (NPs) toward IBD treatment (Collnot et al., 2012; Meissner et al., 2006). A nanoparticle size ~100 nm was found to provide PLGA NPs with a preferential uptake in the inflamed colonic mucosa, in both animals and humans (Schmidt et al., 2013). In addition to particle size, particle surface also plays a crucial role in the interaction with the mucus layer and epithelial cells (Lautenschläger et al., 2014). One of the most extensively evaluated strategies toward particle surface modification in IBD treatment is the enteric-coating, so that the drug release is triggered by the sensitivity of polymer to pH during gastrointestinal (GI) transit (Talaei et al., 2013). On this
regard, Eudragit® (polymethacrylate polymers) are the most commonly used polymers. Hence, we hypothesized that combining conventional PLGA nanoparticles with pH-triggered Eudragit®, might provide both specific accumulation in inflamed tissue and selective drug release in the colon (Makhlof et al., 2009).

Extracted from the turmeric pigment of Curcuma longa, curcumin (CC) is known for decades for its pleiotropic effects: anti-inflammatory, antioxidant, anti-carcinogenic, antimicrobial, hepatoprotective, anti-hyperlipidemic and anti-angiogenic. Anti-inflammatory effects of CC have been reported in several diseases such as Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, epilepsy or cerebral injury (Epstein et al., 2010; Goel et al., 2008). Recently, a prophylactic role of CC in IBD has been reported and explained by its beneficial role in both the regulation of the oxidant/anti-oxidant balance and the modulation of inflammatory mediators release, namely TNF-α and nitric oxide (NO) (Arafa et al., 2009). Sugimoto et al. (2002) reported that CC prevented and ameliorated experimental colitis in mice when mixed with the diet. However, CC presents several limiting factors, such as low solubility in water (i.e., 0.4 μg/mL at pH 7.3) and instability at neutral and alkaline pH values, which results in its low oral bioavailability associated with its high rate of metabolism and its rapid elimination (Memwanga et al., 2013a). Thus, an optimal and IBD-specific CC delivery system should improve its local delivery by protecting CC from fast degradation and by specifically targeting the inflamed colon.

This study aimed to evaluate combined PLGA/Eudragit® S100 pH-sensitive polymeric nanoparticles for a selective colonic CC release in IBD treatment. For this purpose, PLGA/Eudragit® S100 pH-sensitive polymeric CC-loaded NPs were evaluated (i) in vitro, on the basis of their physicochemical behavior, by evaluating their anti-inflammatory potential in reducing TNF-α secretion from activated macrophages (J774 cells) and their transport behavior across Caco-2 cell monolayers; and (ii) in vivo, in a murine dextran-sulfate (DSS)-induced colitis model, by quantitating neutrophil infiltration and TNF-α concentrations, by histologically evaluating inflammation severity in excised colons and by localizing CC-NPs.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals
Curcumin (~80% curcumin), O-dianisidine, hexadecyltrimethylammonium bromide (HTAB), PLGA (L:G 50:50, average Mw 7000–17,000, acid terminated), bovine serum albumin (BSA), hydrogen peroxide (30%), polyvinyl alcohol (PVA average 13,000–23,000), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), Triton X 100, tris(hydroxymethyl)aminomethane and anhydrous sodium carbonate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Eudragit® S100 (ES100) was a gift from Evonik RÖHM GmbH (Darmstadt, DE). Potassium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Merck (Darmstadt, DE). Rhodamine-phalloidin was purchased from Molecular Probes (Eugene, OR). UltraCruz® mounting medium was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dextran sodium sulfate (DSS) was purchased from TdB Consultancy (Uppsala, SE). Complete protease inhibitor cocktail tablets were purchased from Roche Diagnostics (ViVoorde, BE).

2.1.2. Cell culture
Caco-2 cells (clone 1) were kindly provided by Dr. Maria Rescigno, University of Milano-Bicocca (Milano, Italy) (Rescigno et al., 2001). J774 murine macrophages were kindly donated by Prof. Marie-Paule Mingeot (Université catholique de Louvain, LDRI, BE).

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin (PEST), Hank’s balanced salt solution (HBSS), phosphate buffered saline (PBS) and trypsin (0.05%) with EDTA were purchased from Gibco™ (Invitrogen, UK). Heat inactivated fetal serum (Hyclone®) was purchased from Thermo Scientific™ (Erembodegem, BE). Lipopolysaccharide (LPS, Escherichia coli O111:B4) was purchased from Sigma–Aldrich.

2.2. Preparation and characterization of the formulations

2.2.1. Preparation of pH-sensitive nanoparticles
Polymeric pH-sensitive PLGA/ES100 nanoparticles loaded with CC (CC-NPs) were prepared by an adaption of the modified spontaneous emulsification solvent diffusion method (SESD) (Makhlof et al., 2009). Briefly, 45 mg of PLGA, 45 mg of ES100 and 10 mg of CC were dissolved in 3 mL of an acetone/methanol mixture (2:1). Next, the solution was added dropwise in 20 mL of a 0.5% (w/v) PVA solution and centrifuged for 30 min at 45,000 × g and 4 °C. The nanoparticle suspension was then washed twice in distilled water. Supernatants were collected to evaluate encapsulation efficiency of CC using HPLC (Section 2.4).

2.2.2. Curcumin suspension
A CC suspension (CC-sus) was prepared as a control. CC (125 mg) was homogenously dispersed in PBS (10 mL) and the median particle diameter of the CC-sus was measured by laser diffraction (HELOS, Sympatec, Clausthal-Zellerfeld, DE).

2.2.3. Physicochemical characterization and drug encapsulation efficiency
The particle size and polydispersity index (PDI) of CC-NPs were determined by photon correlation spectroscopy (PCS) and the zeta potential was measured using Laser Doppler Velocimetry (LDV) (Malvern Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). The encapsulation efficiency (EE) of CC was estimated by measuring the amount of non-encapsulated CC present in the supernatants by HPLC after the dispersion was centrifuged (45,000 × g, 4 °C for 45 min). The total CC content was quantified by dissolving the CC-NPs in the mobile phase to release trapped CC. The resulting solution was subsequently analyzed by HPLC. The EE of CC is expressed as the percentage of (CC total – CC non-encapsulated)/CC total. All analyses were repeated in triplicate.

2.3. In vitro dissolution profiles

The in vitro release profile of pH-sensitive CC-NPs was evaluated using a pH-changing system (hydrochloric acid pH 1.5, buffer solution pH 4.5 and buffer solution pH 7.2) following the recommendations on methods for dosage forms testing described in the European Pharmacopeia 7.0. To the initial solution of pH 1.5, a dose of a powder mixture containing 2.28 g of tris(hydroxymethyl)aminomethane and 1.77 g of anhydrous sodium acetate was added to reach pH 4.5 and a second dose was added to the buffer solution pH 4.5 to reach pH 7.2. Briefly, 22.5 mg of CC-NPs were suspended in 50 mL of the dissolution medium under magnetic stirring (30 rpm, 37 °C). At prefixed times, withdrawn samples were centrifuged for 30 min at 45,000 × g and 4 °C. The resulting supernatants were collected and analyzed by HPLC in order to quantify non-encapsulated CC (Section 2.4).

2.4. Determination of CC by HPLC
Curcumin was quantified as previously reported by Memwanga et al. (2013a, 2014). HPLC for CC was performed using an Agilent
1100 Series HPLC system and an EC 250/2 Nucleodur 100-5C18 ec column (Macherey-Nagel, DE). The mobile phase contained methanol and 3.6% glacial acetic acid (73:27, v/v) (Cui et al., 2009). The system was run isocratically at a 0.2 mL/min flow rate and the injected sample volume was 50 μL. The sample detection was achieved at 428 nm. The retention time of CC was approximately 6 min under these conditions. The limits of detection (LOD) and of quantification (LOQ) of CC were 5 ng/mL and 15 ng/mL, respectively. The coefficients of variation (CV) for intra- and inter-assay were all within 5%.

2.5. In vitro studies in J774 murine macrophages and Caco-2 cells

2.5.1. Cell culture

Caco-2 cells were maintained in Dulbecco’s Modified Eagle’s Minimal Essential Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone, UK), 1% (v/v) L-glutamine and 1% (v/v) non-essential amino-acids at 37 °C in a 10% CO2 atmosphere. Caco-2 cells were used between passages x + 17 and x + 30, as previously described (Beloqui et al., 2013b; de Rieux et al., 2005; Memvanga and Preat, 2012).

The J774 macrophage cell line was maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were grown in 75 cm² flasks (Corning, MA, USA) at 37 °C in a 5% CO2/95% (v/v) air atmosphere.

2.5.2. Effect of pH-sensitive NPs on cell viability

The cytotoxicity of CC-NPs was evaluated on J774 cells and Caco-2 cells using the MTT method. Briefly, 20,000 cells/well were seeded in 96-well plates (Nunc, Roskilde, DK) and maintained for 24 h at 37 °C. Cells were after exposed to 100 μL of PLGA/ES100-CC NPs dispensed in culture medium for 4 h (J774 cells) or 2 h (Caco-2 cells) at different concentrations. Cells were then washed 3 times with HBSS and incubated for other 3 h with a 0.5 mg/mL MT solution in RPMI. The medium was then removed, and the purple formazan crystals were dissolved in 100 μL of DMSO. The absorbance was measured at 560 nm using a MultiSkan EX plate reader (Thermo Fisher Scientific, MA, USA). Cells with Triton-X 100 and cells with culture medium were considered as positive and negative controls, respectively. The IC50 for the different formulations were calculated using the GraphPad Prism 5 program (CA, USA). All assays were repeated in triplicate.

2.5.3. Permeability studies of pH-sensitive NPs across Caco-2 cell monolayers

Caco-2 cells were seeded at a density of 5 × 10⁵ cells/well on 12-well cell culture inserts (1 μm pore diameter, 0.9 cm² area) (Corning Costar™, NY) and were grown in supplemented DMEM + 1% PEST for 21 days (Beloqui et al., 2013b; de Rieux et al., 2005; Memvanga et al., 2013b; Memvanga and Preat, 2012). The culture medium was replaced every other day. TEER values were measured using an electrode connected to an Eيوم™ volt-ohmmeter (World Precision Instruments, USA). Only Caco-2 cell monolayers with initial transepithelial electrical resistance (TEER) values higher than 400 Ω cm² were used.

The permeability studies across Caco-2 monolayers were carried out by adding a volume of 0.5 mL of CC-NPs (1 mg/mL) dispersed in HBSS on the apical compartment of the inserts. This volume corresponded to 30 μg/mL of CC. A 0.5 mL volume of the CC suspension at a concentration of 150 μg/mL of CC was used as control. The basolateral compartment was filled with 1.2 mL of HBSS + 10 mM HEPES + 1% BSA. After 2 h of incubation, samples were collected from the basolateral compartment and the amount of CC of CC present in the receiver compartment was determined by HPLC (Section 2.4).

CC apparent permeability coefficient (Papp) was calculated according to the following equation (Kunwar et al., 2006):

\[
P_{\text{app}} = \frac{dQ}{dt} \times \frac{1}{C_A}
\]

where \(dQ/dt\) (transport rate) is the amount of CC (μg) appearing per time unit (s) in the receiver compartment, \(C_0\) is the initial concentration in the donor compartment (μg/mL) and \(A\) is the surface area of the monolayer (A = 0.9 cm²).

The integrity of the monolayers was corroborated by measuring the TEER before and after the permeability studies on day 21. TEER values after permeability studies were not significantly different to initial values unless otherwise stated.

2.5.4. Localization of CC-loaded NPs in Caco-2 cell monolayers

pH-sensitive CC-NPs were tested for their cell association behavior across Caco-2 cells monolayers. The localization of CC-NPs in Caco-2 cell monolayers was studied qualitatively by confocal laser scanning microscopy (CLSM). The Transwell inserts fixed in parafomaldehyde (PFA) 4% (v/v) were gently washed in HBSS. Actin was stained with 200 μL of rhodamine–phalloidin (1:50) in buffered HBSS + 0.2% (v/v) Triton-X 100 for 10 min in the dark to reveal cell borders, as described by Beloqui et al. (2013b). Consequently, inserts were cut and mounted on glass slides with an UltraCruz™ mounting medium for fluorescence containing DAPI. Images were captured using a Carl Zeiss™ confocal microscope (LSM 150) using the same settings with a 500 ms exposure time and a laser set at 85% for the 488 nm channel. The fluorescence of CC was detected at 488 nm (Kunwar et al., 2006). Data were analyzed by the Fiji software.

2.5.5. Inhibition of TNF-α production from J774 macrophages by pH-sensitive NPs

One hundred thousand J774 cells/well were seeded in 12-well plates and allowed to adhere for 24 h. The cells were exposed for 4 h to CC-NPs (0.122 mg/mL) or CC-sus (9 μg/mL). These concentration values were based on the cytotoxicity studies (Section 2.5.2). The incubation medium was harvested and stored for the lactate dehydrogenase (LDH) release assay and replaced with fresh medium supplemented with 0.1 μg/mL of LPS for activating macrophages (Fernandes et al., 2012; Kitamura et al., 2008). After 24 h later, supernatants were collected and centrifuged to discharged remaining cells, and stored at –20 °C for cytokine quantitation. TNF-α was quantitated using a Duoset® ELISA kit (R&D Systems, Minneapolis, MN, USA).

The LDH release assay was carried out using a commercial detection kit (Roche Diagnostics GmbH, Mannheim, DE) and used according to the manufacturer’s instructions. The results were expressed as a percentage of the positive control (cells exposed to Triton-X 100).

2.6. In vivo efficacy of CC-loaded pH-sensitive NPs in a murine DSS-induced colitis model

The in vivo study was carried out in C57BL/6 female mice (18–20 g, 8 weeks; Janvier Laboratories, FR) in accordance with the Université catholique de Louvain animal committee (UCL/MD/ 2009-001). Colitis was induced by administration of drinking water containing 3% (w/v) DSS for 5 consecutive days, and colonic inflammation was assessed 8 days after the beginning of DSS treatment (Coco et al., 2013; Larou et al., 2010). The animals were divided into five groups (8 mice per group): untreated control group (healthy mice), PBS-treated DSS group, blank-NPs-treated DSS group, CC-NPs-treated DSS group and CC-sus-treated DSS group. A 15 mg/kg dose was chosen as CC dose for all CC-treated
DSS groups. One hundred microliters of each formulation were administered daily by oral gavage during the 5 days of DSS administration. The untreated control group and the DSS control group received PBS instead of the formulation. At day 8, the animals were sacrificed, and colon samples were collected for evaluation of the severity of colitis.

2.6.1. Clinical activity scoring

The clinical activity score for weight loss, stool consistency and rectal bleeding was calculated as described by Melgar et al. (2005). Inflammation was scored on a scale of 0–4. Inflammatory progression in C57BL/6 mice was evaluated daily during the treatment period. Body weight loss was calculated with respect to the initial body weight (day 0) and expressed in percentage.

2.6.2. Myeloperoxidase activity (MPO)

The tissue-associated MPO assay was performed to quantitate the degree of inflammatory infiltration in the mice (Krawisz et al., 1984). Briefly, colon samples from both the proximal and the distal portion of the colon together (~25 mg) were collected, snap frozen in liquid N2 and stored at −80°C for later assessment. To determine the MPO activity, colon samples were homogenized in HTAB buffer (0.5% HTAB in 50 mM potassium phosphate buffer, pH 6). Ice-cold conditions were maintained all through the homogenization process. Homogenates were first centrifuged at 2000 × g for 10 min at 4°C and subsequently ultracentrifuged (15,000 × g, 20 min at 4°C). The supernatant (7 µL) was added to 96-well plates together with 200 µL of a 50 mM potassium phosphate buffer containing 0.167 mM/2 mg/mL O-diansisidine and hydrogen peroxide (500 ppm). Samples were analyzed in duplicate. MPO activity in the supernatant was measured spectrophotometrically at 460 nm for 30 min (Spectrophotometer Spectramax M2e and program SoftMax Pro Molecular Devices, LLC, USA). The results were expressed as MPO units per gram of protein, and one unit of MPO activity was defined as the amount that degrades 1 mmol/min of hydrogen peroxide at 25°C (Beloqui et al., 2013a).

2.6.3. Tissue cytokine quantitation by ELISA

The concentrations of TNF-α in the colon were determined by a sandwich-type ELISA technique using the Ready-Set-Go! Kit (eBioscience, Vienna, AU) according to the manufacturer’s instructions, as previously described by Beloqui et al. (2013a). The frozen colonic tissue samples were homogenized in 1 mL of extraction buffer (PBS with 1% SDS and 1 tablet of complete protease inhibitor cocktail (Roche Diagnostics, Vilvoorde, BE), per 10 mL of solution) with an UltraTurrax (IKA T18 Basic, Staufen, GE). Homogenates were then centrifuged first at 900 × g for 10 min and 4°C and then at 24,000 × g for 30 min and 4°C. The supernatants were collected and stored at −80°C for later assessment. Total protein concentration was determined using the Lowry method assay and the final TNF-α concentrations were normalized with the total protein of the respected sample.

2.6.4. Histological assessment of colonic inflammation

Small segments of the colon were fixed in 4% buffered formalin and after 24 h embedded in paraffin. Two sets of 3 serial sections (10 µm) were cut at a distance of 100 µm, and 6 sections were evaluated for each mouse. Sections were stained with hematoxylin-eosin, and histological scores were determined according to a scoring procedure as previously reported (Beloqui et al., 2013a).

2.6.5. In vivo localization of CC-NPs in healthy and inflamed tissues

The localization of CC-NPs in the gastrointestinal tract (GIT) in vivo was studied qualitatively by CLSM microscopy, in both healthy and DSS-induced colitis mice, in order to evaluate the selective accumulation of CC-NPs in the inflamed colonic tissues. Colitis was induced in mice as previously described in Section 2.6. After 7 days, mice received orally 100 µL of CC-NPs or CC-sus (3 mg/mL, respectively). Food (but not water) was withdrawn 12 h prior to the administration of the treatment. Mice were sacrificed 12 h after administration, and both proximal and distal colon sections were collected. Colon samples were then embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek, Torrance, CA) and frozen at −80°C for subsequent experiments. Sections were then cut at a thickness of 10 µm using a cryostat (Leica Microsystems, Wetzlar, GE) and mounted on glass slides. Images were captured using a Zeiss® confocal microscope (LSM 150).

2.7. Statistical analyses

Statistical analyses were performed using the GraphPad Prism 5.0 program (GraphPad Software Inc., San Diego, CA, USA). The normal distribution was assessed by the Shapiro–Wilks normality test. A one-way ANOVA test of multiple comparisons followed by Bonferroni’s post-hoc test was applied for the MPO analysis. All other analyses were performed using a Mann–Whitney non-parametric test. Differences were considered statistically significant at *p < 0.05. The results are expressed as mean ± SD (SD: standard deviation).

3. Results

3.1. Physicochemical characterization of nanoparticles

The particle characterization of CC-loaded or unloaded polymeric pH-sensitive NPs is summarized in Table 1. The size, PDI, zeta potential and encapsulation efficiency (EE) and drug loading of each formulation (n = 3; data are expressed as the mean ± SD).

<table>
<thead>
<tr>
<th>Size (nm)</th>
<th>ζ (mV)</th>
<th>PDI</th>
<th>EE (%)</th>
<th>Drug loading (%)(mg CC/100 mg polymer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank-NPs</td>
<td>122 ± 5</td>
<td>-39.8 ± 0.5</td>
<td>0.244 ± 0.04</td>
<td>–</td>
</tr>
<tr>
<td>CC-NPs</td>
<td>116 ± 3</td>
<td>-40.4 ± 0.6</td>
<td>0.261 ± 0.03</td>
<td>67 ± 8</td>
</tr>
</tbody>
</table>

3.2. Release profile of pH-sensitive CC-NPs

In vitro pH-dependent release profile of CC from the pH-sensitive nanocarriers was investigated in a gradually pH-changing buffer in order to assess the protection of the content from the acidity and an effective delivery in neutral pH values (Makhlof et al., 2009). CC was not released in the medium at pH 1.2 and 4.5 (<15% of CC released) (Fig. 1). Once the pH reached neutral values, CC was rapidly released.
3.3. In vitro studies in Caco-2 cells

3.3.1. Assessment of the CC-NPs cytotoxicity

The MTT test was used to evaluate the cytotoxicity of the PLGA/ES100-CC NPs and, thus, select the appropriate range of CC-NPs for the permeability studies in Caco-2 cells and the TNF-α secretion inhibition studies in J774 cells.

In the case of Caco-2 cells, the IC$_{50}$ was $\sim$3.0 mg/mL. In J774 cytotoxic studies, IC$_{50}$ was $\sim$0.5 mg/mL for NPs.

3.3.2. Transport of CC-loaded formulations across Caco-2 cell monolayers

In order to evaluate the apparent permeability ($P_{app}$) of CC from CC-loaded formulations, absorptive transport across Caco-2 cell monolayers was performed for 120 min (Fig. 2). To improve the recovery of CC by enhancing its solubility and its stability, a solution of 1% BSA + 10 mM HEPES in HBSS was used as receiver medium (Krishna et al., 2001; Leung and Kee, 2009). Based on the cytotoxicity studies, Caco-2 monolayers were incubated for 120 min at 37°C with 1 mg/mL of CC-NPs corresponding to a 74 μg/mL CC concentration. CC-sus was incubated at a 150 μg/mL concentration on the apical compartment. The highest $P_{app}$ values for CC were obtained for CC-NPs. CC-sus permeability values were not possibly estimated as the amount of CC in the basolateral compartment was below the limit of detection (LOD<5 ng/mL).

3.3.3. Localization of CC-loaded nanoparticles across Caco-2 cell monolayers

Fig. 3 shows the confocal microscopy images corresponding to the cellular uptake of CC across Caco-2 cell monolayers. CC exhibits green fluorescence. Phalloidin–phallolidin stains actin in red, whereas DAPI stains cell nuclei in blue. Neither CC-sus nor CC-NPs were accumulated within the Caco-2 monolayers.

3.3.4. Inhibition of TNF-α secretion from LPS-activated J774 macrophage cells

The inhibition of TNF-α secretion by J774 macrophages was evaluated by comparing the CC-NPs with blank-NPs and a CC-sus. A significant reduction on TNF-α secretion was only observed for CC-NPs-treated cells (measured by ELISA) when compared to untreated cells (medium) ($^*p<0.01$) (Fig. 4).

3.4. In vivo therapeutic activity of the CC-loaded formulations

3.4.1. Clinical activity scoring

The clinical activity score obtained for healthy and DDS-treated mice confirmed the efficient induction of colitis within the DSS-treated groups (data not shown). Body weight loss is a

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**Fig. 1.** In vitro release profile of CC from pH sensitive PLGA/ES100 nanoparticles in gradually pH-changing buffer (n = 3).

**Fig. 2.** CC $P_{app}$ values from the different assayed formulations: CC-sus (150 μg/ml of CC) and pH-sensitive nanoparticles (CC-NPs, 74 μg/ml of CC). CC levels from CC-sus were found to be below the limit of detection (n.d: no detection) (N = 3; n = 3) ($^{***}p<0.001$).

**Fig. 3.** Representative CLSM images of Caco-2 cell monolayers after 2 h of co-incubation with CC-NPs (left) and CC-sus (right). Cell borders are stained in red with rhodamine–phalloidin and cell nuclei are stained in blue with DAPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
significant symptom of IBD. Compared to the control group, a body weight loss was observed in all DSS-treated groups, demonstrating that colitis was effectively induced. Among the DSS groups, there were no significant differences when compared to the PBS-treated DSS group (Fig. 5).

3.4.2. MPO activity and pro-inflammatory cytokine TNF-α in the colon

MPO activity, which is a measure of neutrophil infiltration, and the concentrations of the pro-inflammatory cytokine TNF-α in the colon are shown in Fig. 6. It is noteworthy that MPO activity and cytokine concentrations for the CC-NPs-treated DSS group were decreased in both cases when compared with the PBS-treated DSS group (**p < 0.01). Interestingly, significant differences were observed when mice were treated with blank-NPs, even if these results were only significant in TNF-α protein expression reduction (**p < 0.05), but not in MPO reduction (p > 0.05).

3.4.3. Histological assessment of colonic inflammation

At day 8, mice were sacrificed, and the severity of inflammation was characterized by colon histology scoring (Fig. 7). The CC-NPs-treated DSS group was the only treatment exhibiting a significant decrease in inflammation when compared to the PBS-treated DSS group (**p < 0.01). In addition, a significantly decreased inflammation was observed when compared to free CC and blank-NPs (**p < 0.001 and **p < 0.01, respectively). Both the CC-sus and PBS-treated DSS groups (Fig. 7C and E, respectively) presented a remarkable submucosa edema, an altered mucosa structure, and increased leukocyte infiltration when compared to the control group (Fig. 7B). Oppositely, CC-NPs-treated DSS group (Fig. 7D) preserved intact the colonic architecture, presenting no structural differences with the control group (Fig. 7B).

3.4.4. In vivo localization of CC-NPs in healthy and inflamed tissues

Twelve hours after orally administered, both untreated and treated mice (healthy and DSS-treated) were sacrificed and the colons removed. Fig. 8 shows the CLSM images of distal colon sections in PBS-treated mice (control), CC-sus-treated and CC-NPs-treated
mice. It is remarkable a higher accumulation of CC (as per fluorescence intensity) in CC-NPs-treated mice in both healthy and DSS-treated mice in comparison to CC-sus-treated mice.

4. Discussion

Nano-sized drug delivery systems to target the inflamed mucosa are a promising strategy in IBD treatment (Collinot et al., 2012; Laroui et al., 2012, 2011; Schmidt et al., 2013). Reducing the particle size of drug delivery systems is believed to increase colonic residence time but can also provide additional benefits for IBD therapy, such as a selective accumulation in inflamed tissues. In this study, polymeric pH-sensitive nanoparticles loading CC were evaluated and compared in vitro and in vivo to a CC suspension to assess a selective and specific delivery of CC in IBD treatment. CC-NPs were obtained using the SESD technique and displayed a mean particle size ~100 nm, a negative charge (~40 mV), and a fairly monodispersed population (Pdl < 0.30) (Table 1). Moreover, CC was efficiently incorporated into the pH-sensitive NPs (~70%). The negative charge of the surface of nanoparticles attract them to positively charged ulcerated tissues (Jubeh et al., 2004). Hence, the nanometer scale size of CC-NPs along with their negative charge, make them good candidates as for IBD treatment.

The pH-sensitive release profile of CC from pH-sensitive CC-NPs in a gradually pH-changing buffer confirmed CC release at pH above 7.2, what makes them suitable for colonic drug delivery (Fig. 1). In acidic pH, a low amount of CC was released from the nanoparticles and found in the medium (CC release ~15%). However, as the pH of the aqueous medium reached 7.2, CC release was ~90%. This phenomenon could be explained by carboxylic function groups of ES100 polymer starting to be deprotonated at these pH values (El-Kamel et al., 2001). ES100 starts then to dissolve and to swell, leading to the release of the encapsulated CC from the pH-sensitive nanoparticles. PLGA/ES100-CC NPs can withstand CC release at gastric and ileal pH (acidic pH), but release CC at higher colonic pH values (pH > 7.2),
ensuring CC release only at the target site: the colon. Chereddy et al. (2013) reported a biphasic pattern in the CC release curve for PLGA-CC NPs presenting an initial burst release within the first 24 h with 40.5 ± 4.5% of the CC released from the PLGA-CC NPs. Afterwards, a sustained release of CC was observed from 40.5 ± 4.5% to 75.7 ± 3.4% over a period of 8 days. Thus, compared to PLGA-CC NPs, pH-sensitive CC-NPs exhibit a selective CC release at basic pH.

Nano-sized particles are taken up by an increased number of immune-related cells in active inflammation (Lamprecht et al., 2001). CC is known to suppress inflammation via multiple pathways, like inhibiting the production of pro-inflammatory cytokines (i.e., TNF-α, IL-1β or IL-8) (Aggarwal and Harikumar, 2009). Hence, the inhibitory effect of CC-NPs on pro-inflammatory cytokines secretion was evaluated in vitro in LPS-activated macrophages (J774 cells) and compared to unloaded NPs and CC in suspension. A significant decrease in TNF-α secretion by LPS-activated macrophages was observed only when cells were pre-treated with CC-NPs (**p < 0.01) (Fig. 2), highlighting the importance of CC encapsulation toward an efficient anti-inflammatory effect.

The pleiotropic effects of CC are counterbalanced by its poor water solubility and low oral bioavailability. The encapsulation of CC in pH-sensitive NPs resulted in a significant permeability enhancement when compared to CC-sus across Caco-2 cell monolayers (**p < 0.001), emphasizing the potential of these NPs also toward increasing CC oral bioavailability. Moreover, CC is known to be retained within the enterocytes (Wahlang et al., 2011). Thus, we evaluated its cell association behavior across Caco-2 cell monolayers. Neither CC-NPs retention, nor free CC retention from CC-sus or CC-NPs was observed within the monolayers after 2 h of incubation (Fig. 3). Taking into account CC permeability values, CC in suspension was not absorbed, whereas CC-NPs increased CC permeability across Caco-2 cell monolayers (***p < 0.001).

The anti-inflammatory efficacy of CC-loaded nanocarriers was evaluated in vitro in LPS-activated macrophages and in vivo in a murine DSS-induced colitis model. In vitro, CC-NPs significantly reduced TNF-α secretion by LPS-activated macrophages (**p < 0.01) (Fig. 4). In vivo, the DSS-induced colitis model was chosen because it exhibits certain characteristics similar to those present in human ulcerative colitis (Wirtz and Neurath, 2007). DSS induces bloody stool, loose feces and weight loss symptoms in mice. Following the latter, the monitoring of body weight loss in our study showed that no treatment was able to ameliorate DSS-related body weight loss (Fig. 5). During the first steps of active intestinal inflammation, an increasing number of leukocytes infiltrate the active inflamed mucosa, resulting in the recrudescence of reactive oxygen species (Holma et al., 2001). MPO is one of the most abundant proteins in neutrophils. MPO level is used as an index of inflammation in clinical practice, animal models of IBD and, especially, in the murine DSS-induced colitis model, where significantly high neutrophil infiltration levels are observed at all stages of DSS treatment, and even after DSS withdrawal (Mendoza and Abreu, 2009; Yan et al., 2009). At day 8, only the CC-NPs DSS-treated group exhibited a reduction in MPO activity when compared with the PBS-treated DSS group (*p < 0.05) (Figure). Along with MPO activity, CC-NPs were

![Fig. 8. In vivo localization of CC 12 h post-administered as suspension or as pH-sensitive nanoparticles in healthy (left) and inflamed (right) colon sections. Green corresponds to CC. Cell nuclei are stained in blue (magnification 10×). Scale bars = 50 µm.](image-url)
able to significantly reduce TNF-α secretion in inflamed tissues when compared to the PBS-treated DSS group (*p < 0.05) (Fig. 6). Interestingly, a significant TNF-α secretion was also observed for the blank-NPs-treated DSS group, remaining unclear whether pH sensitive NPs themselves exert an effect at the colonic site and giving venue to future mechanistic studies toward a better understanding of this effect (*p < 0.05).

The severity of inflammation was also histologically evaluated (Fig. 7). The histological analysis demonstrated a reduction of the submucosa edema, a normalized mucosal structure, and reduced leukocyte infiltration only for the CC-NPs-treated DSS group (Fig. 7D). These reductions can be correlated to the results in MPO infiltration and cytokine quantification in the colon which displayed the same trend toward decreased inflammation in CC-NPs-treated groups.

The local delivery of CC-loaded nanocarriers to the inflamed colon was evaluated in vivo by visualizing curcumin accumulation in both healthy and inflamed tissues (Fig. 8). In comparison to CC-sus-treated mice, CC exhibited a higher accumulation in both healthy and inflamed colon sections, as per fluorescence intensity, when formulated as CC-NPs. These results confirm a higher accumulation of CC when administered as CC-NPs, which could be explained by the large CC release (~90%) at pH 7.2 obtained with these pH-sensitive nanoparticles. It remains unclear whether the enhanced curcumin accumulation in both healthy and inflamed tissues in vivo is due to a higher and selective curcumin release at the intestinal site or to a higher nanoparticle retention and/or nanoparticle penetration, or both.

Meissner et al. (2006) compared conventional sustained release PLGA-NPs with pH-sensitive nanoparticles (Eudragit P-413SF) for the delivery of tacrolimus in IBD. The authors reported that, compared to PLGA-NPs, pH-sensitive NPs exhibit a lack of specificity to the inflamed tissues but presented a higher total amount of tacrolimus delivered to the colon. In contrast, PLGA-NPs increased tacrolimus concentration inside the inflamed tissues with a lower total amount of drug and a more selective accumulation in inflamed tissues. In our study, we observed both a specific CC delivery to the colon and a selective CC release at the colonic site and a lower concentration of drug at the colonic site. The discussed results justified the use of nanoparticles made of PLGA and ES100 combination for CC delivery in IBD treatment.

5. Conclusion

In the present study, CC was encapsulated in polymeric pH-sensitive nanoparticles for a selective and specific delivery of CC to the inflamed mucosa. CC-NPs exhibited suitable physicochemical characteristics (size and surface charge) for colonic delivery. In vitro, encapsulated CC was found to cross through the epithelial barrier better than CC-sus significantly increasing CC permeability across Caco-2 cells. Furthermore, CC-NPs significantly reduced TNF-α secretion in pre-treated LPS-activated macrophages. In vivo, CC-NPs DSS-treated mice showed a significant reduction in both MPO activity and TNF-α secretion, presenting a similar structural colonic pattern in comparison to the healthy group. The localization of CC 12 h post-administration of CC-NPs assessed a specific CC delivery to the colon and an increased CC release at the colonic site.

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References


