Accepted Manuscript

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PII: S0141-8130(15)30152-5
DOI: http://dx.doi.org/doi:10.1016/j.ijbiomac.2015.11.092
Reference: BIOMAC 5806

To appear in: International Journal of Biological Macromolecules

Received date: 9-7-2015
Revised date: 25-9-2015
Accepted date: 22-11-2015

Please cite this article as: Srinivas Mutalik, Neelam A.Suthar, Renuka S.Managuli, Pallavi K.Shetty, Kiran Avadhani, Guruprasad Kalthur, Raghavendra V.Kulkarni, Ranjeny Thomas, Development and performance evaluation of novel nanoparticles of a grafted copolymer loaded with curcumin, International Journal of Biological Macromolecules http://dx.doi.org/10.1016/j.ijbiomac.2015.11.092

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Development and performance evaluation of novel nanoparticles of a grafted copolymer loaded with curcumin

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ABSTRACT

Inflammatory bowel disease (IBD) is an inflammatory condition with mucosal ulceration, edema and hemorrhage of gastrointestinal tract. Curcumin has been shown to mitigate colitis in animal models. However, its usefulness is reduced due to poor pharmacokinetic behavior and low oral bioavailability. To address this, novel pH-sensitive hydrolyzed polyacrylamide-grafted-xanthan gum (PAAm-g-XG) nanoparticles (NPs) loaded with curcumin were prepared for colonic delivery. Optimized nanoparticles (CN20) were spherical, with an average size of 425 nm. A negligible amount of curcumin (≈8%) was released from CN20 NPs in pH 1.2 and 4.5 solutions. When the pH was increased to 7.2, curcumin release was comparatively faster than that observed with pH 1.2 and 4.5 collectively. In pH 6.8 solution, excellent release of curcumin was observed. Highest curcumin release was observed when rat caecal contents were incorporated in pH 6.8 solution, indicating microflora-dependent drug release property of NPs. In acetic acid-induced IBD in rats, curcumin NPs reduced myeloperoxidase and nitrite levels, prevented weight loss and attenuated colonic inflammation. Curcumin was better absorbed systemically in nanoparticulate form with increased $C_{\text{max}}$ (∼3 fold) and AUC (∼2.5 fold) than when delivered as free curcumin. We demonstrate successful development of grafted co-polymeric NPs containing drug suitable for colon targeting.

Keywords: Grafted copolymer; polyacrylamide; xanthan gum; nanoparticles; inflammatory bowel disease; curcumin.
1. INTRODUCTION

The chronic inflammatory bowel disease (IBD), comprising Crohn’s Disease (CD) and Ulcerative colitis (UC), is an inflammatory condition with mucosal ulceration, edema and hemorrhage of gastrointestinal tract [1]. Both CD and UC exhibit a relapsing and remitting pattern with reduced quality of life during exacerbations of disease [2]. The etiology of IBD is still unclear but hypothesized to be due to dysfunction of the mucosal immune response towards the gut flora, autoimmune response to mucosal antigen, genetic or environmental factors [1, 3]. Current IBD management strategy aims to induce and/or to maintain remission with medications such as anti-inflammatory drugs, immunosuppressive agents, antibiotics and biological agents, with beneficial clinical effects. However, serious adverse effects, cost, and the requirement for systemic delivery demonstrate the need for therapies with high local efficacy and low systemic toxicity [3-5]. Orally-delivered nanoparticles (NPs) offer the benefits of improved absorption, solubility, encapsulation of lipophilic actives, protection of encapsulated drugs from metabolism and enzymatic degradation, resulting in improved drug stability and bioavailability [6]. NPs preferentially absorbed in inflamed regions of the gut due to a disrupted intestinal barrier represent a promising alternative for IBD treatment over existing drug delivery systems [7]. One such lipophilic active is curcumin, a naturally occurring polyphenol with anti-inflammatory, antioxidative, anticancer and antiangiogenesis effects. Its anti-inflammatory activity is mediated by scavenging free radicals and inhibition of myeloperoxidase, COX-1, COX-2, LOX, TNF-α, IFN-γ, iNOS and NF-κB [6]. Curcumin has been shown to attenuate colitis in animal models [3, 8]. However, the poor pharmacokinetic behavior and low oral bioavailability of curcumin reduce its usefulness due to its extensive metabolism, poor absorption, limited aqueous solubility and instability [9].

There are limited studies of colon-targeted drug delivery systems (CDDS) based on pH and/or enzyme-mediated drug release for curcumin delivery to the colon in IBD. Previously described formulations include microsponges [10], microspheres [11], solid lipid microparticles [12], cyclodextrin complexes [13] and curcumin-inclusion complex tablets with pH- and enzyme-sensitive polymer coating [14]. While Beloqui et al. [7] formulated curcumin-NPs using conventional
synthetic poly (lactide-co-glycolide) (PLGA) and polymethacrylate (Eudragit® S100), Gugulothu et al. [15] prepared pH-sensitive curcumin-celecoxib-NPs using synthetic Eudragit® S100 polymer. Naturally available polysaccharides may be preferable for colon targeting. Substances such as xanthan gum and guar gum have low toxicity, free accessibility, low cost and biodegradability and are degradable by enzymes in the colon [16]. Furthermore, the diverse structure and water solubility of such polysaccharides makes them ideal materials for the synthesis of grafted polymers [17]. Grafting is a novel technique used for modification of polysaccharide, which combines the properties of each material to create desired properties for drug delivery [17-19]. In our current study, polyacrylamide (PAAm) was grafted onto the backbone of xanthan gum (XG) to obtain a grafted polyacrylamide-grafted-xanthan gum (PAAm-g-XG) copolymer. Further hydrolysis of PAAm-g-XG copolymer converts the amide (-CONH₂) functional group of PAAm to a carboxylic acid (-COOH) group, resulting in a pH-sensitive copolymer [16, 20]. As XG can be activated by colonic microbiota [21], PAAm-g-XG copolymer should confer high specificity for colon targeting due to its precise pH-dependent degradation and microbiota-dependent activation. We previously described the development of microspheres using grafted polymers [16, 18-20]. Development of grafted polymeric NPs is challenging task due to the complexity of the grafted polymer with respect to solubility, gelation or cross-linking issues; but if NP preparation can be achieved using a grafted polymer (such as PAAm-g-XG), then the advantages will be better targetability, solubility, absorption and bioavailability, in comparison with microparticles. The objective of the present study was to develop a PAAm-g-XG copolymer NPs incorporating a lipophilic anti-inflammatory drug for delivery to the colon in IBD. Here, we developed curcumin-loaded NPs, and evaluated them in vitro and in vivo in preclinical studies.

2. MATERIALS AND METHODS

2.1. Materials

Curcumin, polyvinyl alcohol (MW: 30000-70000; PVA), hydrogen peroxide (30% w/v; H₂O₂) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased.
from Sigma Aldrich (St Louis, MO, USA). PAAm and XG were procured from Central Drug House (P) Ltd (New-Delhi, India) and HiMedia Laboratories Pvt Ltd (Mumbai, India) respectively. Ammonium persulfate (APS), dichloromethane (DCM), HPLC grade acetonitrile and sodium acetate were obtained from S D Fine Chemicals (Mumbai, India). Mannitol, potassium chloride, sodium hydroxide, glacial acetic acid and hydrochloric acid (HCl) were obtained from Merck Specialities Pvt Ltd (Mumbai, India). Methanol was procured from Qualigens fine chemicals (Mumbai, India). All other chemicals were of analytical grade and used as received. Vero cell lines (normal green monkey kidney epithelial cells) and HCT116 cell lines were purchased from National Centre for Cell Sciences (Pune, India).

2.2. Synthesis and characterization of PAAm-g-XG Copolymer

PAAm-g-XG copolymer was prepared by free radical polymerization followed by alkaline hydrolysis. Briefly, XG was allowed to hydrate in double distilled water for 4 h with continuous nitrogen gas purging. PAAm and APS were added to XG solution at 80 °C and allowed to polymerize for 60 min with continuous nitrogen gas purging. After cooling, the obtained product was placed in excess methanol for 24 h to de-water. The product was filtered and dried overnight at 50 °C. Then, the dried product was dissolved in 0.9 M sodium hydroxide (2% w/v) and stirred for 60 min at 75 °C in a thermostatic water bath for hydrolysis to occur. After 60 min, the solution was cooled and poured in an excess volume of methanol; the product obtained after filtration was dried overnight at 50 °C and stored in an airtight container free from moisture [18, 20]. The synthesized copolymer was characterized by Fourier Transform Infrared (FTIR) spectroscopy and Elemental analysis to confirm the grafting reaction and alkaline hydrolysis. FTIR Spectroscopy involved KBr pellet press technique using a Shimadzu FTIR 8300 Spectrophotometer (Shimadzu, Tokyo, Japan) in the wavelength range of 400 to 4000 cm⁻¹. Elemental analysis with the help of a Flash EA 1112 CHN analyzer (Thermo Finnigan, Italy) determined the nitrogen content in PAAm, XG and hydrolyzed PAAm-g-XG. ¹H-NMR spectra of acrylamide (AAm), XG and hydrolyzed PAAm-g-XG were recorded using a Bruker 400 MHz NMR spectrometer (Bruker Corporation, MA, USA) with DMSO-d₆ as solvent and trimethylsilane (TMS) as internal standard. The viscosity of 1% w/v solutions of
XG, PAAm-g-XG and hydrolyzed PAAm-g-XG was determined using a Brookfield RVDV-E Viscometer (Brookfield Engineering Laboratories Inc., MA, USA) at room temperature.

2.3. Preparation of PAAm-g-XG NPs loaded with curcumin

The NPs were prepared using a modified version of the solvent evaporation cross-linking technique previously described for NPs [22] and for micro beads [23]. We tried two methods and selected one method for further optimization of NPs.

Method I: Curcumin solution in DCM was emulsified with PVA solution containing 50 mg of polymer under high speed homogenization at 13500 rpm for 15 min (Polytron PT 3100, Kinematica, Switzerland). The emulsion formed was sonicated (Probe sonicator VC 130, Sonica and Material Inc, USA) for 2 min with simultaneous drop-wise addition of aluminium chloride (AlCl₃, 5 mL) followed by further sonication for 2 min. DCM was evaporated under magnetic stirring for 6 h and the dispersion was centrifuged (Sigma Laborzentrifugen-3K30, Osterode, Germany) at 20000 rpm for 45 min for pellet collection.

Method II: PVA solution (7 mL) containing polymer was taken in a glass tube to which curcumin solution in DCM was added drop-wise during sonication. AlCl₃ solution (5 mL) was added over a period of 2 min, and further sonicated for 1 min. The emulsion formed was added to 45 mL of polymeric PVA solution under magnetic stirring. DCM was allowed to evaporate for 6 h followed by centrifugation at 20000 rpm for 45 min for pellet collection.

The pellet obtained in both the methods was re-dispersed in cryoprotectant solution and lyophilized by primary drying at -80 ºC for 4 h and secondary drying at -48 ºC for 48 h using freeze dryer (LFD-5508, Daihan Labtech Co. Ltd., Korea).

2.4. Formulation optimization of curcumin NPs

Factors such as amount of curcumin and polymer, volume of PVA solution and DCM, concentration and volume of AlCl₃ solution, and sonication parameters were varied. Of 18 nanoformulations (Table 1), one appropriate formulation (CN1) was selected for further optimization of the freeze-drying process. Varying concentrations of different cryoprotectants such as mannitol
(5% w/v- CN17; 10% w/v - CN18 and 15% w/v- CN19) sucrose (5% w/v- CN20 and 10% w/v- CN21) and trehalose (5% w/v- CN22 and 10% w/v- CN23) were assessed.

2.5. Characterization of Curcumin-PAAm-g-XG-NPs

2.5.1. Particle size, polydispersibility index and zeta potential determination:

The average particle size, polydispersibility index (PDI) and zeta potential (ZP) of the prepared NPs were determined by a particle size analyzer (Nano ZS, Malvern Instruments, UK) using techniques of dynamic light scattering (in case of size) and combination of Laser Doppler Velocimetry (LDV) and Phase Analysis Light Scattering (PALS) technique (in case of zeta potential).

2.5.2. Practical yield

The freeze dried product was weighed and the practical yield (%) was calculated by dividing the weight of NPs obtained by initial weight of ingredients, multiplied by 100.

2.5.3. Entrapment efficiency (EE)

Freeze dried product (10 mg) was dissolved in pH 7.4 phosphate buffer with the aid of sonication for 15 min and subsequently, ethanol was added and sonicated further for 15 min. The resulting solution was filtered through 0.22 µm syringe filter and analyzed in UV/Visible spectrophotometer at 423 nm after suitable dilution. EE was calculated by considering theoretical and practical amount of curcumin present in nanoparticles.

2.5.4. Shape and surface morphology

Shape and surface morphology of the freeze-dried NPs were studied using scanning electron microscopy (SEM; JEOL JSM 50A, Tokyo, Japan) and transmission electron microscopy (TEM; Morgagni TEM, FEI, The Netherlands). An appropriate amount of the freeze-dried NPs was mounted on aluminium stubs using adhesive tape. The NPs were sputter coated with palladium for 120 sec at 14 mA under argon atmosphere and observed for morphology at an acceleration voltage of 5-20 KV. For TEM, a drop of sample was placed on a copper grid coated with carbon film and after
1 min of air dry, excess was drained off with a filter paper. Sample was then quickly stained with phosphotungstic acid solution (2% w/v, pH 6.0) and air dried followed by examination in TEM.

2.5.5. X-ray diffraction (XRD), FTIR and differential scanning calorimetry (DSC) studies

Curcumin, PAAm-g-XG polymer and prepared NPs were subjected to XRD, FTIR and DSC studies. The XRD pattern was recorded using a Philips, PW-171 X-ray diffractometer with Cu-NF filtered CuKα radiation in the 2θ range of 0-70°. FTIR spectra were recorded by following KBr pellet method. DSC study DSC-60 calorimeter (Shimadzu, Kyoto, Japan) equipped with flow controller (FCL 60), thermal analyzer (TA 60) and operating software (TA 60). The samples were analyzed in a sealed aluminium pan under nitrogen flow (50 mL/min) at a scanning rate of 5 °C/min from 30 °C to 300 °C.

2.5.6. Saturation Solubility

An excess amount of the freeze dried NPs was added separately to each of distilled water, pH 1.2 HCl buffer and pH 7.4 phosphate buffer and samples were mounted on an orbital shaker for 24 h at 37±0.5 °C. The sample was filtered through a 0.22 µm membrane and the amount of drug dissolved was estimated using a UV/Visible spectrophotometer at 423 nm.

2.5.7. In vitro drug release studies

The release of curcumin from the prepared NPs was assessed in vitro using the following pH-changing system: hydrochloric acid pH 1.2 for 2 hours, buffer solution pH 4.5 for 1 h, buffer solution pH 7.2 for 3 h and buffer solution pH 6.8 (+/- 1% w/v rat caecum content) for 3 h. To the initial solution of pH 1.2, a solution containing tris(hydroxyl methyl)aminomethane and anhydrous sodium acetate was added, to reach pH 4.5, and a second aliquot of this buffer mixture was added to the buffer solution pH 4.5 to reach pH 7.2. After 3 h, the pH of the dissolution medium was adjusted to pH 6.8 by adding 2M HCl and the study was continued for 3 more hours. Since curcumin possesses very low aqueous solubility, Tween 80 (3% w/v) was incorporated into the release medium. To investigate the microbiota-activated property of the prepared PAAm-g-XG NPs, a release study was also carried out using 1% w/v rat caecum content in pH 6.8 buffer solution [11]. Briefly, a known
quantity of lyophilized NPs was dispersed in 50 mL of the dissolution medium under magnetic stirring (60 rpm, 37 °C). At prefixed time intervals, 1 ml samples were withdrawn and centrifuged for 30 min at 22,000 rpm and 4 °C. The absorbance of resulting supernatants was measured after appropriate dilution.

**2.5.8. In vitro cytotoxicity**

The cytotoxicity of curcumin-loaded NPs was assessed in Vero and HCT 116 cell lines cultured in Dulbecco’s modified eagle’s medium containing 10% Fetal Bovine Serum, penicillin (100 µg/mL), streptomycin (100 µg/mL) and amphotericin-B (5 µg/mL). Cells were incubated in 5/95 (%v/v) CO₂/air atmosphere at 37 °C (Healforce Incubator, Shanghai, China). Cells were seeded at a density of 1 × 10⁴ cells/well onto 96-well culture plate and allowed to culture in media for 24 h at 37 °C in 5/95 (%v/v) CO₂/air atmosphere for cell adhesion to occur. Next, the medium was removed and cells were treated with various concentrations of plain curcumin and curcumin-loaded NPs for 48 h. After the treatment, the solution from the wells was discarded and 50 µL of freshly prepared MTT (2 mg/mL, in phosphate buffer saline) was added to each well. The plates were shaken gently and incubated for 3 h at 37 °C in 5/95 (%v/v) CO₂/air atmosphere. The supernatant was removed and the formazan crystals formed in the cells were solubilized by addition of 50 µL of iso-propanol. Absorbance was read using a Micro-plate reader (ELx800, BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 540 nm.

**2.6. In vivo studies of optimized curcumin NPs**

**2.6.1. Rats:**

Adult male Wistar rats weighing 200-235 g were bred at Manipal University, Manipal, and housed in polypropylene cages (2 rats per cage), with free access to standard laboratory diet and water. The in vivo experimental protocol was approved by the Institutional Animal Ethical Committee, KMC, Manipal (Approval No: IAEC/KMC/28/2012).
2.6.2. In vivo pharmacodynamic studies of optimized formulations:

Colitis was induced in rats by administration of 2 mL of 0.9% saline containing 4\%\text{v/v} acetic acid (AA). Briefly, rats were deprived of food for 24 h with free access to water. A medical-grade polyurethane cannula was inserted into the anus of anaesthetized rats for enteral instillation of acetic acid (in AA group) or saline (in Normal group) into the colon. During instillation, the tip of the cannula was moved to about 6-8 cm proximal to anal verge. The cannula was then withdrawn and rats were swung in air by holding the tails for about 2 min to prevent spillage of the solution from the rectum [10].

For pharmacodynamic studies, the following groups were tested (6 rats/ group).

Group I: Normal rats (0.5\% w/v of sodium carboxy methyl cellulose (CMC); 1 mL/100 g; p.o.)
Group II: Untreated acetic acid (AA) colitis group (0.5\% w/v of sodium CMC; 1 mL/100 g; p.o.)
Group III: AA colitis group treated with plain curcumin (100 mg/kg in 0.5\% w/v of sodium CMC; p.o.)
Group IV: AA colitis group treated with CN20 NPs (100 mg/kg in 0.5\% w/v of sodium CMC; p.o.)
Group V: AA colitis group treated with the standard drug, sulfasalazine (100 mg/kg in 0.5\% w/v of sodium CMC; p.o.)

From the second day after IBD induction, the rats were administered the test articles orally for 7 days, once daily. Normal rats and IBD control rats were orally administered sodium CMC once daily. Body weight was checked every day of the treatment period. On day 8, rats were sacrificed and length and weight of colon were measured. A section of colon was collected for myeloperoxidase activity, tissue nitrite levels and histopathological examination.

2.6.2.1. Myeloperoxidase (MPO) activity: Colon samples collected previously were homogenized in HTAB buffer (0.5\% w/v in 50 mM potassium phosphate buffer, pH 6) in ice-cold conditions. Homogenate was ultracentrifuged at the speed of 15,000 x g, for 30 min at 4 °C and the supernatant obtained was transferred to 96-well plates. 200 mL of a 50 mM potassium phosphate buffer
containing 0.0167% O-dianisidine and 500 ppm H2O2 was added to wells and the absorbance readings were taken on a microplate reader at 490 nm for 30 min [7].

2.6.2.2. Tissue nitrite levels: Colon sample was homogenized in potassium chloride solution and supernatant (100 µL) was taken into 96-well plate. Nitrate present in supernatant was reduced to nitrite by vanadium(III) chloride before adding Griess reagent (1% sulphanilamide and 0.1% N-(1-napthyl)-ethylenediamine dihydrochloride in 5% H3PO4) and incubated at room temperature for 10 min. Absorbance was measured at 540 nm and nitrite concentration was quantified using a standard curve of sodium nitrite [24, 25].

2.6.2.3. Histopathological examination: Colon sections were washed with normal saline and fixed in Bouin’s fixative. The slides were stained with hematoxylin-eosin and examined under binocular light microscope (CXRIII, Labomed, Mumbai, India).

2.6.3. Pharmacokinetic studies of optimized NP formulations (CN 20)

The overnight-fasted rats were divided into two groups of six rats each and treated orally with plain curcumin or nanoparticulate curcumin at 100 mg/kg [26]:

Group I: Plain curcumin in 0.5% w/v sodium CMC; p.o.
Group II: CN20 NPs containing equivalent amount of curcumin in 0.5% w/v sodium CMC; p.o.

Blood samples were collected from the retro orbital sinus between 0 and 24 h post-treatment into tubes containing EDTA. The plasma was separated by cold centrifugation at 10000 rpm for 10 min and stored at -72°C until further analysis by HPLC.

2.6.4. Bioanalytical method for analysis of curcumin in rat plasma

The bioanalytical method to estimate curcumin in plasma was developed by modifying the reported method by Tsai et al. [27]. A Shimadzu HPLC system (LC-2010 CHT, Kyoto, Japan) equipped with quaternary gradient pump, dual wavelength UV detector and LC solution v.1.24SPI software was used. The protein precipitation method was employed for extraction of curcumin from rat plasma using acetonitrile: methanol mixture (50:50% v/v, 500 µL). Separation of curcumin and lacidipine (internal standard, IS; 100 µg/mL) from rat plasma was achieved by low pressure gradient
elution with phosphate buffer (10 mM; pH = 3.5 ± 0.05) and acetonitrile mixture as mobile phase, and a Genesis C18 (250 × 4.6 mm; 5 µm) column as stationary phase. The flow rate was 0.8 mL/min and detection wavelength 423 nm. The column and auto-sampler temperature was maintained at 25 °C and 4 °C, respectively and the injection volume was set to 100 µL. A low pressure gradient system was used to resolve curcumin and internal standard, wherein the proportion of buffer was varied from 35 to 15% over 2 min after 7 min of run, followed by maintenance at 15% for the next 6 min and then gradual increase to 35% over 3 min, continuing until sample analysis was complete. Blank plasma was analyzed prior to the analysis of curcumin samples. There was no interference from the blank plasma at the retention time (RT) values of curcumin and lacidipine. The peaks were of good shape and were completely resolved from one another. The RT of curcumin and lacidipine were 7.5 min and 16.8 min, respectively. The bioanalytical method was validated with respect to precision (RSD: <15%), accuracy (<15 %) and recovery (%), and was consistent and reproducible. The chromatographic data were analyzed using PK solution’s v2.0™ non-compartmental pharmacokinetic data analysis software to calculate the various pharmacokinetic parameters.

2.7. Statistical analysis

The results were expressed as mean ± standard deviation (SD). Data were analyzed statistically using one-way ANOVA followed by Dunnet’s post-hoc test (in case of comparisons with control group) or Student’s “t” test (to compare two groups) using GraphPad Prism software. A p value less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Synthesis of PAAm-g-XG Copolymer

The grafted polymer PAAm-g-XG was successfully prepared using the free radical polymerization method followed by alkaline hydrolysis. The temperature during synthesis was maintained at 80 °C, as decomposition of APS to sulfate anion free radical takes place at 80 °C. Sulfate anion extracts hydrogen from hydroxyl groups of XG thereby creating alkoxy radicals on the
substrate. These radicals then initiate graft copolymerization of PAAm onto the XG backbone [16]. The viscosity values of 1% w/v solutions of XG, PAAm-g-XG and hydrolyzed PAAm-g-XG were 295±11, 623±15 and 618±17 cp, respectively.

3.2. Characterization of PAAm-g-XG copolymer

3.2.1. FTIR spectroscopy:

The grafting of PAAm onto XG will offer amide functional groups within the graft polymer. This was justified in FTIR spectrum of PAAm-g-XG, which showed peaks at 3399.32 cm⁻¹ and 3194.01 cm⁻¹ as in PAAm, corresponding to the stretching vibration of the –OH group (due to a resonance effect of the lone pair electrons of the nitrogen atom) and the –NH bond of the primary amide group, respectively. The peak observed at 1609.43 cm⁻¹ was due to the C=O group of the primary amide and the peak at 1404.22 cm⁻¹ may be due to CN stretching of the primary amide. These data confirm the grafting reaction. With hydrolyzed PAAm-g-XG, the peak of –NH stretching vibration of the primary amide at around 3194.01 cm⁻¹ was absent, confirming the hydrolysis of the -CONH₂ group to a -COOH.

3.2.2. Elemental analysis and NMR studies:

A minor quantity of nitrogen (1.44%) was observed in XG, which may be due to trace amounts of protein. However, nitrogen was considerably increased in PAAm-g-XG (14.48%) due to the presence of –CONH₂ groups of PAAm on the XG backbone. Nitrogen content in the hydrolyzed PAAm-g-XG copolymer was reduced to 4.43%, which suggests conversion of –CONH₂ groups to –COOH groups and confirms the grafting and hydrolysis reaction in accordance with previous studies [18, 28].

The ¹H-NMR spectra (not shown) of acrylamide (AAm), xanthan gum (XG), and PAAm-g-XG exhibited peaks at 0 and ~2.5 ppm, which could be due to internal standard (TMS) and solvent (DMSO), respectively. The AAm showed the peaks at ~5.6 and ~6.1 ppm because of CH₂ and CH protons, respectively. The specific peaks between ~7.2 and ~7.6 ppm were due to CONH₂ protons of AAm. The XG showed characteristic peak at ~1.4 ppm due to OH protons and at ~1.9 ppm due to
CH$_3$ protons of acetate and pyruvate groups of XG. The peaks between ~3.2 ppm and ~4.8 ppm could be attributed to CH$_2$OH protons of mannosyl groups of XG. On the other hand, in the spectrum of PAAm-g-XG, along with the peaks of XG, new peaks between ~6.8 and ~7.0 ppm were perceived, which could be due to CONH$_2$ protons of acrylamide chain on the backbone of XG. This confirms the grafting reaction of polyacrylamide on xanthan gum. These results are in accordance with earlier reports [18, 29].

3.3. Saturation solubility of curcumin

Curcumin exhibited low solubility in pH 1.2 HCl (1.20 µg/mL), pH 7.4 phosphate buffer (1.60 µg/mL) and distilled water (1.45 µg/mL) (Table 2). Increasing pH resulted in increased solubility. The poor solubility of curcumin justifies the incorporation of a solubilizer (SLS and Tween 80) into the medium [11]. With an increase in surfactant concentration (SLS and Tween 80) from 0.5 to 3% w/v, the solubility of curcumin was also increased. Tween 80 at 3% w/v was selected for dissolution studies.

3.4. Formulation Development

The effect of varying the properties of NPs are shown in Table 3 and discussed below. In view of its outcomes on particle size, PDI, ZP, EE and practical yield, method 1 was most suitable for preparation of NPs.

3.4.1. Optimization of drug polymer ratio

At low polymer content (CN4; 25 mg), the particle size and ZP were high and EE was low, which could be due to the presence of un-entrapped drug. As the polymer content increased, the particle size increased and ZP decreased but drug EE remained the same. Moreover, the particles showed high PDI at 25 mg (CN4) and 100 mg (CN5) of polymer content. The % practical yield was increased when polymer content increased from 25 mg to 50 mg. The ZP values of CN3 and CN5 were lowest, and ZP values of CN1 and CN4 were almost in the same range.

By increasing the amount of drug (CN7), particle size was increased. This may have been because curcumin occupied the interstitial spaces between polymer segments, as has been reported
for polyacrylamide-grafted-xanthan–carboxymethyl cellulose-based hydrogel beads [16]. While a lower concentration of curcumin (CN6) resulted in slightly decreased particle size, ZP, EE and practical yield markedly decreased and PDI increased. The practical yield of CN6 and CN7 was comparatively lower than that of CN1. Based on the overall evaluation of all the parameters, batch CN1, with a drug and polymer content of 5 mg and 50 mg respectively, was selected for further formulation development.

3.4.2. Optimization of organic to aqueous phase volume

Variation in the volume of PVA solution (1% w/v) from 50 mL resulted in increased particle size and PDI, and decreased ZP and EE values. The practical yield of batch CN8 was very low and that of batch CN9 was not appreciably increased in comparison with batch CN1.

3.4.3. Effect of varying AlCl₃ concentration.

As AlCl₃ concentration was increased (to 10% w/v), smaller particles with higher ZP value were obtained. This is because the particles might undergo rapid shrinking that leads to the formation of smaller and more rigid matrix at higher cross-link densities [16]. However, batches CN10 and CN11 showed higher PDI values, lower EE and lower practical yield in comparison with batch CN1.

3.4.4. Effect of volume of cross linking agent

The increase in AlCl₃ volume (up to 10 mL) resulted in increased particle size and decreased ZP, EE and practical yield. Hence, CN1 formulated with 5 mL volume was selected for optimization, with respect to ultra-sonication parameters.

3.4.5. Optimization of probe ultra-sonication parameters

A decrease in EE, practical yield and ZP and an increase in particle size and PDI were observed when the amplitude was varied from 60W (CN1). Use of high amplitude (80 W; CN14) might have resulted in increasing particle size due to swelling of the polymer. At the same time, lower amplitude (40 W; CN15) was not sufficient to reduce the particle size. An amplitude, time and pulse of 60W, 6 min and 6 sec respectively, were optimal.
3.4.6. Selection of suitable cryoprotectant and its concentration

After lyophilisation, CN1 NPs were sticky, with increased size (825 nm). Thus, a suitable cryoprotectant was required, to maintain proper size, to improve flow and to reduce stickiness of the lyophilized product (Table 4). Sucrose and trehalose produced a sticky mass, whereas mannitol produced a free-flowing powder. There was little variation in drug EE (ranging from 15 to 17.49%) between batches. Formulation CN20 with 10% w/v mannitol yielded good particle size with low PDI values, and was selected for further studies.

3.5. Saturation Solubility of curcumin in nanoparticulate form

Curcumin solubility was considerably increased in nanoparticulate form (CN20) in all the media tested, with a 26 - 48 fold increase in water, pH 1.2 HCl buffer and pH 7.4 phosphate buffer (Table 2). This can be explained by Ostwald-Freundlich’s equation, which expresses the dependence of solubility upon particle size. The large surface area of NPs, due to the smaller particle size, allows greater interaction of NPs than free drug with the solvent, leading to an increase in solubility [30-32]. Another possible explanation for increased saturation solubility can be given by Kevin’s equation, which states that the dissolution pressure increases with increasing curvature (i.e., decreasing particle size). The curvature is enormous when the particle is in the nanometer range. High dissolution pressure can be achieved, leading to a shift of the equilibrium towards dissolution [33, 34]. In addition, the hydrophilic nature of the polymer promotes an increase in solubility of curcumin [35]. Hence, PAAm-g-XG NPs of curcumin show enhanced solubility as a result of multiple mechanisms.

3.6. Drug-excipient compatibility studies

3.6.1. FTIR spectroscopy

FTIR spectra of pure curcumin showed characteristic peaks: at 3508 cm⁻¹ for O-H stretching, 2847 cm⁻¹ for CH alkane stretching, 1627.9 cm⁻¹ for C=O stretching, 1276.9 cm⁻¹ for C=O alcohol group and 1425 cm⁻¹ for C=C aromatic stretching. The IR spectra of a physical mixture of curcumin and excipients (curcumin + polymer + mannitol + PVA, 1:1:1:1) and of CN20 NPs demonstrated no
significant changes in curcumin peaks (results not shown), suggesting no chemical interaction between curcumin and excipients used.

3.6.2. Differential Scanning Calorimetry (DSC)

Pure curcumin showed a sharp endothermic peak at 180.57 °C, corresponding to its melting point (Fig. 1). No shift in melting point of curcumin in the presence of the excipients was observed (177.26 °C) indicating no interaction between curcumin and excipients. Also in CN20 NPs (172.56 °C), no shift in the melting point was observed but peak intensity was reduced, which may be due to partial amorphization of curcumin in nanoparticulate form.

3.7. XRD Studies

The XRD studies help to assess the crystalline/ amorphous nature of the drug in nanoparticulate form (Fig. 2). Free curcumin or a physical mixture exhibited a large number of intense peaks, indicative of the highly crystalline structure of curcumin. In contrast, the number and intensity of peaks were decreased in CN20 NPs. This may be due to the formation of an amorphous complex, resulting from the intermolecular interaction occurring within the matrix. Similar observations in the literature provide evidence that crystalline drugs are converted into an amorphous state in NPs [36]. The XRD and DSC results clearly demonstrate the conversion of curcumin into a partially amorphized form in NPs.

3.8. Surface morphology of NPs

The SEM photographs (Fig. 3A) reveal that the CN20 nanoparticles were slightly aggregated with high charge accumulation. Both SEM and TEM (Fig. 3B) demonstrated that CN20 particles have a size of ≈500 nm and spherical shape.

3.9. In vitro drug release studies

The in vitro pH-dependent release profile of curcumin from PAAm-g-XG NPs was investigated in progressively pH-changing buffers in order to assess protection of curcumin from the acidic gastric environment and effective delivery in alkaline pH (Fig. 5). Ideally, no drug should be released in acidic medium as curcumin NPs were intended to target the colon.
Plain curcumin did not show any resistance to either pH 1.2 or pH 4.5 solutions (Curcumin graph in Fig. 4). There was a gradual increase in dissolution of curcumin in its plain form and 100% dissolution was observed within 6 hours. On the other hand, a negligible amount of curcumin was released from CN20 NPs in pH 1.2 and 4.5 buffer solutions (CN20 graph in Fig. 4). Only about 8% of curcumin was released at the end of 3 h (0-2 h in pH 1.2 HCl solution + 2-3 h in pH 4.5 solution). When the pH of the medium was increased from pH 4.5 to 7.2 (3 to 6 h), curcumin release from NPs was comparatively faster than that observed with pH 1.2 and 4.5. This could be due to enhanced solubility of grafted polymer in alkaline pH. However, the release of curcumin was only about 35% at the end of the sixth hour. When the pH of the medium was changed to 6.8 (6 to 9 h), excellent release of curcumin from NPs was observed. About 65% of drug was released during this 3 h period in pH 6.8 solution. The rate of dissolution was good in both pH 7.2 and 6.8 in comparison with pH 1.2 and 4.5. The higher curcumin release in pH 6.8 solution than in pH 7.2 can be explained in the following ways:

i) Kulkarni and Sa [16] reported that beads prepared with PAAm-g-XG copolymer developed pores when incubated in pH 7.4, due to ionization of –COOH groups and electrostatic repulsion of the ionized groups, resulting in the release of entrapped drug. No surface pores were observed at pH 1.2, as –COOH groups were not ionized. In the current study, comparatively better release of curcumin in pH 6.8 solution could be attributed to formation of micro-pores on NPs in pH 7.2 from 4-6 h, followed by higher drug release in pH 6.8 due to these pores.

ii) Comparatively higher drug release in pH 6.8 solution could also be attributed to swelling of NPs in pH 7.2 solution. Subsequently removal or clearing of the swollen mass of NPs in pH 6.8 solution could have released curcumin at a faster rate. Greater release of curcumin in pH 6.8 solution than in acidic pH indicates site-specific targetability of the NPs to colon.

As xanthan gum possesses microflora-activated properties [21], we tested whether PAAm-g-XG copolymer possesses similar properties. A similar dissolution study was carried out up to the pH 7.2 solution, but this time 1% w/v rat caecal content was added to pH 6.8 solution [11]. The drug release profile in this dissolution medium is shown in Fig. 4 (graph: CN20+cecum content). The drug
release profile from CN20 NPs was similar to that observed previously (about 35% curcumin release in pH 7.2 solution; Graph: CN20 in Fig. 4); but when the pH of the solution was adjusted to 6.8 along with addition of rat caecal contents, the curcumin release was very high within 1 h (increasing from 33.56% to 96.98% between the sixth and seventh hours). At the end of eighth hour, 100% curcumin release was observed. These data indicate that the NPs possess microflora-dependent drug release properties. Belogui et al. [7] studied the in vitro release of curcumin from pH-sensitive NPs prepared using conventional synthetic polymers PLGA and Eudragit S100. It was observed that <15% of curcumin was released at pH 1.2 and 4.6; whereas at pH 7.2 rapid release of curcumin (about 80% in 1 h) was observed. NPs prepared with PAAm-g-XG polymer in the present study showed a greater resistance to acidic media, then a slow release of curcumin in pH 7.2, and thereafter a rapid release in pH 6.8. Additionally the grafted copolymer exhibited microflora-dependent drug release properties, which is desirable for colon targeting of bioactive molecules. Thus, we can conclude that PAAm-g-XG copolymer delivers curcumin specifically to the colon.

3.10. In vitro cytotoxicity assay

The IC_{50} values observed with curcumin in this study (Fig. 5A and 5B) were very similar to those observed in previous studies (95 and 25 µg/mL respectively for Vero and HCT116 cells) [37-39]. Plain curcumin and CN20 NPs exhibited similar levels of cell viability in both Vero and HCT116 cell lines, clearly indicating that curcumin did not lose activity when formulated into NPs. Lower IC_{50} values were observed for both plain curcumin and CN20 NPs in HCT116 cells than in Vero cells. Since Vero cells are normal kidney cells and HCT are colon cancer cells, Vero cells are more resistant to drugs such as curcumin as it preferentially induces apoptosis in highly proliferating cells than in normal cells [40].

3.11. In vivo pharmacodynamic studies of the optimized formulation

3.11.1. Change in body weight of rats:

Colitis induction was confirmed by weight loss - a significant symptom in IBD [1, 7]. Rats with colitis lost weight (p<0.05 compared to normal rats). This weight loss was significantly
improved with the delivery of plain curcumin, CN20 NPs and sulfasalazine (p<0.05 compared to untreated AA group) (Fig. 6). The weight of colitic rats delivered CN20s or sulfasalazine was not significantly different from healthy rats. However, there was a significant difference in body weights of the rats treated with plain curcumin and rats treated with CN20 NPs. The similar profile of CN20 NPs and sulfasalazine suggests similar efficacy for the maintenance of healthy body weight of curcumin NPs to that of the standard drug sulfasalazine.

3.11.2. Effect of plain curcumin and CN20 NPs on the levels of myeloperoxidase (MPO)

Tissue necrosis and inflammation are often correlated with an increase activity of MPO in experimental colitis models. MPO assessment provides a measure of the degree of inflammatory infiltration in IBD-affected animals [7, 41]. The colon of acetic acid-induced rats exhibited significantly higher (p<0.05) MPO levels (5.18±0.44 µg/mg) compared to normal colon (0.016±0.01 µg/mg) (Fig. 7). Plain curcumin (1.31±0.21 µg/mg), CN20 NPs (0.18±0.06 µg/mg) and sulfasalazine (0.78±0.14 µg/mg) significantly (p<0.05) reduced MPO levels compared to colitic controls. CN20 NPs alleviated MPO activity very effectively.

3.11.3. Effect of curcumin, NPs and sulfasalazine on tissue nitrite levels

In IBD, inducible isoform of nitric oxide synthase (iNOS) is found to be overexpressed, leading to increased production of nitric oxide (NO) [24]. Excess NO can damage the integrity of the colonic mucosa as it can react with superoxide (O₂⁻) to produce highly toxic reactive peroxynitrite (OONO⁻). This can further lead to cellular injury and necrosis by DNA damage, protein denaturation and peroxidation of membrane lipids [42, 43]. Tissue nitrite level, expressed as mean concentration (±SD) of tissue nitrite in terms of µg/mg of protein, gives a measure of nitric oxide [44, 45]. Colons of untreated colitic rats exhibited significantly higher (p<0.05) nitrite levels (3.22±0.35 µg/mg) compared to normal rats (1.90±0.18 µg/mg) (Fig. 8). Plain curcumin (2.77±0.26 µg/mg), CN20 NPs (2.16±0.22 µg/mg) and sulfasalazine (2.24±0.24 µg/mg) considerably reduced nitrite levels relative to the colitic control group.
3.11.4. Effect of plain curcumin and CN20 NPs on colon length and colon weight

In colitis, due to inflammation and edema, overall length of colon shortens, as an indication of the severity of injury [46]. The decrease in colon length in the colitic control group (11.1±0.75 cm) relative to normal rats (13.0±0.57 cm) was ameliorated significantly by CN20 NPs (12.9±0.81 cm) and sulfasalazine (12.7±0.57 cm) (p<0.05). In contrast, plain curcumin (11.5±0.47 cm) did not restore colon length.

3.11.5. Histopathological studies

The results of histopathological scoring are shown in Table 5 [47]. Colitic control rats showed moderate levels of necrosis, ulceration, congestion, oedema and severe inflammation. All features were reduced in drug- or NP-treated rats. CN20 NPs improved all features, with only mild residual inflammation and oedema. However, neither plain curcumin nor sulfasalazine completely reversed these features. From the histopathological results it is evident that CN20 NPs showed a better effect in ameliorating IBD as compared to curcumin and sulfasalazine treatments.

3.12. Preclinical pharmacokinetics of NPs

The absorption of pure curcumin was very low (Table 6 and Fig. 9). The T_max values indicate delayed release and hence absorption of curcumin from NPs. CN20 NPs showed higher C_max (≈3 fold) and AUC (≈2.5 fold) values compared to plain curcumin, indicating a greater extent of absorption and bioavailability of curcumin from NPs. This could be attributed to i) improved solubility and dissolution rate of curcumin from NPs, ii) amorphous state of the drug in NPs and iii) target specificity of polymeric carrier.

Higher MRT and t_1/2 values for NPs indicated slow elimination of drug from NPs. In vivo, the NPs did not exhibit a completely controlled steady state drug release pattern; however, the plasma concentration values observed at 6-8 hours are much higher than those observed at previous time intervals. A higher T_max for NPs than plain curcumin suggests curcumin absorption after reaching the colon.
4. CONCLUSIONS

In the present study, curcumin-loaded polymeric NPs of PAAm-g-XG polymer were successfully prepared and characterized. The NPs exhibited high gastric resistance and precise pH-dependent solubility along with microflora-activated drug release properties, which are preferred properties for colonic drug targeting. Curcumin-loaded NPs developed in this study alleviated features of colitis in a pre-clinical rat model. This study provides proof-of-concept and methodology for novel grafted drug loaded NPs for the treatment of colitis.

ACKNOWLEDGEMENTS

The authors are thankful to Manipal University, Manipal, India for providing necessary facilities
References


Figure Captions

Fig. 1. DSC Thermograms of pure curcumin, polymer alone, mixture of curcumin and excipients and CN20 NPs.

Fig. 2. X-Ray Diffraction Patterns of different samples A=Pure drug; B=Physical mixture of drug and excipients; C=CN20 Nanoparticles

Fig. 3. Surface morphology of CN20 NPs (A : SEM images; B: TEM image)

Fig. 4. Dissolution profile of pure drug and CN20 NPs in different media A: pH 1.2 HCl buffer; B: pH 4.6 acetate buffer; C: pH 6.8 phosphate buffer; D: pH 7.4 phosphate buffer; E: pH 7.4 phosphate buffer with rat caecum content

Fig. 5. Cell viability results of pure curcumin and CN20 NPs by MTT assay in Vero (A) and HCT116 (B) cells, expressed as % live cells.

Fig. 6. The percentage change in body weight of rats All the values are expressed as Mean ± SD, n=6; a p<0.05 compared to AA control at respective day; b p<0.05 compared to Normal rats at respective day. Statistical analysis: One-way ANOVA with Dunnet’s post-hoc test.

Fig. 7. Effect of curcumin, NPs and sulfasalazine on tissue myeloperoxidase (MPO) levels All the values are expressed as Mean ± SD; a p<0.05 compared to AA control; b p<0.05 compared to Normal rats; Statistical analysis: One-way ANOVA with Dunnet’s post-hoc test

Fig. 8. Effect of curcumin, NPs and sulfasalazine on tissue nitrite levels All the values are expressed as Mean ± SD; a p<0.05 compared to AA control; b p<0.05 compared to Normal rats; Statistical analysis: One-way ANOVA with Dunnet’s post-hoc test

Fig. 9. Plasma concentration vs. time curves of pure curcumin and CN20 NPs All the points are presented as Mean ± SD, n=6. All the concentration values of CN20 NPs are significantly (p<0.05) different from those of plain curcumin at respective time points; Statistical analysis: Two tail p value in Student’s t test.
Fig. 1.
Fig. 4.

[Graph showing the cumulative percentage of drug released over time for Curcumin, CN20, and CN20 + Cecum content.]

Cumulative % of drug released vs. Time (h)
Fig. 5.
Fig. 6.
Fig. 7.
Fig. 8.
Fig. 9.
### Table 1. Composition of different nanoparticle formulations

<table>
<thead>
<tr>
<th>Batch code</th>
<th>Curcumin (mg)</th>
<th>Polymer (mg)</th>
<th>1% w/v PVA (mL)</th>
<th>DCM (mL)</th>
<th>AlCl$_3$ (% w/v)</th>
<th>Vol. of AlCl$_3$ (mL)</th>
<th>Sonication Parameters (Amplitude/ Time/ pulse)</th>
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<td>10</td>
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</table>

*Time in minutes, pulse in seconds; PVA= Polyvinyl alcohol; DCM= Dichloromethane; AlCl$_3$= Aluminum chloride.
Table 2. Saturation solubility of curcumin and curcumin NPs in various media.

<table>
<thead>
<tr>
<th>Media</th>
<th>Solubility (µg/mL)</th>
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<td></td>
<td>Curcumin</td>
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<tr>
<td>Distilled water</td>
<td>1.45</td>
</tr>
<tr>
<td>pH 1.2 HCl buffer</td>
<td>1.20</td>
</tr>
<tr>
<td>pH 7.4 Phosphate buffer</td>
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</tr>
<tr>
<td>pH 1.2 + 3% w/v T 80</td>
<td>1590.20</td>
</tr>
<tr>
<td>pH 7.4 + 3% w/v T 80</td>
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<td>pH 1.2 + 3% w/v SLS</td>
<td>1500.40</td>
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<td>pH 7.4 + 3% w/v SLS</td>
<td>2409.30</td>
</tr>
</tbody>
</table>

T 80= Tween 80; SLS= Sodium lauryl sulphate.
Table 3. The effect of various parameters in optimizing the NPs

| Selection of method of preparation of NPs |
|-----------------|---|---|---|---|---|
| Batch/Method    | Vol. of 5% AlCl₃ (mL) | PS (nm) | ZP (mV) | PDI | EE (%) | PY (%) |
| CN1/I           | 5.0                        | 405.0  | 7.604   | 0.220 | 16.89  | 66.80  |
| CN1A/I          | 2.5                        | 676.0  | 2.760   | 0.565 | 4.00   | 44.40  |
| CN2/II          | 5.0                        | 1784.0 | 7.210   | 0.636 | 4.20   | 44.40  |
| CN2A/II         | 2.5                        | 1672.0 | 11.100  | 0.676 | 4.21   | 63.60  |

<table>
<thead>
<tr>
<th>Optimization of drug polymer ratio</th>
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<tbody>
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<td>Batch</td>
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<td>CN 6</td>
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<table>
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<th>Optimization of organic to aqueous phase volume</th>
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<td>CN8</td>
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<td>CN9</td>
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<table>
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<th>Effect of varying AlCl₃ concentration</th>
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<td>CN 10</td>
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<td>CN 12</td>
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<th>Optimization of probe ultra-sonication parameters</th>
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<tr>
<td>CN 14</td>
</tr>
<tr>
<td>CN 15</td>
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</table>

PS= Particle size; ZP= Zeta potential; PDI= Polydispersity index; EE= Entrapment efficiency; PY= Practical yield; * Sonication parameters: Amplitude/ Time (min) /Pulse (sec).
Table 4. Results of optimization of suitable cryoprotectant.

<table>
<thead>
<tr>
<th>Batch</th>
<th>CP (% w/v)</th>
<th>PS (nm)</th>
<th>ZP (mV)</th>
<th>PDI</th>
<th>PY (%)</th>
<th>Appearance</th>
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<tr>
<td>CN 1</td>
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<td>0.639</td>
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CP= Cryoprotectant; PS= Particle size; ZP= Zeta potential; PDI= Polydispersity index; PY= Practical yield.
Table 5. Histopathological evaluation of colon from rats treated with oral administration of curcumin, NPs and Sulfasalazine.

<table>
<thead>
<tr>
<th>Groups</th>
<th>NEC</th>
<th>INF</th>
<th>ULC</th>
<th>OED</th>
<th>CON</th>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA control</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Plain curcumin</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CN20 NPs</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Histopathological scale: - = negative; + = slight; ++ = moderate; +++ = severe.
NEC= Necrosis; INF= Inflammation; ULC= Ulceration; CON= Congestion; OED= Oedema
Table 6. The pharmacokinetic parameters of curcumin when administered as pure drug and NPs orally in rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pure curcumin</th>
<th>CN20 NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</td>
<td>60.06±5.01</td>
<td>170.30±10.69 *</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>2.00±0.00</td>
<td>6.00±0.00 *</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-t&lt;/sub&gt; (ng.h/mL)</td>
<td>881.70±76.72</td>
<td>1929.90±146.92 *</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (ng.h/mL)</td>
<td>1033.10±93.26</td>
<td>2569.20±161.18 *</td>
</tr>
<tr>
<td>Ke (1/h)</td>
<td>0.093±0.010</td>
<td>0.063±0.006 *</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>7.46±0.51</td>
<td>11.01±0.99 *</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>13.20±1.14</td>
<td>17.80±1.27 *</td>
</tr>
</tbody>
</table>

All values are presented as Mean±SD, n=6; * statistically significant (p<0.05) when compared to respective values of pure curcumin; Statistical analysis: Two tail p value in Student’s t test; AUC= Area under the curve; Ke= Elimination rate constant; t<sub>1/2</sub>= Elimination half-life; MRT= Mean residential time.