CHAPTER-4

CHITOSAN NANOPARTICLES
Introduction

Colon cancer has been reported as a leading cause of mortality and morbidity worldwide [229, 230]. At present, surgical removal is the primary treatment of choice, and early detection and resection be indispensable for curing colorectal cancer [231, 232]. However, the recent chemotherapeutic agents proposed for the cure of this disease are associated with various toxicities. Thus application of non-toxic chemo-preventive agents has captured the concentration of researchers in which one of the main therapeutic can be curcumin (CU).

CU is a yellow natural polyphenol extracted from turmeric (Curcuma longa), which has been subject to a multitude of investigations over the last few decades that demonstrated various health benefits ranging from anti-inflammatory [233], antioxidant [234, 235], anticarcinogenic properties [236, 237]. Furthermore, antidiabetic [238] and anti-HIV [239] activities have also been reported. Clinical study indicates that CU present in Curcuminoids has potential therapeutic value against most chronic disease including neoplastic, neurological, cardiovascular, pulmonary, metabolic and psychological diseases [233, 240, 241]. It has also been established to be effective against adenomas, colon carcinogenesis during progression stage and abnormal crypt foci induced by azoxymethane in rats [242-244]. Nevertheless, therapeutic use of CU has been limited due to poor aqueous solubility, and consequently, minimal systemic bioavailability.

Hence various formulations, particularly CU containing nanoparticles (NPs) are being investigated in an effort to improve its oral bioavailability, including the use of poly (butyl) cyanoacrylate NPs, [245] poly (lactic-co-glycolic acid) (PLGA) NPs [246]. The literature provides partial information regarding the association between CU and biopolymers i.e. chitosan (CS) [247].

CS, the 60-100% N-deacetylated derivative of chitin (a naturally occurring polysaccharide found in the shells of naval crustaceans) having the structure of β-[1–4]-linked D glucosamine (deacetylated unit) and N-acetyl-D-glucosamine is natural, biocompatible, bioadhesive polymer, whereby it produces non-toxic and easily removable degradation products. CS being cationic in nature offers huge advantages for ionic interactions. In alkaline media, the amino groups of CS lose their protons as they interact with the OH groups from the sodium tripolyphosphate (TPP) and this interaction of CS with TPP leads to development of biocompatible CS-NPs [248].

The aim of present investigation has been to study the targeting ability of Eudragit S 100 (ES) coated CS-NPs loaded with CU (ES-CS-NPs-CU) to colon after oral administration and
restricting the size of the formulation up to few nanometres. ES, the pH sensitive enteric polymer has been used for selective colon targeting, as ES dissolves at the pH 7.0 by ionization of its carboxylic functional group and protect the drug loss in the upper gastrointestinal (GI) tract, resulting the release of CU in the colon. The CU exhibits an innumerable biological actions like anti-inflammatory and anti-oxidant effects which work to advantage during chemotherapy [249] [250]. The CU loaded CS-NPs (CS-NPs-CU) have been prepared by ionic gelation method. The coating of ES on CS-NPs-CU (ES-CS-NPs-CU) has been performed by oil-in-oil solvent evaporation method using coat: core ratio (2:1). Preparation of CS-NPs and its ES coating with the release of CU from it at different pH of simulated GI fluid has been represented in Fig 4A.
Materials and Method

Materials

Curcumin (CU), Chitosan (CS) (70% deacetylated), Sodium azide-flourescin isothiocynate (FITC), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Tween 80 and Span 85 were purchased from Sigma Aldrich (St. Louis, USA). Eudragit S100 (ES) was gifted from Evonik Roehm Pharma polymers. Triethylcitrate, Dialysis bag (cut off mol. wt. 12000 Dalton) were purchased from Himedia (Mumbai, India). Sodium tripolyphosphate (TPP), HPLC grade acetonitrile and methanol were purchased from Spectrochem India. All other chemicals used were of analytical grade. Milli Q water was used throughout the study was prepared by Milli- Q plus 185 purification system (Bedford, Massachusetts)

Preparation of CS-NPs-CU

CS-NPs have been prepared by the procedure based upon ionic gelation between positively charged CS and negatively charged TPP as reported earlier by Calvo et al. (1997). Briefly, the CS was dissolved in 2% acetic acid solution (50 mL) containing 1% of Tween-80 for 12 h and 2% (w/v) TPP solution prepared in triple distilled water was added drop-wise to CS solution and stirring was continued for 2 h thereby leading to formation of CS-NPs, a opalescent suspension at ambient temperature (25°C). The prepared CS-NPs were separated by centrifugation and washed 3 times with 10 % aqueous methanol and lyophilized. CS-NPs-CU was similarly prepared by adding a TPP solution and CU (5 mg) in ethanol simultaneously into the CS solutions.

Fig 4A: Schematic representation of preparation of polysaccharide nanoparticles by ionic gelation method.
**ES coating on CS-NPs-CU**

The above prepared CS-NPs-CU was further coated with ES using oil-in-oil solvent evaporation method using coat: core ratio (2:1) [251]. CS-NPs-CU was dispersed in 10 mL of coating solution prepared by dissolution of ES in ethanol: acetone (2:1). The dispersion was then poured in 50 ml of light liquid paraffin containing 1% w/v Span 85. This dispersion was maintained under agitation speed of 600 rpm at room temperature for 3 hours, after which the organic phase was removed under vaccum. The resultant solution was washed with 3×50 ml of n-hexane to remove liquid paraffin and dried to obtain ES-CS-NPs-CU. ES-CS-NPs without CU have also been prepared by the aforementioned method and the schematic diagram 4 B shows release of coating in GIT.

![Schematic diagram](image)

**Fig 4B.** Schematic diagram representing the preparation of ES-CS-NPs-CU and the release of CU from it at different pH of gastric fluid and colonic fluid.

**Parameters Optimization**

Various formulations and process variables that could affect the preparation and properties of the NPs were identified and optimized to get small, discrete, uniform, spherical and smooth surfaced nanospheres.

**Effect of polymer concentration**
Initially the effect of CS concentration on the formation was examined. The general appearance, size and zeta potential of NPs containing different CS concentration (1.0 - 2.5 mg/ml) were measured after 24h storage at room temperature.

**Effect of TPP concentration**

TPP (Sodium tripolyphosphate) acts as cross linking agent for chitosan polymeric chains. TPP concentration if too high and too low then size and PDI of the NPs containing different TPP concentrations (0.8 - 2.0 mg/ml) were measured after 24h storage at room temperature.

**Characterization of nanoparticles**

**Fourier transforms infrared spectroscopy (FTIR)**

The cross linking of prepared CS with TPP were confirmed by FTIR spectrum which has been recorded on FTIR multiscope spectrophotometer (Perkin-Elmer, Seer Green, Beaconsfield, and Buckinghamshire, United Kingdom) equipped with spectrum v3.02 software.

**Particle size and zeta potential**

The mean particle size, size distribution and zeta potential of CS-NPs, CS-NPs-CU and ES-CS-NPs-CU were determined by a Malvern Zetasizer NanoZS (Malvern 3000HS, France). Each sample was measured in triplicate.

**Scanning Electron Microscope (SEM)**

For morphological characterization of CS-NPs-CU and ES-CS-NPs-CU, SEM (Leo Electron Microscope Ltd, Cambridge, UK.) studies were performed. Gold and palladium coated samples were placed over a copper grid and subjected to SEM analysis.

**Transmission Electron Microscope (TEM)**

A drop of the fresh CS-NPs-CU and ES-CS-NPs-CU was placed onto a carbon-coated copper grid, forming a thin liquid film. The films on the grid were negatively stained with drop of 1% (w/v) phosphotungstic acid. The excess staining solution was drained off with a filter paper, and then air-dried. The stained films were viewed under a transmission electron microscope and photographed.
% Entrapment efficiency (%EE)
% EE has been calculated to estimate the amount of CU loaded in CS-NPs-CU and ES-CS-NPs-CU. The CS-NPs-CU and ES-CS-NPs-CU were centrifuged at 45,000 rpm for 3 h. After centrifugation, the supernatant was collected and the amount of un-entrapped CU was analyzed by HPLC method described below. The % EE was given by the following equation [252].

% E.E = (weight of CU used - weight of un-entrapped CU × 100) / Weight of CU used

Drug loading in CS-NPs-CU and ES-CS-NPs-CU has also been determined by:
% Drug loading = (amount of encapsulated CU / amount of NPs) ×100

HPLC Analysis
A HPLC method was developed and validated for estimating CU. The HPLC system was equipped with two 10 ATVP binary gradient pumps (Shimadzu), Rheodyne (Cotati, CA, USA) model 7125 injector fitted with a 20 µl loop and SPD-M10 AVP UV detector (Shimadzu). HPLC separation was achieved on a RP-C18 column (250mm, 4.6mm, 5 µm, Merck). Column effluents were monitored at 425 nm. Data was acquired and processed using Shimadzu class VP software. The mobile phase was a mixture of acetonitrile: triple distilled water: glacial acetic acid (65:34:1) v/v. The solution was filtered and degassed before use. Chromatography was performed at 25°C; at a flow rate of 1.0 ml/minute. Calibration curve of CU was in the range of 1-10 µg/mL. The same method was used to analyze the drug during in-vitro release studies. Under these conditions the drug shows retention time of about 4 minute.

In vitro release study
In vitro release study of CU, CS-NPs-CU and ES-CS-NPs-CU was performed in simulated gastro intestinal fluids i.e in simulated gastric fluid (SGF) at pH 1.2 (2 h), in intestinal fluid (SIF) at pH 7.4 (6 h) and colonic fluids (SCF) of pH 6.8 (24 h) at 37°C to evaluate the effect of different pH on CS-NPs-CU and ES-CS-NPs-CU. The volume of SGF [253] (pH 1.2) containing sodium chloride (1.0 g), pepsin (1.6 g) and hydrochloric acid (3.5 ml) was made up to 500 ml with triple distilled water. The volume of SIF [254] (pH 7.4) consisting of monobasic potassium phosphate (3.4 g), 0.2N sodium hydroxide (90 ml) and pancreatin (5 g) was made up to 500 ml with triple distilled water. SCF was prepared by a method reported earlier [254], [255], [256]. The release studies were carried out in a USP dissolution test apparatus (Apparatus II, 100 rpm, 37±0.5°C) using the method reported earlier with slight modification [257], [258]. Briefly 500
ml of dissolution medium was taken in the container, immersed in the water bath of the apparatus. The treated dialysis bags containing CS-NPs-CU and ES-CS-NPs-CU were immersed separately in the dissolution medium containing SGF, then into SIF and finally in to SCF. Tween 80 (1%, v/v) was added to the dissolution medium to facilitate the solubilization process of CU released from CS-NPs-CU and ES-CS-NPs-CU. Samples (2 ml) were withdrawn from each dissolution medium at a predetermined time intervals (0.25h, 0.5h, 1h, 2h, 3h, 4h, 5h, 6h, 8h, 10h, 12h, 18h, 24h) replacing with same volume of respective dissolution medium after each sampling to maintain the sink condition. The sample was centrifuged at 10,000 rpm for 15 min, supernatant was filtered through 0.4 µm membrane filter and the filtrate was subjected to HPLC analysis after appropriate dilution.

Cytotoxicity studies

In vitro cytotoxicity of CU, CS-NPs-CU, ES-CS-NPs-CU along with CS-NPs and ES-CS-NPs have been analyzed in Caco-2 cell lines by (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) MTT assay. Caco-2 cell line was maintained in sulforhodamine B growth medium supplemented with 10% heat inactivated in FBS, 2mM L-Glutamine, Penicillin 100 U/ml, Streptomycin 100 µg/ml. Cells were grown in incubator (5% CO₂, 37°C) for the stated length of time. The Caco-2 cells were seeded onto 96 well plates at a density of 4×10⁵ cells/well for 24 h and CU, CS-NPs-CU, ES-CS-NPs-CU at a concentration of CU 10µM and 20µM and 40µM were incubated for 24 h [259-261] and CS-NPs, ES-CS-NPs have also been incubated at equivalent concentrations to that of CS-NPs-CU, ES-CS-NPs-CU; to know the cytotoxic effect of blank CS-NPs, ES-CS-NPs. Cytotoxicity was estimated by staining live cell by 0.5 mg/mL MTT for 3 h (formazan crystal formation), the optical density values were determined at 540 nm using ELISA plate reader. Triplicates of each sample were analyzed. The data has been reported as a mean values from three different experiments.

In vivo studies

The study was carried out according to the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Experiments were conducted after ethical clearance by the Institutional Animal Ethics Committee of the institute (IAEC approval no. 2012/40/Pharmaceutics/IAEC).
Animals
Male Wistar rats (150-200 g) were obtained from ‘central animal facility’ of the institute. The study was conducted in accordance with current legislation of institute on animal experiments and protocol was approved by the ‘Institutional Animal Ethical Committee’. All rats were individually housed at 21°C, for 12 cycle of 12-h/12-h light-dark. Normal chow diet and water were provided ad libitum.

Histopathological study
Histopathological study was performed to visualize the localization of the carrier system in Peyer’s patches and enterocytes. Wistar rats weighing about 150–200 g were divided into three groups having 6 rats each and kept on overnight fasting prior to dosing with Fluorescein isothiocynate (FITC) loaded CS-NPs (FITC-CS-NPs) and FITC loaded ES-CS-NPs (FITC-ES-CS-NPs), which were prepared by the same method used for the preparation of CS-NPs-CU and ES-CS-NPs-CU as described above, FITC was taken instead of CU. All the protocol was performed in dark conditions. 1 ml of FITC loaded formulation (~15mg) was given orally with the help of feeding cannula to each rat. The uptake of FITC-CS-NPs and FITC-ES-CS-NPs in stomach tissues was assessed by sacrificing rat after 2 h of post dosing. About 4-6 cm long part of stomach was collected and washed with saline and fixed in formalin for further sectioning using cryostat. Similarly, small intestine and colon sections were collected after 5 h and 9-10 h of post dosing respectively and washed with saline fixed, processed and embedded in paraffin wax and 5-µm thick sections were cut using microtome (LEICA RM 2155, Nussloch, Germany) and photographs were taken using fluorescent microscope (Leica, DMRBE, Bensheim, Germany)
Pharmacokinetic study
Pharmacokinetic studies were carried out to analyze the colon specific delivery of CU in ES-CS-NPs-CU, CS-NPs-CU and CU. Wistar rats were randomly divided into three groups and kept on overnight fasting prior to dosing. CU, ES-CS-NPs-CU and CS-NPs-CU containing equivalent amount of CU (10 mg/kg body weight) were administered orally to different groups. Blood (500 μl) was collected from retro orbital plexus under mild ether anaesthesia at 0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h and 24 h into heparinised microcentrifuge tubes. The samples were collected for 24 h as the gastric emptying time in rats is about 22 h [262]. Plasma was immediately prepared by centrifugation at 1000 rpm for 10 min at 4 °C and stored at −80 °C until use. After protein precipitation, concentration of CU in plasma was determined by HPLC method.

Statistical analysis
All data are expressed as mean ± standard deviation. Statistical analysis was done with one-way analysis of variance (ANOVA) followed by the Turkey-Kramer multiple comparison test, using Graph Pad Instat software (Graph Pad Software Inc., San Diego, California). P<0.01 was considered to be statistically significant.
Results

Optimization of Process Variables

Effect of polymer concentration
The increase in particle size with increased CS concentration could be attributed to higher gel content as more gelatin chains are available inside the droplets, which can swell more after re-dispersion in the aqueous phase. The data has been shows in Table 4A. The PDIs were in the range of 0.5−0.2 for the redispersed samples, indicating a relatively narrow size distribution. Membrane permeability of the CS NPs would be theoretically lower with increasing CS concentration due to increased chain packing and rigidity, as well as inter chain bonding.

<table>
<thead>
<tr>
<th>Polymer conc. (mg/ml)</th>
<th>Size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>837 ± 15.34</td>
<td>0.57 ± 0.02</td>
</tr>
<tr>
<td>2.0</td>
<td>776 ± 13.54</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>1.6</td>
<td>525 ± 6.64</td>
<td>0.47 ± 0.06</td>
</tr>
<tr>
<td>1.2</td>
<td>368 ± 5.51</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>1.0</td>
<td>194 ± 5.01</td>
<td>0.26 ± 0.04</td>
</tr>
</tbody>
</table>

Effect of TPP concentration
TPP (Sodium tripolyphosphate) acts as cross linking agent for CS polymeric chains. TPP concentration if too high and too low then size and PDI of the nanoparticles is increased simultaneously. So, optimum conc. must maintained (1mg/ml). The data has been shows in Table 4B.

The electrostatic repulsion prevents the polymer chains from uncontrolled agglomeration. After the NPs are formed, their surface has a sufficient zeta potential to prevent further agglomeration of the particles.
Table 4B: Effect of TPP concentration on size and PDI of nanoparticles

<table>
<thead>
<tr>
<th>TPP concentration (mg/ml)</th>
<th>Size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>976 ± 16.37</td>
<td>0.67 ± 0.04</td>
</tr>
<tr>
<td>1.6</td>
<td>576 ± 11.34</td>
<td>0.55 ± 0.05</td>
</tr>
<tr>
<td>1.2</td>
<td>368 ± 6.63</td>
<td>0.41 ± 0.06</td>
</tr>
<tr>
<td>1.0</td>
<td>200 ± 5.51</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>0.8</td>
<td>431 ± 7.01</td>
<td>0.52 ± 0.03</td>
</tr>
</tbody>
</table>

*Values shown are mean ± SD (n=3)

Particle size, poly dispersity Index (PDI) and zeta potential of NPs

The mean particle size of CS-NPs, CS-NPs-CU and ES-CS-NPs-CU was found to be 112±4.2 nm, 173±4.5 nm and 236±3.2 nm with PDI of 0.29, 0.16 and 0.22 respectively. The zeta potential of CS-NPs, CS-NPs-CU and ES-CS-NPs-CU was found to be 48.1±2.1 mV, 31.7±1.7 mV and -29.8±2.2 mV respectively. The data has been illustrated in Table 4C.

Table: 4C. Particle Size, PDI, zeta potential and % EE of CS-NP, CS-NP-CU and ES-CS-NP-CU.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle Size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>%EE</th>
<th>% Drug loading</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-NP</td>
<td>112 ± 4.2</td>
<td>0.29</td>
<td>48.1 ± 2.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CS-NPs-CU</td>
<td>173 ± 4.5</td>
<td>0.16</td>
<td>31.7 ± 1.7</td>
<td>44 ± 1.3</td>
<td>3.9 ± 1.4</td>
</tr>
<tr>
<td>ES-CS-NPs-CU</td>
<td>236 ± 3.2</td>
<td>0.22</td>
<td>-29.8 ± 2.2</td>
<td>42 ± 1.9</td>
<td>4.2 ± 1.5</td>
</tr>
</tbody>
</table>

Note: Where, n=3

FTIR characterization of cross linked NPs

The FTIR spectrum for CU, CS, TPP and the CS-NPs-CU is shown in (Fig 4C.A). The FTIR CU spectrum (Fig 3A) shows characteristic absorption bands at 3434 cm\(^{-1}\) which represent phenolic OH, at 1628 cm\(^{-1}\) the C=O stretching peak of conjugated ketone, at 1512 cm\(^{-1}\) and 1441 cm\(^{-1}\) represents aromatic and aliphatic C=C stretching respectively and the C–O–C stretching peak of ether at 1029 cm\(^{-1}\) [263].
The FTIR spectrum for CS (Fig 4C.B) shows characteristic absorption bands at 3448 cm\(^{-1}\), which represent the presence of an OH group. The NH\(_2\) bending peak at 1587 cm\(^{-1}\) associated with the glucosamine functional group were also present [264]. While C=O stretching peak at 1656 cm\(^{-1}\) represents the structure of N-acetylated group. Stretching vibrations at 1214 and 900 cm\(^{-1}\) have been reported for phosphate groups present in the spectra of TPP (Fig 4C.C). The FTIR spectra of CS-NPs-CU (Fig 4C.D) shows disappearance of peak at 1656 cm\(^{-1}\) and 2 new peaks at 1651 cm\(^{-1}\) and 1572 cm\(^{-1}\) appeared that is attributed to linkage between the phosphoric and ammonium ions. The appearance of new band at 1116 cm\(^{-1}\) confirms the presence of TPP [265] linked to the CS through intermolecular interactions.

![Fig 4C. FT-IR spectra of (A) CU, (B) CS, (C) TPP, and (D) CS-NPs-CU.](image)

**Drug entrapment efficiency, % Drug loading**

The % EE and drug loading in ES-CS-NPs-CU was found to be 42±1.9 and 4.2±1.5 respectively while in case of CS-NPs-CU was found to be 44±1.3 and 3.9±1.4 respectively (Table.4C)
SEM and TEM

The SEM and TEM image of developed formulations has been represented in Fig 4D and Fig 4E. As shown in Fig 4D.A and Fig 4E.A, CS-NPs-CU has uniform spherical shape with smooth texture while ES-CS-NPs-CU have rough surface (Fig 4 D.B and Fig 5E.B) that could be due to coating with ES.

![SEM images of (A) CS-NPs-CU (B) ES-CS-NPs-CU](image)

**Fig 4 D. SEM images of (A) CS-NPs-CU (B) ES-CS-NPs-CU**

![TEM images of CS-NPs-CU (B) ES-CS-NPs-CU](image)

**Fig 5 E. (A) TEM images of CS-NPs-CU (B) ES-CS-NPs-CU**

**In vitro release study**

The *in vitro* release profile of CU from CU, CS-NPs-CU and ES-CS-NPs-CU has been carried out in USP dissolution apparatus II, by using dialysis bag (Sigma; cut off MW-12 KD). The release of CU from CU, CS-NPs-CU and ES-CS-NPs-CU at pH 1.2, 7.4 & 6.8 were monitored
by analyzing the samples through HPLC method described previously. The in-vitro release of CU, CS-NPs-CU and ES-CS-NPs-CU exhibited fairly controlled release profile; the amount of CU released in SGF at pH 1.2 was 54.3 ± 2.4 % and 21.2 ± 2.1 % for CU and CS-NPs-CU. In case of ES-CS-NPs-CU no CU release has been observed in SGF, this might be due to the coating of ES; CU begins to release after 2 h when it was exposed to SIF pH 7.4 which can be attributed to carboxyl groups of ES that ionize from neutral to alkaline media. As the ionization precedes the integrity of the coating gets disturbed and the CU releases.

However, release study conducted in SIF pH 7.4 at 37°C up to next 6 h showed 53.8 ± 4.1 % and 31.6 ± 4.2 % of cumulative CU release from CS-NPs-CU and ES-CS-NPs-CU respectively whereas CU, as a plain drug was readily available and released immediately through the membrane 90.8 ± 4.8 % within 4 hrs (Fig 4F). The cumulative release of CU from CS-NPs-CU at pH 7.4 may be due to diffusion through nonporous polymer matrix. The cumulative release profiles in the presence of rat caecal and colonic contents in SCF were 77.9 ± 7.8 % and 69.3 ± 6.4 % for CS-NPs-CU and ES-CS-NPs-CU respectively. 27% of CU was released from ES-CS-NPs-CU in SCF; whereas only 18% of CU was released from CS-NPs-CU in SCF. This profile indicated that more CU was available in colon from ES-CS-NPs-CU than CS-NPs-CU. The results clearly shows that ES-CS-NPs-CU releases the maximum amount of CU in the colonic environment, possibly due to degradation of polymer matrix by colonic pH and the microbial flora residing in the colon.
Fig 4F. *In vitro* drug release profile of CU, CS-NPs-CU and ES-CS-NPs-CU in different simulated GI fluids [SGF (1.2), SIF (7.4) and SCF (6.8)]

**Cytotoxicity studies**

The dose dependent cytotoxicity of the formulations was determined by MTT assay [266]. The cell viability was found to be more than 93% at all equivalent concentrations for both CS-NPs and ES-CS-NPs; which do not include CU, after 24h of incubation in Caco-2 cell lines revealing the safety of the excipients used in the formulations while the MTT assay unveiled that CS-NPs-CU and ES-CS-NPs-CU when exposed to different equivalent concentrations reduce the viability of Caco-2 cell lines. The cell viability at 10µM, 20µM and 40µM concentrations was estimated and the results revealed that the viability of cells exposed to ES-CS-NPs-CU was less than the cells exposed CS-NPs-CU but the difference was not significant. At higher concentration (40µM) ES-CS-NPs-CU and CS-NPs-CU showed 24.4 ± 4.5 % and 26.1 ± 2.9 % of cell viability respectively, at 20µMol ES-CS-NPs-CU and CS-NPs-CU showed less than 40% of cell viability whereas at lower concentration (10 µM) ES-CS-NPs-CU showed 47.3 ± 3.6 % and CS-NPs-CU showed more than 50% of cell viability. The data has been represented in Fig 4G.
Fig 4G. Cell viability of Caco-2 cells exposed to different concentrations are 10 µM, 20 µM, 40 µM. Cell viability of Caco-2 cell line in the presence of CU, CS-NPs-CU, ES-CS-NPs-CU, CS-NPs and ES-CS-NPs. The cell viability was measured via the MTT assay. Values shown are means and standard deviations (n=3).

**Histopathological study**

The histopathological studies of FITC-CS-NPs and FITC-ES-CS-NPs in various part of GI tract were carried out after oral administration. The control did not show any kind of fluorescence (Fig 4H.A, 4H.D and 4H.G). Fig 4H (B, E, H) shows the uptake of FITC-CS-NPs in stomach, intestine and colon respectively. The Fig 4H.B shows the fluorescence of FITC-CS-NPs to the apical part of the stomach, which was absent in stomach sections of FITC-ES-CS-NPs (Fig 4H.C) i.e., possibly due to the effect of ES coating which prevents the release. Section of intestine Fig 4H.E shows uptake of FITC-CS-NPs to some extent than Fig 4H.F i.e. FITC-ES-CS-NPs. Furthermore, in colon section fluorescent points could be observed in the connective axis of the villi representing FITC-ES-CS-NPs inside vascular structures (Fig. 4H.I) as compare to FITC-CS-NPs (Fig. 4H.H). This reveals enhanced uptake of FITC-ES-CS-NPs in comparison with that of FITC-CS-NPs in colon sections.
Fig 4H. A section of stomach, small intestine and colon has been represented as Fig 4H.A, Fig 4H.D and Fig 4H.G respectively after oral administration. Section of stomach Fig 4H.B and Fig 4H.C shows uptake of FITC-CS-NPs and FITC-ES-CS-NPs respectively. Fig 4H.E and Fig 4H.F shows uptake of FITC-CS-NPs and FITC-ES-CS-NPs in section of small intestine. The colon section Fig 4H.H shows that FITC-CS-NPs and Fig 4H.I FITC-ES-CS-NPs-CU reach the colon in decent quantities.
Pharmacokinetics

Calibration curve of CU was found to be linear in the range of 50-300 ng and the coefficient of correlation was found to be 0.993. The plasma concentration time profiles of different CU formulations are given in Fig 4I. The relevant pharmacokinetic parameters including $C_{\text{max}}$, $T_{\text{max}}$, MRT and $AUC_{0-\infty}$ are listed in Table 4D. Higher MRT and higher $AUC_{0-\infty}$ has been observed in ES-CS-NPs-CU (21.12±2.30 h, 16906.62±103.87 h*ng/mL) as compared to CS-NPs-CU (18.59±3.50 h, 9817.14±87.00 h*ng/mL) and CU (12.252±2.42 h, 4722.55±76.98 h*ng/mL) respectively. CS-NPs-CU exhibited a rapid increase in the plasma level, which reached a maximal plasma concentration ($C_{\text{max}} = 1102.38 \pm 25.31$ ng/mL) than that of ES-CS-NPs-CS ($C_{\text{max}} = 904.74 \pm 38.46$ ng/mL) and CU ($C_{\text{max}} = 514.12 \pm 14.09$ ng/mL).

Table: 4D. Pharmacokinetic parameters of CU upon oral administration of CU, CS-NPs-CU and ES-CS-NPs-CU.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>$C_{\text{max}}$ (ng/ml)</th>
<th>$T_{\text{max}}$ (hrs)</th>
<th>$AUC_{0-\infty}$ (ng/h/ml)</th>
<th>MRT (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CU</td>
<td>514.12± 14.09</td>
<td>3.16 ± 0.15</td>
<td>4722.55 ± 76.98</td>
<td>12.252 ± 2.42</td>
</tr>
<tr>
<td>CS-NPs-CU</td>
<td>1102.38 ± 25.31</td>
<td>4.1 ± 0.1</td>
<td>9817.14 ± 87.00**</td>
<td>18.59 ± 3.50</td>
</tr>
<tr>
<td>ES-CS-NPs-CU</td>
<td>904.74 ± 38.46</td>
<td>8.33 ± 1.30</td>
<td>16906.62 ± 103.87** ##</td>
<td>21.12 ± 2.30</td>
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** p<0.01, Significances vs. CU, ## p<0.01 denotes ES-CS-NPs-CU vs. CS-NPs-CU

$C_{\text{max}}$: maximal plasma concentration.

$T_{\text{max}}$: time to reach maximal plasma concentration.

AUC: area under plasma concentration vs. time curve.

MRT: Mean residual time.
Fig 4I. *In-vivo* bioavailability of CU from CS-NPs-CU and ES-CS-NPs-CU. Value are means ± SD (n = 3).
Discussion
It has been postulated that delaying the drug release could result in higher localization of drug in to the colon with subsequent reduction in dose of drug with increased specificity. CU with a strong anti-proliferative activity and low toxicity has attracted much interest and has been preferred as potential agent for adjunct chemotherapy for the conditions of colon cancer [237, 267]. CS, a natural polymer has been chosen as a carrier for the preparation of the NPs and delivering the CU due to its attractive biodegradable and biocompatible properties with varied pharmaceutical applications. The CS molecules have been converted into NPs with TPP molecules via electrostatic interaction by ionic gelation method and the formation of which has been indicated by its opalescence appearance [229, 268]. Furthermore, by ionic gelation method, the development of CS-NPs-CU has been carefully controlled to achieve the preferred property like uniformity in NPs with low polydispersity. The formulation of CS-NPs-CU has been intended to deliver the CU in the colon which is not achievable without protecting them from being released in the stomach (pH 1.2). Exploring the unique features of colon (pH 6.8) can be one of the ways for maximum drug release in the vicinity of colon. ES having the desired properties has attracted our interest in preparation of such pH dependent targeted formulation, which can deliver the drug to the specific targeted area. In the present investigation, the pH-dependent approach was exploited for the specific delivery of ES-CS-NPs-CU to the colon. The ES coating was achieved by oil-in-oil solvent evaporation method using coat: core ratio 2:1. The selective dissolution of ES at the colonic pH by the ionization of its carboxylic functional groups was exploited to achieve the suitable action [269]. The process provides the particles in the nano-size range with low polydispersity index and appropriate zeta potential. In addition, zeta potential has been shown to affect the intra-cellular localization of the NPs [270]. However, the particle size of CS-NPs-CU (173±4.5) was more with decreased zeta potential than that of CS-NPs (112±4.2), this may be due to loading of CU into CS-NPs. CS-NPs-CU initially had a positive zeta-potential (+31.7 ± 1.7 mV). This positive zeta-potential of CS-NPs-CU was found to be reversed by ES-CS-NPs-CU and reached to (-29.8 ± 2.2 mV). This indicates that coating of CS-NPs-CU by ES loses its positive charge and was completely masked by the negative charge of ES resulting in ES-CS-NPs-CU. The characterization with FTIR confirms that the phosphate groups of TPP have been linked to the amino groups of CS through intermolecular interaction. The entrapment efficiency of CS-NPs-CU was found slightly higher than ES-CS-NPs-CU this
might be due to small amount of CU loss in the process of coating. Particle size and surface morphology was further confirmed by SEM and TEM studies which have evidently shown ES coating on CS-NPs-CU. The formulation intended for colon-targeted delivery system requires minimum release of the drug in the stomach and the upper small intestine to ensure maximum drug reaches the colon during its passage which has been successfully demonstrated by the in vitro release results, which clearly depicts that the formulations has a differentiated pattern of release at different pH. Fig.4F shows that the release of CU was instantaneous at pH 1.2 as compared to pH 7.4 and 6.8. However, in case of CS-NPs-CU biphasic release profile was observed. The initial burst release was due to CU adsorbed on the surface of the particles followed by diffusion controlled phenomenon due to swelling of polymeric matrix caused by protonation of primary amino groups of CS [271, 272].

In case of ES-CS-NPs-CU, there was no release of CU at pH 1.2 as anticipated, however the release was observed when the pH of the medium was adjusted to 7.4 or 6.8. To appraise the CU release in the colonic environment, SCF (pH 6.8) containing 4% rat cecal contents with enzyme induction (Fig. 4F) at 37°C was used. After 12 h the cumulative percent CU released was found to be 67 ± 5.2 % from CS-NPs-CU in which more than 20 % of the drug release was observed in SGF and up to 54 ± 2.4 % in SIF however, in case of ES-CS-NPs-CU there was no release of CU at pH 1.2 (SGF) as mentioned above though 56 ± 5.2 % of CU was released after 12 h when kept in SCF. The results are expressed as cumulative percent released in all simulated gastro intestinal fluid (SGIF). As shown in Fig 4F, 27% of CU was released from ES-CS-NPs-CU in SCF while it was only 18% from CS-NPs-CU. This clearly demonstrates that maximum fraction of CU was available in colon from ES-CS-NPs-CU as compared to CS-NPs-CU [273], this pH-dependent solubility of this polymer was exploited to avoid the rapid dissolution of CU during the initial transit of the NPs through the gastric cavity. The data has been represented in Fig 4G. This profile indicates that more amount of CU is available in colon from ES-CS-NPs-CU than CS-NPs-CU in cell viability of Caco-2 cells exposed. The same was observed in in-vivo studies (Fig. 4I). The blood levels were proportional to the amount of CU available for absorption at a particular part of GIT. Very small amount of the CU was absorbed in colon from CU or CS-NPs-CU (Tmax at 3.16 and 4.1 h respectively), while most of the CU was absorbed from colon (Tmax 8.33 h) and will remain at higher concentrations till 24 h as compared to CU or CS-NPs-CU.
These results indicate that more CU is available in colon from ES-CS-NPs-CU than CS-NPs-CU or CU for pharmacological action.

ES-CS-NPs-CU has shown a synergistic effect on concentration dependent study in reduction of cell viability. The cell cytotoxicity of ES-CS-NPs-CU has been found to be higher than CS-NPs-CU at all concentrations (10µM, 20µM and 40µM) but the difference was not significant. The CU alone shows higher cell viability as compared to other CU loaded NPs which may be assigned to their size influenced improved cellular internalization.[274] The increased permeability of the NPs may also be attributed to the inhibition of P-glycoprotein effluence pump by D-alpha-tocopheryl polyethylene glycol succinate (TPGS), leading to inhibition of multi-drug resistance (MDR) effect of cancerous cells resulting in increased retention of formulation inside the cancerous cells thus leading to higher cytotoxicity. [275] To confirm the targeted delivery of CU to colon cancer further uptake studies of the developed NPs in vivo models has been carried out which results in opaque staining with the FITC in the colonic tissues indicating specificity of the ES coating to NPs in the colon. This study indicates the potential of polymer matrix to retard the release in the upper GI tract and prolonged release in the regions of colon, its application for the targeted delivery of bioactive is in offing.

CU alone and CS-NPs-CU exhibited rapid increase in plasma profile then ES-CS-NPs-CU, this may be due to the immediate initialization of release after administration. The significantly higher (p<0.01) AUC\textsubscript{0-\infty} was observed in case of ES-CS-NPs-CU in comparison to CU and CS-NPs-CU. The AUC\textsubscript{0-\infty} of ES-CS-NPs-CU increased about 2.01 folds compared to that CU and 1.72 folds compared to CS-NPs-CU respectively. It may be due to the fact that the drug release from ES-CS-NPs-CU occurs after entering the intestine, indicating improved bioavailability of CU as a nanoparticulate formulation. Thus, it is clear from the pharmacokinetic evaluation that ES-CS-NPs-CU are targeted formulations and they release very small amount of the drug in the stomach and release most of the drug in colon.
Conclusion

From the results of the present study, it can be concluded that CS-NPs-CU prepared by ionic gelation method coated with ES (ES-CS-NPs-CU) could be used for colon targeting. These composite NPs have a suitable size distribution, zeta potential and CU encapsulation efficiency. *In vitro* release studies of the CS-NPs-CU and ES-CS-NPs-CU show that CU is sustained. Most importantly, the intracellular uptake and cell viability assays also demonstrated efficient uptake and its enhanced systemic bioavailability. The significant improvement in the pharmacokinetic study demonstrated by the ES-CS-NPs-CU may reduce the effective dose of CU required to inhibit colon cancer. The improved bioavailability of the developed formulation could be due to the nano-structure and the targeting potential of the developed system. The experimental results demonstrate that ES-CS-NPs-CU is the potential candidate which can be used as a drug carrier for the effective colon-targeted delivery.