CHAPTER-6

EUDRAGIT S-100 COATED FOLIC ACID

GLIADIN NANOPARTICLES
Introduction

*In vivo* study plays a major role in validating the effectiveness of a designed drug delivery system. The *in vivo* performance is the preliminary step for clinical evaluation of a drug. There are many ways to assess the *in vivo* performance viz. measuring the intact drug or metabolite level in the blood or urine, assessing the pharmacological or biological response in laboratory animals, or by measuring tissue or organ distribution of the drug. A successful colon targeted drug delivery system is one, which remains intact in the physiological environment of stomach and small intestine but releases the drug in the colon. Different animal models can be used for evaluating colon specific drug delivery systems, but the anatomy and physiology of experimental animals and humans should be almost same in order to extrapolate animal data to clinical trials. Most published studies on the development of colon specific drug delivery systems have been performed in rats, dogs, rabbits, pigs and guinea pigs however studies have also been performed in humans [339-341].

Different *in vivo* methods are used to evaluate the carrier systems for their ability to deliver drugs specifically to the colon. Pharmacokinetic data can also be used indirectly to assess the potential of colon targeted delivery systems. Measurement of drug in isolated organs of GI tract after oral administration is another technique to check the efficacy of the site specific colon targeted delivery system. Gamma scintigraphy and high frequency capsule are currently preferred methods to evaluate colon specific delivery formulations [342]. γ-scintigraphy is the technique of choice for monitoring the transit of pharmaceutical dosage forms through the GI tract [343]. The procedure involves labelling of formulation with an γ-emitting radionuclide. Following administration, an image of the distribution of the radiolabelling within the body is obtained using a gamma camera. The combination of γ-scintigraphy with conventional pharmacokinetic studies facilitates the identification of sites of drug absorption. The radioisotope is normally incorporated into a dosage form or compound and administered to the subject via various routes of administration. The γ-radiation emanating from the subject is collimated and then detected by a crystal. The energy is transformed to light scintillation and amplified so that the information can be digitalized in a quantifiable manner. γ-scintigraphy has been used to monitor physiological processes such as the gastric emptying of pharmaceutical dosage forms, clinical evaluation of new preparations designed to release drugs in the human colon and the evaluation of oral colon targeted drug delivery systems [344]. The procedure is non-invasive and results in
low radiation doses to the patients. In addition to monitoring the transit of food, $\gamma$-scintigraphy can also be used to investigate the behaviour of pharmaceutical dosage forms within the GI tract. Such studies are particularly relevant in assessing formulations designed to target specific regions of the gut or to release active ingredients over prolonged periods.

On the basis of ex-vivo and in vitro characterization of Folic acid conjugated Gliadin nanoparticles (FA-CU-GdNP) the in vivo performance of the developed colon targeted Eudragit S-100 coated Folic acid conjugated Gliadin nanoparticles (ES-FA-CU-GdNP) and uncoated nanoparticles was assessed using gamma scintigraphy, measurement of drug in different segments of GI tract and calculation of pharmacokinetic parameters after oral administration.
Materials and Method

Materials
Curcumin (CU) and Gliadin (Gd) were purchased from Merck Ltd., Mumbai (India). Sodium azide-flourescin isothiocynate (FITC), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Tween 80 and Sorbitan trioleate (Span85), Folic Acid (FA), 1-(3-dimethylaminopropyl)-3-ethylocarbodiimide hydrochloride (EDC), Sodium chloride, N, N'-Dicyclohexylcarbodiimide (DCC) were purchased from Sigma Aldrich (St. Louis, USA). Triethylcitrate, Eudragit S100 (ES) was gifted from Evonik Roehm Pharma polymers. All other chemicals were of analytical grade. Triple distilled water used throughout the study was prepared by Milli- Q plus 185 purification system (Bedford, Massachusetts).

HCT 116 Cell Line
Human colorectal carcinoma cell line HCT116 was kindly gifted by Dr Bert Vogelstein, Johns Hopkins University. The cells were grown in McCoy’s 5A media (Sigma Aldrich) supplemented with antibiotics, L-glutamine and 10% Fetal Bovine Serum (Gibco BRL) at 37 ℃.

Synthesis of FA conjugated Gd
FA conjugated Gd was synthesized by simple carbodiimide coupling chemistry [131]. A solution of FA and 1-(3-dimethylaminopropyl)-3-ethylocarbodiimide hydrochloride (EDC) in anhydrous DMSO (molar ratio was 1:1) was prepared and stirred at room temperature until FA and EDC were well dissolved (2 h). It was then added slowly to a solution of Gd (100 mg) in a mixture of ethanol: water (7:3 v/v). The resulting mixture was stirred at ambient temperature under dark up to 24 h. This mixture was brought to pH 9.0 by drop wise addition of 1M NaOH solution and the precipitate was collected; the precipitate obtained was dialyzed in two steps; against phosphate buffered saline (PBS pH 7.4) for 3 days and then against water for 4 days. The FA-Gd conjugate was collected by lyophilization and kept for further use.

Development of CU-GdNP and FA-CU-GdNP formulations
CU-GdNP were prepared by desolvation method as reported previously with slight modifications [102]. Briefly plain Gd and CU were dissolved in a 20 mL mixture of an ethanol: water (7:3 v/v), this solution was added drop wise into a stirred saline phase (0.9% NaCl) containing 0.5% Pluronic F-68 as stabilizer, after addition, ethanol was removed by evaporation under reduced pressure, centrifuged at 40,000g for 30 min, the supernatant was removed and the pellets were
re-suspended in water, centrifuged again and finally, the precipitated CU-GdNP was freeze-dried to obtain purified CU-GdNP. CU-GdNP batches were hardened by the addition of 2 mg gluteraldehyde per mg CU-GdNP followed by stirring for 2 h at room temperature. The same method was followed for the preparation of FA-CU-GdNP in which FA conjugated Gd have been used instead of plain Gd.

**Eudragit S-100 coating of CU-GdNP and FA-CU-GdNP formulations**

The above prepared CU-GdNP was further coated with ES using oil-in-oil solvent evaporation method with coat: core ratio (2:1) [251]. CU-GdNP was dispersed in 10 mL of coating solution prepared by dissolution of ES in a mixture of ethanol: acetone (2:1). The above dispersion was then poured in 50 ml of light liquid paraffin containing 1% w/v Span 85, maintained under agitation speed of 600 rpm at room temperature for 3 hours, after which the organic phase was removed under vacuum. The resultant solution was washed with 3×50 ml of n-hexane to remove liquid paraffin and dried to obtain ES-CU-GdNP. ES-FA-CU-GdNP were also been prepared by the same method; mentioned above.

Radiolabeled enteric FA conjugated Gd nanoparticles bearing technetium 99m–labeled diethylene triamine pentacetate (\(^{99m}\)Tc-DTPA) were prepared similar to the method as discussed above, and all the components were used in the same quantity, except that the CU was replaced with sodium chloride having radioactive (\(^{99m}\)Tc-DTPA) tracer adsorbed on its surface.

**Characterization**

**Average Size and Size Distribution**

The mean particle size & particle size distribution of ES-CU-GdNP and ES-FA-CU-GdNP were determined by a Nano-ZS zeta sizer (Malvern Instruments, Malvern, UK) at 25°C. The analysis was carried out on various formulations within 2 hr after their preparation. The formulations were diluted with distilled water prior to analysis, and each measurement was carried out in triplicate.

**Zeta Potential Studies**

Electrophoretic mobility of nanoparticles was measured using a Malvern zetasizer NanoZS (Malvern 3000HS, France). Optical properties of the sample were defined on the basis of refractive index (1.450), viscosity (0.8872) and absorption (0.00). The mobility (u) was converted into zeta potential (\(\xi\)) values using the Smoluchowski relation,
Zeta potential, $\xi = \eta/\varepsilon$

Where $\eta$ and $\varepsilon$ are the viscosity and permittivity of the solution, respectively. All $\xi$-potential measurements were performed without added electrolyte. Finally the data of optimized formulations are recorded. The average of three readings per sample is presented as the zeta potential at 25 °C. Each sample was measured in triplicate.

**Particle morphology**

ES coated NPs were characterized for their particle size and morphology using Scanning Electron microscope (Quanta 200, FEI, Oregon, and USA). For SEM characterization, NPs in aqueous suspension were first dried to obtain a dry powder by leaving undisturbed at 4°C. The powder was then mounted on a sample holder followed by coating with gold, using a sputter coater (Hind Hivac Sputtering unit, Model 6SPT, Hind high Vacuum Co., and Bangalore). The sample was then scanned with a focused fine beam of electrons. The surface characteristics of the sample were obtained from the secondary electrons emitted from the sample surface.

**HPLC Analysis**

A validated RP-HPLC method was developed for estimating CU content in ES-CU-GdNP and ES-FA-CU-GdNP. 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: water: glacial acetic acid (650:340:10) v/v. The solution was filtered and degassed before use. Chromatography was performed at 25°C with a flow rate of 1.0 ml/min. Calibration curve of CU was in the range of 1-10 µg/mL. Under these conditions the drug shows retention time of about 4 min.

**In-vitro release study**

In vitro release study of CU, ES-CU-GdNP and ES-FA-CU-GdNP was performed in simulated gastrointestinal 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 ES-CU-GdNP and ES-FA-CU-GdNP. 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 900 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]. 900 ml of dissolution medium was taken in the container, immersed in the water bath of the apparatus. The treated dialysis bag containing ES-CU-GdNP and ES-FA-CU-GdNP 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 ES-CU-GdNP and ES-FA-CU-GdNP. Samples (2 ml) were withdrawn from each dissolution medium at a predetermined time intervals (0h, 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h, 12h, 24h) and replaced 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.

**In-vitro haemolytic assay**

The fresh rat blood was collected in tube containing heparin and the undamaged erythrocytes were removed by centrifugation at 4000 rpm for 10 min. Cells/pellets were washed three times with cold PBS (pH 7.4) by centrifugation at 4000 rpm for 10 min and re-suspended in the PBS. In order to prepare the samples for haemolytic study, red blood cells and PBS were added to free CU, ES-CU-GdNP and ES-FA-CU-GdNP. Then, samples were incubated for 60 min at 37°C. The release of haemoglobin was determined after centrifugation (4000 for 10 min) and followed by addition of 50 µl of supernatant to the PBS in a 96 well plate. Finally, absorbance was measured at 540 nm by using ELISA plate reader, (Bio-TEK Power Wave XS) using Blank PBS as negative control (0% haemolysis standard) and triple distilled water as positive control (100% haemolytic standard). Less than 10% haemolysis was regarded as non-toxic effect level in NPs experiments. The experiments were run in triplicate and were repeated twice.

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\text{% Haemolysis} = \frac{\text{Actual Reading}-\text{Negative Control reading}}{\text{Positive Control reading}} \times 100
\]
Annexin V/Propidium Iodide staining for apoptotic cells
Quantitation of apoptotic cells by Annexin V staining was performed according to the manufacturer’s instructions (Calbiochem). The caco-2 cells (5×10^5 cells/well) were seeded in 6 well plates and treated with 5mM of formulations i.e. control, plain drug, ES-CU-GdNP and ES-FA-CU-GdNP for 24 h. After the incubations, cells arranged as a suspension in 500mL of cold PBS were centrifuged for 5 min at 1000 ×g then resuspended in 500 mL cold × binding buffer and added 1.25mL of Annexin V-FITC and incubated for 15 min at RT in dark, centrifuged at 1000 × g for 5 min at RT, supernatant was discarded, gently resuspend in 500 mL cold × binding buffer and added 10mL PI. Flow cytometry was performed using a FACS Flow cytometer, equipped with a single 488 nm argon laser. Annexin V-FITC was analyzed using excitation and emission settings of 488 nm and 535 nm (FL-1 channel); PI, 488 nm and 610 nm (FL-2 channel). Debris and clumps were gated out using forward and orthogonal light scatter. The experiment was repeated three times independently and the values have been expressed as mean ± SEM.

Cytotoxicity studies
In vitro cytotoxicity of CU, ES-CU-GdNP and ES-FA-CU-GdNP were 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 as described previously. The caco-2 cells were seeded onto 96 well plates at a density of 4×10^5 cells/well for 24 h and CU, ES-CU-GdNP and ES-FA-CU-GdNP at a equivalent concentration of CU i.e. 1 µM, 10µM, 25µM and 50µM were incubated for 24 h [260]. Blank formulations ES-Gd-NP and ES-FA-GdNP at all equivalent concentration were also incubated [259]. 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.

In vivo studies
Animals
Eight to ten weeks old nude mice (male, 30-35 g) were obtained from INMAS New Delhi, India and were acclimatized in a climate- and light cycle-controlled environment for 2 days before investigation. The animals were maintained at the controlled temperature of 23 ± 1ºC, humidity of 55 ± 5%, in a 14 h light/10 h dark cycle. Throughout the study, the animals were provided
with soy-free and filtered drinking water. The animal experiments have been carried out by following experimental protocol 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 (INM/DASQA/IAEC/09/015).

**In vivo tumor xenograft study**

The *in-vivo* anti-tumor cancer targeting efficacy of the CU laden ES-GdNP. ES-FA-GdNP formulation was assessed in the tumor bearing Male nude mice of 3-4 weeks by assembly these mice immunodeficient via irradiation so that they using HCT-116 cancer cell lines $2 \times 10^6$ cancer cells/mice were applied intraperitoneally in 1 mL culture medium by sterile injection. After 2 weeks, all of the animals had developed intra abdominal tumor nodes. They were used as tumor bearing mice for the solid colon cancer model [345, 346]. All procedures were performed in a laminar flow hood using aseptic techniques. The initial tumor size was taken approximately 100 mm$^3$ in size. The tumor bearing mice were randomly divided into four treatment groups (control, free CU, ES-CU-GdNP and ES-FA-CU-GdNP) for treatment with 25 mg/kg body weight dose equivalent to CU. At predetermined time intervals the tumor volume (mm$^3$) was measured by measuring its dimension (major and minor axis) using electronic digital Vernier Caliper and the tumor volume was estimated according to the following formula: tumor volume (mm$^3$) = $L \times W^2 / 2$, where L is the length and W the width. The study was terminated 45 days post treatment and the results were recorded as the mean ± SD. All animals were accommodated in a pathogen-free laboratory environment during the tenure of the studies.

**Plasma and tissue distribution study**

The drug distribution profiles of CU–loaded NP (free drug, ES-CU-GdNP, ES-FA-CU-GdNP) in various organs/tissues after oral administration were investigated. Healthy Nude tumorigenic mice (male, 30–35 g) were assigned randomly into four groups with 18 mice in each group for this study. The first group served as control. Plain CU was given to all mice in the second group, whereas mice of the third and fourth groups received ES-CU-GdNP, ES-FA-CU-GdNP, respectively, by oral administration. The dose of CU given to animals was equivalent to 25 mg/kg body weight of animals. Three animals from each group were killed at each time point (i.e., 0, 2, 4, 6, 8, 10, 12, 16, and 24 hours). The GI tract (GIT) was removed, and the mesenteric
and fatty acid tissues were removed. The GIT was fragmented into the stomach, small intestine, and colon. Tumor was also isolated, washed with Ringer’s solution to separate any adhered debris and dried using tissue paper and simultaneously the blood was also collected from the heart puncture.

Organs and luminal contents were weighed and homogenized with 2 mL PBS (pH 7.4) using tissue homogenizer (MAC Micro Tissue Homogenizer; New Delhi, India) and vortexed after addition of chloroform (CHCl3) and methanol (CH3OH) mixture and centrifugation for 5 min at 2000 × g on Sigma 3-16K (Frankfurt, Germany). After centrifugation, obtained supernatant was decanted into another eppendorf and evaporated to dryness under vacuum in speed vaccum concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 50 µL of the mobile phase, vortexed and 20 µL was injected onto analytical column. Concentration of CU in tissues was determined by HPLC, using the method reported previously. Similarly, the blood samples were prepared and analyzed for the drug content using the HPLC method.

γ-Scintigraphic study
This study was performed to investigate the behavior of drug dosage form and to estimate the release pattern of the NP qualitatively within the GIT of mice. Mice (male) weighing between 30-35 g was selected and was fasted overnight before administration of the enteric-coated radiolabeled NP. In order to evaluate the presence of the radiolabeled NP in different parts of GIT, a high dose (1 mCi) of 99m Tc-DTPA was encapsulated in NP and radioactivity was measured using radioisotope dose calibrator (CRC-127R; Capintech Inc., Pittsburgh, Pennsylvania). These enteric-coated radiolabeled NP were orally administered to the mice with 2 mL of water. The dosed mice were kept in a restraining cage that was located under the scintillation camera. Transit of the radiolabeled NP through the GIT was supervised at different time intervals using a gamma camera (E. Cam Siemens, Germany) fitted with low energy all-purpose collimator for scanning. The camera was connected to a computer system for attainment and storage of data. The useful field of view was 256 × 256 mm, and the mean energy window of 99mTc was 140 ± 14 keV. The camera was set for 100 K counts for each acquisition, and anterior images were recorded with the rodents in supine position. Images were recorded after one hrs administration of the NP and at 10 min intervals for 3 h and then at 20 min intervals up to 6 hrs and later on at 60 min intervals up to the gamma images were recorded for 24 hr study period.
using an online computer system and analyzed to determine the distribution of activity in the GI tract as demonstrated and archived onto optical disk for subsequent analysis.

**Statistical analysis**

All data have been 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. *In vivo* data were statistically analyzed by One way ANOVA followed by Newman-Keüls post analysis test using Graph Pad Prism version 5.00.
RESULTS

Average Size and Size Distribution
The mean particle size & particle size distribution of ES-CU-GdNP and ES-FA-CU-GdNP were determined by a Nano-ZS zeta sizer (Malvern Instruments, Malvern, UK) at 25°C. The polydispersity index (PDI) was obtained by the in-built software of the instrument. The results are shown in Fig 6A and Table 6A.

Zeta Potential Studies
The result of the study shows that the size and PDI increased with polymer concentration. Zeta potential after coating with Eudragit S-100 increased negatively. This may be due to the negative charge of acrylates. Among the four investigated batches the second batch (01:02) was considered as optimized formulation due to its narrow size distribution. This batch was evaluated for drug release and in vivo activity.

Table 6A: Particle Size, PDI and zeta potential of ES-CU-GdNP and ES-FA-CU-GdNP.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Mean ± SD</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES-CU-GdNP Coat 1:1</td>
<td>244.43±5.03</td>
<td>0.127</td>
<td>-17.2</td>
</tr>
<tr>
<td>ES-CU-GdNP Coat 1:2</td>
<td>374.43±6.01</td>
<td>0.235</td>
<td>-25.9</td>
</tr>
<tr>
<td>ES-FA-CU-GdNP Coat 1:1</td>
<td>253.13±12.3</td>
<td>0.149</td>
<td>-15.5</td>
</tr>
<tr>
<td>ES-FA-CU-GdNP Coat 1:1</td>
<td>391.9±82.33</td>
<td>0.363</td>
<td>-25.1</td>
</tr>
</tbody>
</table>
Fig 6A: zeta potential distribution analysis for the ES-FA-CU-GdNP coat 01:02 batch formulation.
Particle morphology
The SEM image of developed NPs has been represented in Fig 6B. ES-FA-CU-GdNP has irregular shape with smooth texture that could be due to coating with Eudragit S-100 (ES).

![SEM image of ES-FA-CU-GdNP](image)

**Fig 6B: SEM images of ES-FA-CU-GdNP**

**In Vitro Release Study**
The *in vitro* release profile of CU from ES-CU-GdNP and ES-FA-CU-GdNP was studied by pre-activated dialysis bag (Sigma; cut off MW-12 KD) at different pH 1.2, 7.4 and 6.8 while the temperature of the medium was kept at 37°C. The CU was released instantly within 6 h. It was observed that there was no drug release up to 2-3 hours in case of both the ES- CU-GdNP and ES-FA-CU-GdNP, whereas drug initiates to be released only after 3 hours at SIF pH 7.5. This can be explained by the fact that the ES polymer contains carboxyl groups that ionize from neutral to alkaline media. As the ionization takes place, integrity of the coating is disturbed and releases the NPs. Further the release conducted in the presence of rat caecal and colonic contents having pH of 6.8, where the total cumulative amount of CU released from ES-CU-GdNP, drug release was found to be 64.09% ± 2.32% and 75.33% ± 3.14% at the end of 8 and 24 hours, respectively; in the case of the ES-FA-CU-GdNP, drug release was found to be 58.16% ± 2.17%
and 68.12% ± 2.32% at the end of 8 and 24 hours, respectively. Results indicate that ES-CU-GdNP and ES-FA-CU-GdNP releases the maximum amount of CU in the colon environment due to the sustained release from the matrix system.

Figure 6C: *In vitro* drug release profile of CU, ES-CU-GdNP and ES-FA-CU-GdNP in different gastrointestinal simulated fluids (SGF, SIF, and SCF).

**In-vitro haemolytic assay**

Haemolytic assay showed that the formulations are safe and Haemocompatible i.e. less than 10% as shown in Table 6B

**Table 6B:** Haemolytic assay of NPs equivalent to 10 mg/ml

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Positive control</th>
<th>Negative control</th>
<th>Plain drug</th>
<th>ES-CU-GdNP</th>
<th>ES-FA-CU-GdNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>1.205</td>
<td>0.063</td>
<td>0.129</td>
<td>0.173</td>
<td>0.152</td>
</tr>
<tr>
<td>% Hemolysis</td>
<td>1.205</td>
<td>0.063</td>
<td>5.47</td>
<td>9.1</td>
<td>7.3</td>
</tr>
</tbody>
</table>
Apoptosis studies

Toward finding the possible reason of how ES-CU-GdNP and ES-FA-CU-GdNP could kill cancer cells, we have analyzed the apoptosis induction in ES-CU-GdNP and ES-FA-CU-GdNP treated colon cancer cells such as caco-2. Apoptosis is one of the major cell death pathways induced by anticancer agents and we have evaluated apoptosis inducing effects of ES-CU-GdNP and ES-FA-CU-GdNP by Annexin V/Propidium iodide (PI) binding. As can be seen in Figure.6D, the fraction of apoptotic cells was higher in case of cells treated with ES-FA-CU-GdNP than with ES-CU-GdNP or with Plain CU, the ES-FA-CU-GdNP increased the percentage of apoptotic cells in caco-2 cells from 0.40% in control, while CU had very few cells i.e. 9.69% in apoptotic phase, the ES-CU-GdNP and ES-FA-CU-GdNP had shown to 16.82% and 54.70% in treated cells. From the above data it is evident that ES-FA-CU-GdNP truly induced apoptosis specifically in colon cancer cells irrespective of ER status and did not induce apoptosis in normal cells.

Figure 6D: Apoptotic cells detected by flow cytometry with Annexin V conjugated with PI staining on caco-2 cells were treated with different formulation (A) control (B) Plain CU (C) ES-CU-GdNP and (D) ES-FA-CU-GdNP.
Cytotoxicity studies

The dose dependent cytotoxicity of the NP was determined by MTT assay [266]. Caco-2 cells were plated with plain CU, ES-CU-GdNP and ES-FA-CU-GdNP at different concentrations and cultured for 24 hr with 5% CO\textsubscript{2} at 37°C and the cytotoxicity for cells was determined by MTT assay. The percentage cell viability of different NP with different concentration was found to be significantly less in comparison to plain CU. The blank formulations ES-GdNP and ES-FA-GdNP showed only slight cytotoxicity (less than 10%) revealing the safety of the excipients used. The cell viability at 5µM, 10µM, 20µM and 40µM concentrations were estimated and the results revealed that the viability of cells exposed to ES-FA-CU-GdNP was less than the cells exposed ES-CU-GdNP. At higher concentration (40µM) ES-FA-CU-GdNP and ES-CU-GdNP showed 16.4±0.92 % and 22.7±1.13 % of cell viability respectively, at 20µMol ES-FA-CU-GdNP and ES-CU-GdNP showed less than 40% of cell viability whereas at lower concentration (10 µM) ES-FA-CU-GdNP showed 33.5±1.67 % and ES-CU-GdNP showed more than 45% of cell viability. The data has been represented in Figure 6E.

![Figure 6E](image)

**Figure 6E:** % Cell cytotoxicity of Caco-2 cells exposed to 5 µM, 10 µM, 20 µM and 40 µM equivalent concentration of CU in the plain CU, ES-CU-GdNP and ES-FA-CU-GdNP. Values shown are means and standard deviations (n=3), **p<0.001.
Analysis of pharmacokinetic parameter after oral administration

The overall pharmaceutical targeting efficacy of the CU loaded surface engineered FA-CU-GdNP were evaluated in HCT-116 tumor bearing nude mice. The plasma concentration profiles of the CU after oral administration of the free CU, ES-CU-GdNP and ES-FA-CU-GdNP (25.0 mg/kg body weight) and pharmacokinetic parameters were determined using non-compartment modeling as summarized in Table 6C (Figure.6F). Calibration curve of CU was found to be linear in the range of 50-200 ng and the coefficient of correlation was found to be 0.993. Higher MRT and higher AUC$_{0-\infty}$ of ES-FA-CU-GdNP were approximately 6.38 folds higher, respectively as compared to free CU i.e. (20.65571±2.41 h, 16906.62±103.87 ng/h/mL) as compared to ES-CU-GdNP (16.16822±3.52h, 8998.07±85.1ng/h/mL),approximately 3.1 folds higher as compared to free CU and CU (5.605±2.57 h, 2837.44±61.13 ng/h/mL) respectively. ES-CU-GdNP exhibited a rapid increase in the plasma level, which reached a maximal plasma concentration (C$_{max}$=1102.95±24.89 ng/mL) than that of ES-FA-CU-GdNP (C$_{max}$=747.067±29.67 ng/mL) and CU (C$_{max}$=537.47± 15.05 ng/mL). It clearly evinces the enhanced pharmacokinetics with better bioavailability and more prolonged retention in systemic circulation than that obtained on administration of ES-CU-GdNP and plain CU to nude mice.

Table: 6C. Pharmacokinetic parameters of CU upon oral administration of CU, ES-CU-GdNP and ES-FA-CU-GdNP.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cmax (ng/ml)</th>
<th>Tmax (hrs)</th>
<th>AUC$_{0-\infty}$(ng/h/mL)</th>
<th>MRT (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CU</td>
<td>537.47± 15.05</td>
<td>3±014</td>
<td>2837.44±61.13</td>
<td>5.605±2.57</td>
</tr>
<tr>
<td>ES-CU-GdNP</td>
<td>1102.95±24.89</td>
<td>4±0.09</td>
<td>8998.07±85.1**</td>
<td>16.16822±3.52</td>
</tr>
<tr>
<td>ES-FA-CU-GdNP</td>
<td>747.067±29.67</td>
<td>8±1.28</td>
<td>18110.68±102.34**##</td>
<td>20.65571±2.41</td>
</tr>
</tbody>
</table>

Cmax: maximal plasma concentration, Tmax: time to reach maximal plasma concentration, AUC: area under plasma concentration versus time curve, MRT: mean residual time. ** p<0.01 Significances vs. CU, ## p<0.01 denotes ES-FA-CU-GdNP vs. ES-FA-CU-GdNP
**Figure 6F: In-vivo bioavailability of CU, ES-CU-GdNP and ES-FA-CU-GdNP. Values are mean ± SD (n = 3)**

**Tissue distribution study**

A tissue distribution study was carried out to evaluate the *in vivo* CU release in different parts of the GIT from enterically protected enteric coated NP. The results of drug distribution from ES-CU-GdNP and ES-FA-CU-GdNP in the upper GIT after oral administration are displayed in Figure 6G. Entire GIT parts were homogenized, and the content of CU was quantitatively evaluated by using an HPLC assay previously described. The free CU accumulates progressively in stomach, where up to 72.90.14% of dose is localized after 2 h administration whereas only 1.91% of CU was found after 6 h. In all cases the CU concentrations were considerably higher for the ES-FA-CU-GdNP than for the ES-CU-GdNP, CU concentration in colon and tumor tissue after 12 hours was observed to be 9.34 and 11.6 (ES-CU-GdNP) and 8.26 and 30.44 μg CU/g tissue (ES-FA-CU-GdNP), respectively.
Figure 6G: Distribution profiles of CU, ES-CU-GdNP and ES-FA-CU-GdNP in various tissues as a function of time after oral dose (25mg/kg) to mice represent mean ± SD obtained from three animals per time point. Colon (+C)*, includes colon contents and tissue, cecal contents, and tissue; CU; curcumin, SI; small intestine.

γ-Scintigraphic studies
The gastric transit and colon arrival time of designed NP bearing $^{99}$mTc-DTPA in nude mice were recorded using γ-scintigraphy. The mean gastric emptying time of the formulation was found to be 1.50±0.17 hrs, mean intestinal transit time was calculated to be 3.20±0.22 hrs and mean colon arrival time was 6.60±0.27 hrs (Figure.6H) after the administration of the NP.
Assessment of anti-tumor cancer targeting efficacy

The percentage body weight changes of the HCT-116 tumor bearing nude mice model after oral administration of the CU loaded formulations was calculated upto 35 days as showed in Figure 6I(A). It clearly suggests that the developed formulations do not affect the body weight of the nude mice, while in case of the normal saline treated group the loss of body weight was observed. These results further confirmed that the higher tumor treatment potential infatuated by the ES-FA-CU-GdNP.

The in vivo tumor targeting efficacy was assayed on HCT-116 tumor bearing nude model and the starting tumor size was approximately 100 mm$^3$ for all dose receiving groups of the developed ES-CU-GdNP and ES-FA-CU-GdNP as well as normal saline and plain CU. The tumor volume (mm$^3$) was 129.01, 118.7, 102.5 and 90.5 in case of normal saline, free CU, ES-CU-GdNP and ES-FA-CU-GdNP at 5th day. The size of the tumor volume (mm$^3$) was reduced to 79.8 and 43.89 in 30th days after treatment with ES-CU-GdNP and ES-FA-CU-GdNP formulations, respectively [Figure. 6I (B)].
At the experimental terminal, the tumor sizes of the CU treated groups were all notably smaller than that of the control group and plain CU [Figure 6I(C)], followed the order: control group > Plain CU > ES-CU-GdNP > ES-FA-CU-GdNP and subsequently after 30 days no significant tumor (p<0.05) was seen in the nude mice colon. From the results it is clear that ES-FA-CU-GdNP causes a marked improvement in therapeutic efficacy and growth-inhibitory effect on tumor.

![Figure 6I](image)

**Figure 6I:** (A) Body weight changes in tumor bearing nude mice after administration of CU solution and CU loaded NP formulations, (B) Tumor regression analysis after oral administration of control, CU, ES-CU-GdNP and ES-FA-CU-GdNP (25 mg/kg body weight dose). The ES-FA-CU-GdNP treated group showed suppression of tumor growth compared with the other groups (lower) (n=3) (C) Representative photos of tumors in each treatment group with controls.
DISCUSSION

The FA-Gd conjugate was further used for preparing the FA-CU-GdNP, a FA rich delivery system prepared by desolvation method [102]. To examine the targeting specificity of ES-FA-CU-GdNP and ES-CU-GdNP has also been prepared by the using oil-in-oil solvent evaporation method with coat: core ratio (2:1) [251] in uniform size with low poly dispersity index. The hydrodynamic diameters and zeta potentials of GdNP, CU-GdNP and FA-CU-GdNP were measured to further verify the presence of FA on the NP. Table 6A shows that the hydrodynamic diameter of NP increases by enhanced coating. The change in the amount of surface charge alters the dielectric state of the NP, consequently resulting increases of the zeta potential values. SEM micrographs (Figure.6B) confirmed the nanosize range of ES-FA-CU-GdNP depicting irregularly shape with smooth texture of FA-CU-GdNP. The in vitro CU release profiles of the ES-FA-CU-GdNP and ES-CU-GdNP in 24 h are shown in Figure 6C. In ES-FA-CU-GdNP and ES-CU-GdNP, 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. This clearly exhibits that maximum fraction of CU was available in colon from ES-CU-GdNP as compared to ES-FA-CU-GdNP, 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 drug release from the ES-FA-CU-GdNP shows similar behavior to that of ES-CU-GdNP apart from the little slower rate due to FA conjugated on the surface of CU-GdNP which provides the possibility to often fight against cancer cells, resulting in the decreased cancer cell viability (shown in the segment of in vitro cytotoxicity below). The cumulative release percent almost achieved 65% within 24 hrs, showing a good releasing ability of the NP in different pH in different simulated fluids [332]. Conventional dissolution testing is less likely to accurately calculate in-vivo performance of colon specific delivery systems triggered by bacteria residing in the colon due to factors related with colon’s environment such as insufficiency of fluid, decreased motility, and presence of micro flora [316]. Hence, release studies were performed in an alternate release medium (PBS 6.8 with rat cecal contents). After 12 h the percent CU released in the SCF (pH 6.8) containing 4% rat cecal contents with enzyme initiation at 37 °C was found to be 75% and 68% for the formulation ES-CU-GdNP and ES-FA-CU-GdNP respectively. Thus, the step release of CU from matrix at pH 6.8 of colonic fluid was due to diffusion through nanopores of polymer network or degradation of polymer matrix by colonic bacteria. Significantly higher (p<0.001)
uptake of ES-FA-CU-GdNP over ES-CU-GdNP in caco-2 cell lines generates interest that still there is scope of enhanced localization of NP in colon macrophages. ES-FA-CU-GdNP was able to localize higher intensity of fluorescence inside the cells compared to ES-CU-GdNP because of better interaction with FRs on macrophages confirmed by apoptosis. Apoptosis is a key regulator of physiological growth control and regulation of tissue homeostasis but it is highly deregulated in cancer. Implementation of apoptosis is regulated by excess of cell signaling pathways and recent chemotherapeutic agents solely exhibit their action by targeting these signaling pathways. The enhanced therapeutic ability of dual effect of formulation with distinct cellular targets is able to hit signaling cascades simultaneously, thus ultimately causing enhanced apoptosis.

Cytotoxicity of ES-FA-CU-GdNP was found to be significantly (p < 0.001) higher than ES-CU-GdNP and CU both. This effect could have been attributed to preferential binding of FA with FRs on the caco-2 cells thus inhibiting up-regulated cell growth. The results illuminate low toxicity of the blank NP as well as ability of ES-FA-CU-GdNP to preferentially target CU to colon cancer cells. The cell cytotoxicity studies reveal negligible cytotoxicity of both blank ES-FA-GdNP and GdNP in caco-2 cells lines indicating that the FA is not adding any kind of further cytotoxicity to the formulation instead facilitating the uptake without any damage. Having dual advantage of the surface modified NP by releasing the CU in the colon as well receptor mediated uptake by the cancer cells, its application for the targeted delivery of bioactive is in offing. Our experiment concludes that FA causes ligand specific internalization and enhanced endocytosis of NP which leads to superior cytotoxicity can also are supported by related findings of various research groups. Folate decorated poly (lactide)–vitamin E TPGS nanoparticles for the targeted delivery of paclitaxel was synthesized by the group of Pan et al. in which they have showed the improved advantages of the NP versus the pure drug in achieving enhanced therapeutic effect [347].

The behaviour of NP in vivo correlated well with their in vitro interaction with tumor cells. Indeed, the in vitro antitumor activity of NP were tested by using MTT method after cell directly interacted with free CU or NP formulation. In this process, the cellular uptake and the resulting intracellular release play important role. But in vivo, NP having anti-tumor effect is a complex biological process, in which CU first should be targeted to
the tumor site and then use inhibitory effect on tumor cells, determined factors that are greatly different from that \textit{in vitro}.

One of the major interests lying in formulating NP of CU is to improve \textit{in vivo} bioavailability of CU. Interestingly, ES-FA-CU-GdNP and ES-CU-GdNP showed 6.38 and 3.1-folds more AUC \( \infty \) compared to plain CU respectively, which improved bioavailability.

The biodistribution studies indicated that ES-FA-CU-GdNP reached the tumor and were more effective in treating colon tumors as compared to ES-CU-GdNP and the free CU. This may be due to ligand mediated uptake of ES-FA-CU-GdNP in the tumor and releases the drug in tumor instead of colon. The reason for this small amount of drug in colon may be due to the larger number of NP uptake from the tumor surface via receptor mediated endocytosis and few of the NP are also expected to reach through tumor leaky vasculature by FRs, a human colon adnenocarcinoma, could be as large as 400 nm and the NP size well below this pore size i.e. in the size range of 150 to 335 nm, hence it could be the reason for successfully achieving the targeting strategy.

The most useful technique, to date, to evaluate \textit{in vivo} behaviour of dosage forms in animals and humans is external scintigraphy or \( \gamma \)-scintigraphy, After testing the \textit{in vitro} colon targeting behaviour of the developed NP, it was worthwhile to evaluate the \textit{in vivo} behaviour of NP in terms of residence time of NP in different parts of GI tract. The interesting fact in the transit of the material is that during the initial hours of the study the NP remains firm which may cause fast transit and as the time passed the rigidity of the NPs became weaker due to penetration of solvent and that led to slow transit through rest of the GI tract. [348] also observed gastric emptying values from 0.4 to 4.4 hrs with multiparticulate radiolabeled NP administered to human volunteers.

It is clearly seen from the captured scintigraphs that little amount of the radiolabelling was released in the stomach (Figure 6H.a). This corresponds to effective enteric coating to the NP. It is evident from the scintigraph taken after 3 hrs (Figure 6H.b) that small quantity of tracer was released in the small intestine during 1.85 to 3.20 hrs time period. The NP remains intact as it descends down through the small intestine. The radiolabeled tracer activity, which was released in small intestinal environment, is visible around the NP. After 3.0 hrs as the formulation entered into ascending colon, release of the tracer was increased (Figure 6H.c). It is due to a change in the pH of the lower GIT leading to dissolution of enteric coating and thus release of the tracer.
After 5 and 6 hrs, the formulation remained in the ascending colon but the release of radioactivity from the tablet was considerably improved as the coating dissolved with time in the colon but its shape was distorted with the liberation of considerably higher amount of tracer. After 11-12 hrs (Figure 6H.d) the formulation entered into transverse colon leaving the ascending colon filled with the tracer released. The NP was completely disintegrated after 10 hrs (Figure 6H.e) and after 24 hrs; the movement of the radiolabelling NP in intact form from stomach and small intestine to the colon without the tracer being released and complete degradation of the network in the colon reveals the efficiency of this pH dependent system. The in vivo anticancer study once again revealed that the CU, the ES-CU-GdNP and ES-FA-CU-GdNP exhibited a notably enhanced anticancer efficacy. A significant difference was observed between mice treated with saline and CU.NP mice (Figure.6J A and B) showed that the relative body weight and the tumor volume in the tumor bearing nude mice were obvious different among the various experiment groups. Both saline and CU showed similar anticancer activities within 20-25 days. Neither saline nor CU had any measurable influences on tumor growth, in both groups the tumor volumes and weights increased rapidly. For nude mice treated with ES-FA-CU-GdNP, the decrease in body weight was limited and a complete tumor regression was observed for over 50% of mice compared with the saline or CU groups. The increases of body weights in saline-treated mice and CU-treated mice were because of the incredible increases in tumor volume. The design of appropriate ES-FA-CU-GdNP and ES-CU-GdNP and its effect on regulatory policies for the beneficial NP was obstructed by the incomplete information about the health and the environmental safety of the NP. This hinders their routine usage as therapeutics.
Conclusion
It is observed from the gamma scintigraphic images that enteric coating to the NPs prevents the release of radiolabeled substance in the GIT. Induction of tumor in mice is successfully achieved in the colonic region by the implementation of a new concept of minimizing immunity by irradiation. It is also observed that there is a regression in tumor size for a longer period of time when they are administered ES-FA-CU-GdNP. Experimental observations also suggest that ES-FA-CU-GdNP are more effective against the HCT-116 solid tumor than free CU. From the plasma and tissue distribution studies, it is seen that the maximum amount of drug is recovered from colon and tumor indicating the efficacy of colon specific drug delivery. In addition, since the surface of the ES-CU-GdNP could be similarly functionalized with other targeting ligand besides FA, they should serve as an effective carrier for targeted drug delivery to different types of cancer. ES-FA-CU-GdNP might have potential in cancer diagnosis and therapy which is more affected in FRs expressed on the tumor surface offers the dual advantage of localizing tumors by non-invasive imaging and also treating them. FA can prove to be good candidates for drug release to achieve a localized treatment in the colon.