CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

Chitosan (Molecular weight(Mw)-100-150 KDa and Degree of deacetylation (DDA)-80%) was obtained from Koyo chemical Co Ltd., Japan, acetic acid, isopropanol, curcumin(CRC), thioglycolic acid(TGA), and hydrochloric acid(HCl), potassium chloride(KCl), disodium hydrogen phosphate(Na₂HPO₄), potassium dihydrogen phosphate( KH₂PO₄ ) and phenolphthalein was purchased from Merck, chloroacetic acid from Nice chemicals, pentasodium tripolyphosphate (TPP), 5-fluorouracil(5-FU), bovine serum albumin(BSA), dialysis tubings (Mw cut-off 12K Da), triton X-100, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], xylene, paraffin wax, hematoxylin solution(Harris Modified), eosin solution, rhodamine 123(Rhod 123), propidium iodide(PI), sodium hydroxide (NaOH), sodium chloride(NaCl), Ellman’s reagent (5, 5’-dithio-bis-2-nitrobenzoic acid or DTNB), para formaldehyde (PFA) and Ribonuclease (RNase) was purchased from Sigma Aldrich. Roswell Park Memorial Institute medium 1640(RPMI 1640), fetal bovine serum (FBS) trypsin-EDTA and Penicillin-Streptomycin (P/S) was obtained from Gibco Life technologies. Human colon adenocarcinoma (HT 29) and mouse intestinal epithelial cells (IEC 6) were purchased from NCCS Pune, India. Tetramethylrhodamine- 5-(and 6)-isothiocyanate (TRITC) conjugated Phalloidin, live dead assay kit, and mitochondrial membrane potential assay kits (JC-1) were purchased from Invitrogen. All other chemicals used are of analytical grade and used without further purification.
2.2 Methods

2.2.1 Synthesis of N, O-CMC from chitosan

N, O-CMC was prepared from chitosan through the carboxymethylation reaction of chitosan using monochloroacetic acid in alkaline medium (354, 355). Chitosan, 2g was mixed with isopropyl alcohol and made into slurry. The resulting slurry was treated with 5M NaOH (10ml) solution. Then monochloroacetic acid was added in 5 minutes interval, after this the temperature of the reaction mixture was raised to 60°C with continuous stirring for 3 hours. Resulting mixture was filtered and the residue was washed with methanol, and then dried in vacuum oven at room temperature for 24 hours. The dried sample was used for further characterization and nanoparticles synthesis. The reaction scheme for N, O-CMC synthesis from chitosan is depicted in Fig.2.1.

![Reaction scheme for N, O-CMC synthesis from chitosan](image)

**Figure 2.1.** Representing the reaction scheme for the synthesis of N, O-CMC from chitosan.

2.2.2 Synthesis of TCS from chitosan

TCS was synthesized based on the reported literatures (316, 346, 356, 357) and the reaction scheme for same is shown in Fig. 2.2. The coupling reaction of chitosan with thioglycolic acid (TGA) was mediated by a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Briefly 500mg of chitosan was dissolved in 50ml of 1% acetic acid. Thereafter EDC dissolved in 1ml de mineralized water was added to a final concentration of 125mM. After proper mixing of these two, 500mg of TGA was added and the pH of the medium was adjusted to 5. This was performed to avoid the formation of disulfide bonds between the polymer chains by oxidation during the coupling reaction. At that pH, the amount of the reactive form for the oxidation of
thiol groups (thiolate anions) is low and the formation of disulfide bonds can be minimized (348). Then the reaction mixture was incubated for 4 hours in dark at room temperature under constant stirring. In order to isolate the TCS from the reaction mixture, the polymer solutions were dialyzed in tubings of cellulose membrane with a molecular weight cut off 12KDa for 3 days in dark against 5mM HCl, then twice against the same medium but containing 1% NaCl to reduce the ionic interactions between the cationic polymer and the anionic sulphhydryl groups. For maintaining the pH of the medium as 4, the samples were again dialyzed against 5mM HCl for two days. During dialysis, the sample containers were kept in dark at 4°C for avoiding the oxidation of sulphhydryl groups. After dialysis, the samples and controls were lyophilized by drying the frozen aqueous polymer. The lyophilized product was used for further characterization and studies.

![Figure 2.2](image.png)

**Figure 2.2.** Representing the reaction scheme for the synthesis of TCS from chitosan using TGA.

### 2.2.3 Characterization of N, O-CMC and TCS using FT-IR

FT-IR spectral analysis has been carried out to characterize the prepared N, O-CMC, and TCS along with control chitosan. The procedure involves the mixing of the dried samples (N, O-CMC, and TCS; 2mg) with KBr (175mg), the resulting mixture was made into a pellet using a pelletizer and the pellet was scanned by Perklin Elmer Spectrum RXI Fourier Transform Infrared spectrophotometer with a resolution of 4 cm\(^{-1}\) and 100 scans per sample.
2.2.4 Determination of degree of carboxymethylation of N, O-CMC using titration method

The degree of carboxymethyl substitution in N, O-CMC was determined based on the protocol which was described in literatures (345, 349). A measured amount of modified polysaccharide i.e. N, O-CMC (0.02 g) was stirred with 2ml of water, 0.5 ml of methanol and 0.5 ml of 0.40 M NaOH for 4 hours. The resulting solution was titrated with 0.440 M HCl using phenolphthalein as the indicator. The degree of carboxymethyl substitution (D.S) was calculated based on the formula (Eq.1).

\[
D.S = \frac{0.162 A}{(1-0.058A)} \quad \text{Eq. (1)}
\]

Where A = Amount of NaOH required per gram of samples.

2.2.5 Determination of degree of thiol substitution/determination of free sulphhydryl groups) of TCS using Ellman’s method

The degrees of thiol group substitution in the modified thiol functionalized polymer were determined spectrophotometrically using Ellman’s reagent as described in literature (356). Ellman’s reagent; 5, 5’-dithio-bis-(2-nitrobenzoic acid) or DTNB is a water-soluble compound used for quantitating free sulfhydryl groups in solution. A solution of this compound produces a measurable yellow-colored product when it reacts with sulfhydryls groups. Consequently, Ellman’s reagent is very useful as a sulfhydryl assay reagent because of its specificity for -SH groups. At neutral pH, DTNB reacts with free sulfhydryl groups to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB) as shown in Fig. 2.3. The target of DTNB in this reaction is the conjugate base (R-S⁻) of a free sulfhydryl group. Therefore, the rate of this reaction is dependent on several factors such as the reaction pH, the pKa of the sulfhydryl and steric and electrostatic effects. TNB is the ‘colored’ species produced in this reaction and has a high molar extinction coefficient in the visible range.
Sulfhydryl groups can be estimated by comparing with a standard curve composed of known concentrations of a sulfhydryl-containing compound such as TGA.

![Reaction scheme showing Ellman’s method for the quantification of degree of thiol substitution.](image)

**Figure 2.3.** Reaction scheme showing Ellman’s method for the quantification of degree of thiol substitution.

### 2.2.6 Synthesis of bare and drug loaded nanoparticles

#### 2.2.6.1 Synthesis of $N$, $O$-CMC nanoparticles

$N$, $O$-CMC nanoparticles were obtained as a result of the ionic cross linking reaction of $N$, $O$-CMC with TPP as described in literatures (359, 360). $N$, $O$-CMC solution (1mg/ml) and TPP solution (10mg/ml) was prepared in Millipore water. Nanoparticles were obtained by the interaction of $N$, $O$-CMC solution with TPP for a volume ratio of 1: 0.1. The resulting nanoparticles were separated from the stable suspension by centrifuging the sample at 20,000rpm for 30 minutes at 15°C (Hermle Labortechnik). The resulting nanoparticle pellets were dispersed in saline and was used for further studies.
2.2.6.2 Synthesis of 5-FU loaded \( N, O\)-CMC nanoparticles (5-FU-\( N, O\)-CMC NPs)

The methodology of 5-FU-\( N, O\)-CMC NPs synthesis was based on our own reported method (286) in which a slight modification was carried out to increase the redispersibility of the 5-FU-\( N, O\)-CMC NPs. The methanolic 5-FU solution (1mg/ml) was incubated with \( N, O\)-CMC solution in water, (22.5 mg of \( N, O\)-CMC and 10mg of drug) for overnight. The drug loaded nanoparticles were obtained by TPP cross-linking (1%) for a volume ratio of 45: 1(\( N, O\)-CMC: TPP), followed by incubating with 0.1ml of 1% BSA solution for 30minutes. The drug loaded nanoparticles were separated by centrifugation (Hermle Labortechnik) at 15000 rpm for 30 minutes at 15°C. The nanoparticle pellet was collected, redispersed in saline and used for further studies. The resulting nanoparticle pellets were dispersed in saline and was used for further studies.

2.2.6.3 Synthesis of CRC loaded \( N, O\)-CMC nanoparticles (CRC-\( N, O\)-CMC NPs)

The methodology of CRC-\( N, O\)-CMC NPs synthesis was based on our own reported method (285) in which modifications were carried out to improve the redispersibility of the CRC-\( N, O\)-CMC NPs. CRC incorporated \( N, O\)-CMC solution (22.5 mg of \( N, O\)-CMC, and 5mg of drug) was cross-linked with TPP (1%) followed by incubating with 0.1ml of 1% BSA solution for 30minutes. The drug loaded nanoparticles were separated by centrifugation at 15000 rpm for 30 minutes at 15°C. (Hermle Labortechnik). The resulting pellet was collected and used for further characterization and studies.

2.2.6.4 Synthesis of TCS NPs

TCS NPs were obtained as a result of the ionic cross-linking reaction of TCS solution with TPP (283, 359). TCS solution was dissolved in water and cross linked with TPP solution for a weight ratio of TCS to TPP as 3:1. The resulting turbid suspension was kept for half an hour stirring and the formed TCS NPs were separated from the stable suspension by centrifugation at 13000rpm for 30minutes at 15°C.
(Hermle Labortechnik). The pellet was redispersed in saline and was used for further characterization and studies.

### 2.2.6.5 Synthesis of 5-FU loaded TCS nanoparticles (5-FU-TCS NPs)

5-FU was dissolved in methanol (1mg/ml) and it was incubated with TCS solution, 45ml (22.5 mg of TCS and 5mg) for overnight. Further, the drug incubated TCS samples were cross-linked with TPP (1%) solution for a volume ratio of (TCS: TPP, 45: 1), followed by incubating with 0.1ml of 1% BSA solution for 30minutes. The drug loaded nanoparticles were separated from the suspension by centrifugation at 15000 rpm for 30minutes at 15°C (Hermle Labortechnik). The supernatant was discarded and the pellet was redispersed in saline. It was used for further characterization and studies.

### 2.2.6.6 Synthesis of CRC loaded TCS nanoparticles (CRC-TCS NPs)

CRC incorporated TCS solutions [CRC in ethanol, 1mg/ml for a ratio of 45ml (22.5 mg of TCS, 5mg of drug)] was cross-linked with TPP (1%) followed by incubating with 0.1ml of 1% BSA solution for 30minutes. The drug loaded nanoparticles were separated by centrifugation (15000 rpm for 30 minutes). The supernatant was discarded and the pellet was redispersed in saline and was used for further characterization and studies.

### 2.2.7 Characterizations of bare and drug loaded nanoparticles

The particle size and zeta potential for all the nanoparticles systems were determined by DLS-ZP /Particle Sizer Nicomp™ 380 ZLS. The size and morphological characteristics of nanoparticles were further confirmed by SEM (JEOLJSM-6490LA) and AFM (JEOL JSPM-5200). For SEM, the nanoparticle suspension was diluted in Millipore water and it was spread on an aluminium disc and dried at room temperature. The dried nanoparticles were then coated with platinum and were used for imaging. For AFM, the nanoparticle suspension was successively diluted in Millipore water and it was spread on an aluminium disc and dried at room temperature. The resulting samples were used for AFM imaging. FT-IR spectral analysis has been carried out to analyze the potential interaction happening between the carriers, drugs and the cross-linkers using Perkin Elmer Spectrum RX1 Fourier.
transform infrared spectrophotometer by KBr pellets (1% w/w of product in KBr) with a resolution of 4 cm\(^{-1}\) and 100 scans per sample.

### 2.2.8 In vitro cytocompatibility of N, O-CMC and TCS nanoparticles using MTT assay

The cytocompatibility of the carrier system was proven by MTT assay for a range of concentration (0.1 to 5mg/ml) in HT29 and IEC6 cells for 48 hours and the protocol followed from our own earlier reports (283, 285, 286). HT29 and IEC 6 cells were maintained in CO\(_2\) incubators with 5% CO\(_2\) at 37°C in RPMI media (with 10% FBS and 100 units/ml of antibiotic). After confluence, the cells were detached from the flask with trypsin-EDTA; the cell pellets were collected via centrifugation at 1500 rpm for 3 minutes (Eppendorf Centrifuge 5702 R), resuspended in complete media, counted using a hemocytometer and used for further cell culture studies. The underlying principle of MTT involves the reduction of tetrazolium component of MTT in to purple colored formazan crystals by the mitochondrial reductase enzymes of viable cells. The formazan crystals produced have absorption maxima of 570 nm. So the percentage of viable cells or viability can be quantified using the OD values at 570nm. The OD values were measured using a Beckmann Coulter Elisa plate reader, BioTek Power Wave XS and it is directly proportional to the number of viable cells. Based on that, a graph was plotted i.e., the percentage cell viability versus concentration of nanoparticles in mg/ml.

For MTT, the cells were seeded on 96 well plates with a density of \(10^4\) cells/well and kept for 24 hours incubation for attaching the cells. Different concentrations of the \(N, O\)-CMC nanoparticles (0.1 to 5 mg/ml) and TCS nanoparticles (0.1 to 5 mg/ml) were prepared by dilution with the media. After 24 hours of incubation, the media was removed and the cells were incubated with different concentration of the nanoparticles (100 µl) samples in media and incubated for 48 hours. Cells in media alone devoid of nanoparticles acted as the positive control and wells treated with triton X-100 (1%) acts as the negative control. After the incubation period, media was removed and the cells were incubated with MTT solution for 4 hours followed by 1hour incubation with solubilization buffer. Then the
optical density of the solution was measured at a wavelength of 570 nm. Triplicate samples were analyzed for each experiment and each experiment was repeated thrice. Cell viability was expressed as the percentage of the positive control (Eq. 2).

\[
\text{Absorbance of the cells treated with the samples} \\
\text{Cell viability (\%) = } \frac{\text{Absorbance of the untreated cells}}{} \times 100 \quad \text{Eq. (2)}
\]

2.2.9 \textit{In vitro} blood compatibility assessment of \( N, O \)-CMC and TCS nanoparticles using hemolysis and clotting (coagulation) assays

The blood compatibility studies were performed to evaluate the influence of developed NPs on RBC and coagulation (clotting) cascade. This has been performed by hemolysis and coagulation assay measurements. The detailed protocols for each of these experiments are appended below.

2.2.9.1 \textit{In vitro} hemolysis assay

The \textit{in vitro} hemolytic assay of \( N, O \)-CMC/TCS nanoparticles was done based on the protocol as explained in the literatures (361-365). Fresh blood was taken from human volunteers and collected into ACD (Acid Citrate Dextrose) containing tubes. 0.1 ml of NPs samples of \( N, O \)-CMC and TCS (0.1 to 5 mg/ml) were incubated with human blood (0.9 ml) containing ACD for 3 hours at 37 °C under shaking. The resulting plasma was collected by centrifugation at 4500 rpm for 10 minutes (Thermo Scientific Microcentrifuge). 0.1 ml of plasma sample was diluted with 0.9ml of 0.1% sodium carbonate, and analyzed for the presence of plasma hemoglobin (Hb) using optical density measurements. The positive and negative controls in the current experiment involve saline and triton-X (1%) treated blood. Plasma hemoglobin concentration was quantified spectrophotometrically and it is directly proportional to the concentration of lysed blood cells, which can be directly correlated to the hemolytic activity of the material. The plasma Hb concentration and the percentage hemolysis values can be determined using the following equations 3 and 4.

\[
\text{Plasma Hb} = \frac{[(2A_{415}-(A_{380}+A_{450})\times1000\times\text{Dilution factor})]}{E \times 1.655} \quad \text{Eq. (3)}
\]
Here $A_{415}$ corresponds to the absorption band of hemoglobin, $A_{380}$ and $A_{450}$ are the correction factors applied for the uroporphyrin absorption falling in the same wavelength range. $E$ corresponds to the molar absorptivity value of Hb (79.46), 1.655 corresponds to the correction factor accounting for plasma sample turbidity. The plasma and total Hb values can be used for calculating the percentage hemolysis.

\[
\text{Plasma hemoglobin content in test} = \frac{\text{Hemolysis} \ (%)}{\text{Total hemoglobin content}} \times 100 \quad \text{Eq. (4)}
\]

Triplicate samples were analyzed for each experiment and each experiment was repeated thrice.

**2.2.9.2 Coagulation assay via prothrombin time (PT) and activated partial thromboplastin time (aPTT) measurements**

The interaction of the $N, O$-CMC and TCS nanoparticles with the plasma coagulation factors was analyzed by coagulation time measurements by two tests, i.e., PT and aPTT. The protocol for the same is explained below. Fresh blood was collected into ACD containing tubes. It was then centrifuged at 4000 rpm at 25°C for 15 minutes (Ependorf centrifuge 5810 R) to obtain the platelet-poor plasma (PPP). The experimental protocol involves the incubation of 0.9ml of PPP with 0.1ml of different concentrations of NPs samples (0.2 to 2 mg/ml) for 20-30 minutes at 37°C. 100µl of prothrombin reagent (Diagnostica stago, France) was added to 50 µl of treated plasma and the time taken for the plasma to coagulate, i.e., PT was measured. In the case of activated partial thromboplastin time (aPTT) measurement, 50 µl of aPTT activator (Diagnostica stago, France) was added to 50 µl of plasma and incubated for 180 seconds before the addition of 50 µl of 0.025 M CaCl$_2$. After CaCl$_2$ treatment, the time taken by plasma to coagulate was measured as aPTT. The experiments were carried out using the saline treated PPP as the negative controls and it was done in triplicates to confirm the data.
2.10 Preparation of standard graphs of 5-FU and CRC

2.10.1 Preparation of standard graph of 5-FU

Standardization of 5-FU was done spectrophotometrically at an absorbance value of 266 nm with an absorption spectrophotometer (UV-VIS Spectrophotometer Shimadzu). It was done for a range of concentrations from 0.01 to 0.06mg/ml by diluting the initial stock of 5-FU (0.06mg/ml) in methanol with PBS. Then the prepared samples were quantified spectrophotometrically at an absorption maximum of 262nm. A plot was drawn by putting the drug concentration on the X axis and corresponding absorbance values along the Y axis. The equation of straight line from the plot was used for further 5-FU quantification for entrapment and release studies using the nanoformulation.

2.10.2 Preparation of standard graph of CRC

Standardization of CRC was done spectrophotometrically at an absorbance value of 429 nm with an absorption spectrophotometer (UV-VIS Spectrophotometer Shimadzu). It was done for a range of concentrations from 0.01 to 0.06mg/ml by diluting the initial stock of CRC (0.06mg/ml) in ethanol. Then the prepared samples were quantified spectrophotometrically at an absorption maximum of 429nm. A plot was drawn by putting the drug concentration on the X axis and corresponding absorbance values along Y axis. The equation of straight line from the plot was used for further CRC quantification for entrapment and release studies using the nanoformulation.

2.11 Entrapment efficiency (EE) and loading efficiency (LE)

The EE and LE of the drug loaded NPs samples were quantified spectrophotometrically. The protocol involves the complete extraction of 5-FU/CRC from the nanoparticle pellet using methanol/ethanol followed by spectrophotometric quantification of CRC/5-FU. The EE and LE values were calculated with respect to the amount of entrapped drug and the nanoparticle yield (yield of nanoparticles obtained after lyophilization) respectively. The EE and LE values were calculated using the following equations (Eq.5 and Eq. 6) as reported earlier (285, 286).
Total amount of CRC/5-FU present in the pellet
\[ \text{EE (\%)} = \frac{\text{Total amount of CRC/ 5-FU present in the pellet}}{\text{Initial amount of 5-FU/CRC used for drug loading}} \times 100 \] Eq. (5)

Amount of CRC/5-FU within the pellet
\[ \text{LE (\%)} = \frac{\text{Amount of CRC/5-FU within the pellet}}{\text{Yield of nanoparticles}} \times 100 \] Eq. (6)

2.12 In vitro drug release profile
The in vitro release profile of 5-FU/CRC from 5-FU-N, O-CMC NPs, CRC-N, O-CMC NPs, 5-FU-TCS NPs and CRC-TCS NPs was studied using the reported protocols (105, 285, 286) by dialysis and eppendorf method respectively for 5-FU (105, 286) and CRC(285). The experiments were carried out in phosphate buffered saline (PBS) of pH 4.5 and 7.4 at 37°C.

2.12.1 5-FU release from the 5-FU-N, O-CMC NPs and 5-FU-TCS NPs
For the 5-FU release studies, the 5-FU-N, O-CMC NPs/5-FU-TCS NPs was dispersed in 3ml of water, transferred to dialysis tubes, and kept in a vessel containing PBS of pH 4.5 and 7.4. The whole system was incubated at 37°C under shaking. At fixed time intervals, 0.75ml of release media was taken out and replenished with fresh PBS. The collected PBS at each time point was analyzed for the quantification of releasing 5-FU by an absorption spectrophotometer at 262 nm. The concentrations of drug at each time point was calculated based on the standard graph of 5-FU. All the experiments were repeated thrice, and triplicates were analyzed in each set. The percentage of drug release was quantified using the following equation (Eq. 7). Here, the experiments were repeated with direct dispersion method as well.

\[ \text{Drug Release (\%)} = \frac{\text{Released drug at a definite time point}}{\text{Total amount of entrapped drug within the NPs}} \times 100 \] Eq. (7)

2.12.2 CRC release from the CRC-N, O-CMC NPs and CRC-TCS NPs
In CRC release studies, the CRC-N, O-CMC NPs/CRC-TCS NPs pellets were dispersed in a definite volume of PBS (pH 4.5 and 7.4), homogeneously mixed, transferred to a beaker and kept in a water bath shaker at 37°C. At definite time intervals, triplicate samples (0.5ml) were taken out, replenished with fresh PBS, the
released CRC was separated out, (through ethanol extraction followed by centrifugation) and quantified spectrophotometrically at 429nm. The concentrations of drug at each time point was calculated based on the standard graph of CRC. The percentage of drug release was quantified using the following equation (Eq. 7). All the experiments were repeated thrice, and triplicates were analyzed in each set.

2.13 In vitro blood compatibility assessment of drug loaded nanoparticles

2.13.1 Hemolysis and clotting assays

The blood compatibility studies were performed to evaluate the influence of drug loaded NPs on RBC and coagulation (clotting) cascade. The experiments were performed using hemolysis and clotting assays for a range of concentrations of drug containing nanoparticles (10-80 μM of CRC- N, O-CMC NPs /5-FU- N, O-CMC NPs and CRC-TCS NPs/5-FU-TCS NPs) using the reported protocols(285, 286).

For the hemolysis studies, 0.1ml of NPs samples (5-FU-N, O-CMC NPs, CRC-N, O-CMC NPs, 5-FU-TCS NPs, and CRC-TCS NPs) were incubated with human blood (0.9 ml) containing ACD for 3hours at 37 ºC under shaking. The resulting plasma was collected, diluted with 0.1% sodium carbonate, and analyzed for the presence of hemoglobin (Hb) using optical density measurements. The positive and negative controls in the current experiment involve saline and triton-X (1%) treated blood. The plasma Hb concentration and the percentage hemolysis values were calculated using the equations 3 and 4. The clotting studies were carried out through PT and aPTT measurements. The experimental protocol involves the incubation of 0.9ml of platelet poor plasma (PPP) with 0.1ml of NPs samples(5-FU-N, O-CMC NPs, CRC-N, O-CMC NPs, 5-FU-TCS NPs, and CRC-TCS NPs) for 20-30 minutes at 37 ºC followed by measuring the PT and aPTT using the coagulation analyzer and reagent kits (CK Prest and Neoplastin from Diagnostica Stago, France). The experiments were carried out using saline treated PPP as the negative controls keeping triplicates samples and it was repeated thrice to confirm the data.

2.14 Cell culture experiments

HT29 and IEC 6 cells were maintained in CO2 incubators with 5% CO2 at 37ºC in RPMI media (with 10% FBS and 100 units/ml of antibiotic). After
confluence, the cells were detached from the flask using trypsin-EDTA; the cell pellets were collected via centrifugation at 1500 rpm for 3 minutes (Eppendorf Centrifuge 5702R), resuspended in complete media, counted using a hemocytometer and was used for further cell culture studies.

2.14.1 Evaluation of the cellular internalization of drug loaded nanoparticles

Cellular internalization of CRC-\textit{N}, \textit{O}-CMC NPs, & 5-FU-\textit{N}, \textit{O}-CMC NPs (system1) and CRC-TCS NPs, & 5-FU-TCS NPs (system 2) were carried out using confocal imaging. As confocal microscopy gives a better understanding of cellular internalization of nanoparticles, this was used to confirm the uptake of nanoparticles in HT29 cells. The confocal imaging has been carried out by incubating the Rhod 123 labeled drug loaded nanoparticles (Rhodamine-123; excitation at 511nm and emission at 534 nm). The protocol for Rhod 123 labelling in to the nanoparticles are depicted below.

2.14.1.1 Labeling of Rhod 123 to CRC-\textit{N}, \textit{O}-CMC NPs and 5-FU- \textit{N}, \textit{O}-CMC NPs

Rhod 123 was used for labeling the CRC-\textit{N}, \textit{O}-CMC NPs and 5-FU- \textit{N}, \textit{O}-CMC NPs and the protocol involves the overnight incubation of Rhod 123(5mg/ml, 0.5ml) with CRC-\textit{N}, \textit{O}-CMC NPs and 5-FU- \textit{N}, \textit{O}-CMC NPs individually in the dark. The unbound Rhod 123 was removed using centrifugation at 15000 rpm for 5 minutes followed by washing with water for 3 times. The resuspended pellet in saline was used for the cellular internalization studies.

2.14.1.2 Labelling of Rhod 123 to CRC-TCS NPs and 5-FU-TCS NPs

Rhod 123 was labeled to the CRC-TCS NPs and 5-FU-TCS NPs using ionic interaction and physical adsorption. The redispersed nanoparticle pellet of 5-FU-TCS NPs and CRC-TCS NPs was separately incubated with Rhod 123 (5mg/ml, 0.5ml) for overnight under stirring in the dark. The resulting Rhod 123 labelled nanoparticles were centrifuged (15000 rpm for 15minutes), and washed (3 times with water). The washed pellet was redispersed in saline and used for internalization studies.
2.14.1.3 Confocal laser scanning microscopy (CLSM)

The cellular internalization using confocal imaging involves the following protocol. HT 29 cells were seeded in acid etched cover slips kept in 12 well plates with a density of 75000 cells/cover slip. Once the cells were attached to the cover slips (after 24 hours), the medium was removed, cells were washed with PBS and incubated with Rhod 123 labeled samples 1mg/ml; CRC-N, O-CMC NPs, 5-FU- N, O-CMC NPs, CRC-TCS NPs and 5-FU-TCS NPs for 6 hours. Thereafter, the media containing NPs were removed and the cover slips were processed for imaging. The processing involves, the washing the cover slips using PBS, fixing the cells in 3.7% para formaldehyde (PFA) for 20 minutes followed by a final PBS wash. Further the cells were stained with TRITC conjugated Phalloidin (excitation: 540-545nm and emission: 570-573nm). TRITC conjugated Phalloidin dye will bind to the actin filaments of cells and give red fluorescence. The staining procedure is briefed as follows. After treating the cells with the samples, the cover slips were processed for imaging and the processing involves the fixing of the cells using 3.7% PFA (0.3 ml) for 20 minutes followed by a final PBS wash. Then the cells were permeabilized using 0.5% Triton in PBS (0.1 ml) for 5 minutes followed by staining with TRITC conjugated Phalloidin dye (0.05 ml) for 1 hour. Then the cover slips were air dried under dark and fixed using DPX [(Diethyl (phenyl) xanthine)] as the mountant medium, and dried. Further the internalization of nanoparticles was confirmed by the Z-stack analysis by CLSM (358, 359). Approximately 10 confocal sections from the basal to the apical cell side were captured for the Z-stacking analysis.

2.14.2 In vitro combinatorial anticancer effects of CRC-N, O-CMC NPs & 5-FU- N, O-CMC NPs(system 1) and CRC-TCS NPs & 5-FU-TCS NPs(system 2) towards HT29 cells

The in vitro combinatorial anticancer effects of CRC- N, O-CMC NPs, and 5-FU- N, O-CMC NPs and CRC-TCS NPs, and 5-FU-TCS NPs were evaluated in HT29 cells using MTT and live dead assay. Further the combinatorial anticancer effects were confirmed and quantified by mitochondrial and cell cycle analysis measurements.
2.14.2.1 MTT assay

MTT assay was carried out using the reported literatures (276, 277). The HT 29 cells were seeded in 24 well plates (seeding density of 37500 cells per well) and incubated for 24 hours. The cells after incubation was treated with media containing nanoparticle samples of different drug concentrations (5 to 60µM) of 5-FU, CRC, combination of bare drugs, 5-FU- N, O-CMC NPs, CRC- N, O-CMC NPs, combination of 5-FU- N, O-CMC NPs, and CRC- N, O-CMC NPs, and incubated for 24, 48 and 72 hours. After the incubation, the media containing NPs samples were removed and incubated with 1% MTT solution in media for 4 hours. The formazan crystals formed were dissolved, and the optical density values were measured at 570 nm using a Beckmann Coulter Elisa plate reader, BioTek Power Wave XS. The positive and negative controls in the current experiment include triton-X 100(1%) treated, and normal tissue cultured cells. The experiments were carried out in triplicates and corresponding cell death values were calculated.

The MTT experiments were repeated for the TCS nanoparticle system as well. The experimental protocols were repeated for the HT 29 cells treated with the different concentration of drug containing NPs samples individually as well as in combination along with the bare drugs. The MTT assay was performed after 24, 48 and 72 hours.

2.14.2.2 Examination of the effect of drug combination

The percentage cell death induced by CRC, 5-FU, combination of bare drugs, 5-FU-N, O-CMC NPs, CRC-N, O-CMC NPs, combination of 5-FU-N, O-CMC NPs/CRC-N, O-CMC NPs, 5-FU-TCS NPs, CRC-TCS NPs, and combination of 5-FU-TCS NPs/CRC-TCS NPs were calculated for 24, 48 and 72 hours with varying concentrations of 5-FU and CRC. The corresponding data was subsequently analyzed by the median effect method (139, 369, 370). This method enables the calculation of combination indices of (CIs) which determines whether the effect is synergism, summation (additive effect), or antagonism as follows; CI <1, CI = 1, CI >1 indicate synergism, summation (additive effect) or antagonism of the two drugs, respectively. CIs were calculated by the following formula:
Where \((D_x)_1\) and \((D_x)_2\) are the concentrations of CRC/CRC-NPs and 5-FU/5-FU-NPs alone, respectively, resulting in growth inhibition of HT29 cell line (in X %). \((D)_1\) and \((D)_2\) are the drug concentrations that inhibit the cell growth at same percentage (X) when the drugs are used in combination, and \(a = 1\) or \(0\), depending on whether the two drugs are assumed to be mutually non-exclusive or mutually exclusive, respectively. The CI values were calculated in each case, and each time point. Out of these, for the 48hours incubated cells with a drug concentration of 20µM drugs in combination showed a CI value of <1 proving the synergistic anticancer effects. Thus confirmatory studies were performed with 20µM of drug concentration in combination for a time point of 48hours.

2.14.2.3 Live dead assay

The basic principle of live dead assay involves the usage of two fluorescent dyes acridine orange and ethidium bromide which specifically binds with the live and dead cells, giving green and red fluorescence respectively. The excitation and emission maxima for acridine orange and ethidium bromide are 500/530, and 510/595 respectively.

HT 29 cells seeded in acid etched cover slips kept in 12 well plate were seeded with a density of 75000 cells per well. After 24 hours, the cells were treated with the drug loaded nanoparticle samples; 20 µM 5-FU, 20 µM CRC, combination of bare drugs, 20 µM 5-FU-N, O-CMC NPs, 20 µM CRC-N, O-CMC NPs, combination of 20 µM 5-FU-N, O-CMC NPs and 20 µM CRC-N, O-CMC NPs and incubated for 48 hours. After the incubation, the NPs samples were replaced with 100 µl of PBS containing dye mixtures, and incubated in dark for 20-30 minutes. The incubated cover slips were viewed under a fluorescent microscope (Olympus BX-51) to visualize the green and red fluorescence of live and dead cells. The experiments were performed for the TCS nanoparticle system as well. The experimental protocols were repeated for the HT 29 cells treated with the NPs samples; 20 µM 5-FU, 20 µM CRC, a combination of both, 20 µM CRC-TCS NPs, 20 µM 5-FU-TCS NPs,
combination of 20 µM 5-FU-TCS NPs/20µM CRC-TCS NPs. After 48 hours of incubation, the cells were incubated with the PBS containing dye mixtures and were imaged under a fluorescent microscope (Olympus BX-51) to visualize the green and red fluorescence of live and dead cells.

2.14.2.4 Confirmation of cell death evaluation

The cell death induced by the combined treatments of CRC-N, O-CMC NPs, and 5-FU- N, O-CMC NPs and CRC-TCS NPs, and 5-FU-TCS NPs was quantified and confirmed using mitochondrial membrane potential and cell cycle analysis measurements. The protocols for each of these experiments are depicted below.

2.14.2.4.1 Mitochondrial membrane potential measurements

During apoptosis different key events happening in the mitochondria of cells leading to cytochrome c release, results in loss of mitochondrial membrane potential ($\Delta \Psi_m$). The mitochondrial membrane potential changes happening during the cell death forms the basic principle of the current experiment. JC-1(5, 5', 6, 6'-tetraethylbenzidazolylcarbocyanine iodide) is a lipophilic cationic dye used for evaluating the status of $\Delta \Psi_m$. Polarized mitochondria in healthy live cells form JC-1 aggregates, whereas remains as monomers in the mitochondria of dead or apoptotic cells. The JC-1 aggregates and the monomers have fluorescence in the red and green regions respectively. Thus the changes in the mitochondrial membrane potential happening during apoptosis were measured using flow cytometry (371) by measuring the emission from the JC-1 monomers and aggregates. The green emission, corresponds to the dead cells, thus by measuring the percentage of cells having green emission, the percentage of dead/apoptotic cells can be quantified.

HT 29 cells were seeded in 6 well plates with a density of $1.5 \times 10^5$ cells/well. After 24 hours, the cells were treated with the nanoparticle samples; 20 µM 5-FU, 20 µM CRC, combination of bare drugs, 20 µM 5-FU- N, O-CMC NPs, 20 µM CRC- N, O-CMC NPs, combination of 20 µM 5-FU- N, O-CMC NPs and 20 µM CRC- N, O-CMC NPs and incubated for 48 hours. After the desired incubation, the media was collected, trypsinised and the cell pellets were collected. The resulting cell pellet was incubated with JC-1 reagent in buffer at 37°C for 15 minutes in the dark.
The cell pellets were centrifuged at 5000 rpm for 5 minutes, washed and resuspended in the assay buffer (0.5ml) and analyzed using flow cytometry with the green and red channel of flow cytometry instrument.

The experiments were performed for the TCS nanoparticle system as well. The experimental protocols were repeated for the HT 29 cells treated with the NPs samples of TCS system (20 µM CRC-TCS NPs, 20 µM 5-FU-TCS NPs, combination of 20 µM 5-FU-TCS NPs/20µM CRC-TCS NPs) along with the control bare drugs. After 48 hours of incubation, the cells JC-1 assay was performed.

2.14.2. 4.2 Cell cycle analysis using propidium iodide (PI) staining

In the current experiment, the cells after sample treatments were fixed using 70% ethanol at 4°C. The fixed cell samples were stained with PI (PI can intercalate with the DNA) in the dark for 30 minutes and the PI stained cells were analyzed using flow cytometry. The resulting cell cycle analysis data, describes the percentage of cells in different phases of cell cycle, G0/G1, G2/M and S phase with DNA contents as 2n, 2n-4n and 4n respectively. In addition, the cell cycle analysis data also describe the cells with apoptotic/dead phase as well. In the current experiment, we have quantified the percentage of cells in the apoptotic/dead phase with the use of a flow cytometry instrument.

Here the experiments were carried out in 6 well plates in HT 29 cells with a density of 1.5 × 10^5 cells /well. After 24 hours, the cells were treated with the samples; 20 µM 5-FU, 20 µM CRC, combination of bare drugs, 20 µM 5-FU- N, O-CMC NPs, 20 µM CRC- N, O-CMC NPs, combination of 20 µM 5-FU- N, O-CMC NPs and 20 µM CRC- N, O-CMC NPs and incubated for 48 hours. After the desired incubation, media was collected, trypsinised and the cell pellets were collected. The cell pellets were washed with PBS (three times) to remove the media components. The resuspended cell pellets in PBS (0.3ml) were fixed using 0.7ml of ethanol in cold and incubated in ice for overnight. The ethanol fixed cell pellets were centrifuged, washed with cold PBS (2 times), resuspended in PBS, incubated with RNase for 30 minutes at 37°C. The resulting samples were stained with PI for 15 minutes and analyzed flow cytometrically. The experimental protocols were repeated for the TCS
nanoparticle system as well. HT 29 cells were treated with the NPs samples; 20 µM 5-FU, 20 µM CRC, a combination of both, 20 µM CRC-TCS NPs, 20 µM 5-FU-TCS NPs, combination of 20 µM 5-FU-TCS NPs/20µM CRC-TCS NPs. After 48 hours of incubation, the cells were fixed using 70% ethanol, stained with PI and analyzed using flow cytometry instrument.

2.15 In vivo studies

2.15.1 Drug quantification using high performance liquid chromatography (HPLC)

The in vivo drug quantification experiments were carried out using HPLC (LC 2010A HT SHIMADZU) with C18 columns, Qualisil BDS C18 chromatography column (5 µm, 4.6 × 250 mm) and Luna 5µm C18 column (Phenomenox) respectively for 5-FU and CRC. A calibration graph of 5-FU and CRC was made using the reported methods (123, 124, 372-374). Different concentrations of 5-FU and CRC standards were made in methanol and ethanol respectively from the lowest to highest. The different samples were analyzed in triplicates using HPLC and the same experiments were repeated on different days to validate the protocol. The protocols for the HPLC quantification are depicted below.

For 5-FU, the HPLC system was set in reverse phase with a C18 column under isocratic conditions and the solvent system consists of methanol water mixture (1: 9). The flow rate was adjusted to 1ml/minute and the retention time (Rt) obtained was found to be 4.2 ± 0.8 seconds. For CRC, the HPLC system was set in reverse phase with a C18 column under gradient elution and the solvent system consists of acetonitrile: acetic acid (75: 25). The flow rate was adjusted to 1ml/minute, and the retention time (Rt) obtained was found to be 3 ± 0.5 seconds. The area under the curve (AUC) was measured in each case and calibration graphs were plotted based on the concentration and AUCs. Further these calibration graphs were used for drug quantifications.

2.15.2 In vivo studies in Swiss albino mice

The animal experiments were carried out in Swiss Albino mice with the prior approval from the Institutional Animal Ethical Committee AIMS Kochi.
The experiments were performed by trained technicians and the animals were maintained in the Animal House Facility with the free access to food and water. Swiss Albino female mice of adult stage 4-5 months (25-30g) was used, the animals were injected with the samples through the tail vein. The samples include; 5-FU-\(N\), \(O\)-CMC NPs (NPs containing 20mg/Kg), CRC-\(N\), \(O\)-CMC NPs (NPs containing 25mg/Kg of CRC), a combination of CRC-\(N\), \(O\)-CMC NPs and 5-FU-\(N\), \(O\)-CMC NPs, 5-FU-TCS NPs (NPs containing 20mg/Kg of 5-FU/mice), CRC-TCS NPs (NPs containing 25 mg/Kg of CRC/mice), combination of CRC-TCS NPs and 5-FU-TCS NPs, bare CRC and 5-FU in combination, and control saline. Blood samples were collected in ACD containing tubes after sample administration at 0.25, 1, 6, 12, 24, 48 and 72 hours. At 72 hours, the animals were euthanized by the overdose injection of Ketamine and Xylazine. The mice organs; brain, heart, lungs, kidney, liver and spleen samples were collected in saline and formalin. The organ in saline and formalin was used for biodistribution and histopathological assessment respectively.

### 2.15.2.1 Pharmacokinetic evaluation

The pharmacokinetic studies were carried out using the following protocols. The blood samples collected in ACD at specified time points were centrifuged, and the plasma was collected. The plasma samples were processed for protein precipitation using methanol addition followed by incubation in \(-20^\circ\text{C}\) for overnight. The resulting samples were centrifuged (4000rpm, 15minutes), and the supernatant was collected. Both the drugs were purified based on the above method (123, 124, 374) and the supernatant in both cases were analyzed using HPLC with the calibrated protocols. The AUC was measured in each case and the concentrations of 5-FU/CRC were calculated at each time point using the AUCs and calibration graphs (123, 124, 373, 374). The pharmacokinetic parameters such as concentration maximum (\(C_{\text{max}}\)), time of maximum concentration (\(T_{\text{max}}\)), and area under the curve (AUC) were determined using a recently published Microsoft add-in tool, PK Solver (375). In \textit{vivo} PK modeling was done by considering the whole body as a single compartment and AUC was calculated using the linear trapezoidal rule (376).
2.15.2.2 Biodistribution analysis

The collected organs in saline were dried using a blotting paper, weighed. The weighed organs were homogenized using a tissue Homogenizer (T 10 basic ULTRA-TURRAX, with S 10 N - 8 G - ST Dispersing element) after adding 1ml of saline. The resulting tissue homogenisate was used for extracting 5-FU/CRC by methanol addition (1ml) for overnight. The overnight incubated samples were centrifuged at 2000 rpm for 5 minutes, the supernatant was collected. The amount of the CRC/5-FU present in the supernatant was quantified using an HPLC system.

2.15.2.3 Histological analysis

The histological studies were carried out using the formalin fixed tissue samples. The tissue samples were grossed, kept in tissue cassettes, washed with water for overnight and processed in different grades of isopropyl alcohol and xylene. The resulting samples were embedded in paraffin wax, and tissue sections were taken at a thickness of 5 \( \mu \)m. Further the tissue sections were stained with Harris’s Hematoxylin-Eosin (H and E) reagent for histopathological observations (377). Eosin (pink) binds to the eosinophilic tissue, cytoplasm, muscle fibres, connective tissue fibers, secretory granules, erythrocytes, fibrous tissue and protein precipitate whereas Hematoxylin binds to basophilic tissues, nucleus, RNA and proteins in the cytoplasm, mucus and mucoprotiens and cells with large amounts of ribosomal material on smooth endoplasmic reticulum.

2.16 Statistical analysis

All the in vitro experiments were done in triplicates and each experiment was repeated thrice. The in vivo experiments were done with an ‘n’ value of 4. The results obtained were analyzed using statistical test. A Student’s t-test was conducted to determine the statistical significance. A probability level of \( p < 0.05 \) was considered to be statistically significant.