CHAPTER 4
CONCLUSIONS AND FUTURE PERSPECTIVES

The current thesis work focuses on an effective strategy to improve the efficacy of 5-FU assisted chemotherapy against colon cancer. This has been addressed by combinatorial approach in which CRC was used in combination with 5-FU. The potential of both drugs were improved by nanoencapsulation, wherein non-toxic polymeric carrier systems; water soluble chitosan derivatives; N, O-CMC and TCS were employed as drug delivery systems (283, 348, 351, 357, 359, 360, 378). Thus the chemically modified water soluble chitosan derivatives; N, O-CMC and TCS was synthesized and characterized. The degree carboxymethylation and thiol substitution was shown to be 57 ± 8, and 60 ± 2% respectively for N, O-CMC and TCS. TPP being a well established non-toxic cross-linker for chitosan based systems (283, 359, 360, 378), cross-link with N, O-CMC and TCS resulting in the formation of N, O-CMC and TCS NPs. The stable 100 nm sized systems of N, O-CMC and TCS NPs were shown to be cytocompatibile (in vitro) and hemocompatibile (in vitro) up to 5mg/ml concentration. The cytocompatibility results of N, O-CMC NPs and TCS NPs suggest the potential of these NPs systems in biomedical applications. Since cytocompatibility is an essential property of material to be used in medical field (283, 285, 286). The hemocompatibility of the N, O-CMC NPs and TCS NPs pinpoints the potential of these carrier systems in intravenous administration applications (379-381). The developed N, O-CMC (system 1) and TCS (system 2) were individually used as nanocarrier systems for both 5-FU and CRC. The carrier systems were evaluated for the encapsulation of both drugs, followed by characterization, in vitro
combinatorial anticancer evaluation and \textit{in vivo} studies (pharmacokinetics). The major conclusions from the research work are depicted below,

- 5-FU-\(\text{N, O-CMC NPs, CRC-N, O-CMC NPs, 5-FU-TCS NPs and CRC-TCS NPs were developed by TPP cross-linking.}
- Since TPP is a well known biocompatible cross-linker, undergoes ionic gelation with \(\text{N, O-CMC and TCS, results in drug loaded nanoparticle systems (283, 359, 360, 378).}
- The particle size of the 5-FU-\(\text{N, O-CMC NPs, CRC-N, O-CMC NPs, 5-FU-TCS NPs and CRC-TCS NPs were measured as 150 ± 20, 100 ± 20, 150 ± 40 and 150 ± 20 nm respectively.}
- Since the developed NPs systems were of size below 200 nm, passive targeting allows more uptake of the drug loaded NPs into the cancer cells through EPR effect of tumor microenvironment, which allows more uptake of the drug loaded NPs into the cancer cells in comparison with normal cells (60-70).
- The zeta potential values of 5-FU-\(\text{N, O-CMC NPs, CRC-N, O-CMC NPs, 5-FU-TCS NPs, and CRC-TCS NPs were measured as +38.7 ± 6.32, +23.4 ± 5.08, +48.2 ± 2, and +35.7 ± 3 mV which confirmed the good stability and positive surface charge of the developed nanoparticles.}
- The zeta potential values of all the four NPs systems were in the stable range which suggests the good colloidal stability (383, 384). Studies had shown that cancer cell surfaces are usually negatively charged (due to the translocation of negatively charged constituents of the inner layer of the cell membrane) (419, 420). So the nanoparticles with positive surface charge will interact electrostatically with the cell membrane, this leads to increased uptake of the nanoparticles inside the cancer cells. This has been proven in a study in which positively charged chitosan-modified paclitaxel loaded PLGA nanoparticles showed increased cancer cellular uptake (419-421).
- FT-IR spectral analysis confirmed the potential chemical interaction between the carriers; TCS NPs, \(\text{N, O-CMC NPs and the drugs; 5-FU, CRC.}
Our FT-IR results are comparable with the reported literatures (87, 102, 122, 157, 285, 286, 385, 386, 422) where the characteristic peaks of drugs (5-FU, CRC) and carrier systems (TCS and N, O-CMC) were present in the final drug loaded NPs system.

The hemocompatibility of the developed nanoformulations; 5-FU-N, O-CMC NPs, CRC-N, O-CMC NPs, 5-FU-TCS NPs, and CRC-TCS NPs were shown up to 80µM drug containing nanoparticles. The hemocompatibility is a critical factor for a biomaterial to be used for intravenous administration applications (379-381). The hemocompatibility results of our nanoformulations suggest the potential of same in intravenous administration in animal models. Based on this result, we further moved on to animal studies in Swiss Albino mice.

The drug entrapment efficiency of the nanoformulations was shown as 47.96 ± 2.34, 84.26 ± 1.46, 46.83 ± 3.2, and 85.53 ± 6.81% respectively for 5-FU-N, O-CMC NPs, CRC-N, O-CMC NPs, 5-FU-TCS NPs and CRC-TCS NPs. Similarly the drug loading efficiencies were shown as 33.78 ± 7.59, 50.63 ± 3.83, 16.55 ± 3.06, and 28.80 ± 3.89 % respectively for 5-FU-N, O-CMC NPs, CRC-N, O-CMC NPs, 5-FU-TCS NPs and CRC-TCS NPs respectively. The loading and entrapment efficiency values of the four nanoformulations were comparable with the reported literatures (82, 166, 167, 175, 196, 391-396). Also the drug content in the 5-FU-NPs and CRC-NPs is sufficient to produce required therapeutic activity (in vitro anticancer activity) in colon cancer (HT29) cells. The loading as well as the entrapment efficiency values of 5-FU loaded system (5-FU-N, O-CMC NPs and 5-FU-TCS NPs) was shown to be lesser than the values of CRC-N, O-CMC NPs and CRC-TCS NPs. The hydrophilicity of 5-FU makes the system with low encapsulation and loading content (82, 391-396).

The in vitro drug release profile confirmed the sustained and pH dependent drug release from both systems over a period of 108 hours.

The sustained in vitro drug release profile of the drug loaded nanoformulations under physiological pH are in well correlation with the reported literatures (82,
These sustained drug delivery systems are advantageous in the *in vivo* conditions in terms of the improvement in the pharmacokinetic profile of poorly bioavailable drugs (106, 122, 123, 155-158, 190-207). The enhanced release pattern in acidic pH is also in correlation with the reported literatures. Since chitosan based systems undergoes acid dependent swelling, followed by degradation and drug release (378, 387, 388). This property of the nanoformulation is advantageous in terms of the selective release of drugs in acidic pH such as in endocytic vesicles (endosome and lysosome) and tumor tissues (389, 390).

- The cellular internalization of the 5-FU-\(N\), \(\text{O-CMC}\) NPs, CRC-\(N\), \(\text{O-CMC}\) NPs, 5-FU-TCS NPs, and CRC-TCS NPs were shown by labeling with Rhod 123. The CLSM stacking proved the internalization of the nanoparticles inside the HT 29 cells. The cellular internalization of the NPs inside the cancer cell has clinical relevance, since these internalized particles will slowly release the drugs inside the cell (397). This improves the specificity and reduces the nonselective exposure of drug loaded NPs. The cellular internalization of the drug loaded nanoformulations are facilitated by the positive surface charge of the NPs. Studies had shown that positively charged chitosan based NPs are easily up taken by the cancer cells (419-421). Here in our results, we could prove the cellular internalization of drug loaded NPs, where the CLSM stacking showed that the NPs are inside the cells, not on to the cell surface.

- The *in vitro* combinatorial anticancer effects of the systems (1 and 2) by MTT, mitochondrial membrane potential and cell cycle analysis showed the enhanced anticancer effects of the combinatorial treatment unlike the individual treatments. Also the results proved that CRC/CRC-NPs can synergize the anticancer effects of 5-FU/5-FU-NPs for a concentration of 20\(\mu\)M of CRC and 5-FU. Earlier reports had proven that CRC and 5-FU individually induces colon cancer cell death by apoptosis through the arrest of cells in the G2/M and S phase.
respectively (108, 130, 403). Our results of the individual treatments are in correlation with these literatures. In combinatorial treatment, there is significant enhancement in the percentage of cells with apoptosis. The effect is more and it is higher than the additive effects of the individual treatment. Thus CRC sensitize 5-FU to induce apoptosis in HT 29 cells which in turn improves the anticancer effects of 5-FU. The reduction in COX-2 expression and the synergistic anticancer effects of CRC with 5-FU had been well proven (138-142, 162-164). Based on our in vitro results, we are suggesting that in future our system will also behave in similar aspects in colon cancer xenograft models. Finally to prove, the in vivo experimental results (in colon cancer models) in tumor reduction are mandatory.

- The in vivo pharmacokinetics data of the nanoformulations of (1) 5-FU-N, O-CMC NPs, CRC-N, O-CMC NPs (2) 5-FU-TCS NPs, CRC-TCS NPs individually and combination (co-administration) in Swiss Albino mice shown the following points,

  - The bioavailability of both 5-FU and CRC were improved after making the NPs. From the data, we could confirm the retention of drug (from the drug loaded nanoparticles) in blood for 3 days in µg/ml quantities, implying its improved systemic retention in the circulation unlike the bare drugs.
  - The AUC_{0-t} values of 5-FU-N, O-CMC NPs, and 5-FU- N, O-CMC NPs from the co-administered system, 5-FU-TCS NPs, and 5-FU-TCS NPs from the co-administered system were found to be 15-25 folds higher than the AUC_{0-t} values of bare 5-FU solution.
  - Similarly the AUC_{0-t} values of CRC-N, O-CMC NPs, and CRC- N, O-CMC NPs from the co-administered system, CRC-TCS NPs, and CRC-TCS NPs from the co-administered system were found to be 7 fold higher than the AUC_{0-t} values of bare CRC solution.
  - The improved/enhanced AUC_{0-t} values of the drug loaded nanoparticles systems in comparison with the bare drugs in blood,
suggest the improved bioavailability of both CRC and 5-FU after entrapment into the nanosystems.

The *in vivo* pharmacokinetic results had shown the improved bioavailability of the CRC and 5-FU nanoformulations, which is not achievable with the bare drugs. The improved pharmacokinetic effect of CRC and 5-FU from the nanoformulation results from the slow release of the drugs from the carrier system into the circulation (392). Our results on the improved bioavailability of CRC and 5-FU were well correlated with the reported literatures (123-125, 158, 189-191, 197, 198, 406).

The pharmacokinetic data of 5-FU system at 48th hour showed a plasma 5-FU concentration of 25-35 µg/ml (both the individual and the co-administered system). In an earlier study, a plasma 5-FU concentration of 40-60 µg/ml was obtained at 48th hour from a block copolymer. The system was proven to inhibit the growth of colon cancer in colon cancer xenograft model (104). Thus we are suggesting that, our 5-FU-NPs system in combination with CRC-NPs will produce anticancer effects in colon cancer xenograft model. Finally to prove, the *in vivo* experimental results in tumor reduction are mandatory.

- The biodistribution and of 5-FU-N, O-CMC NPs, 5-FU-N, O-CMC NPs from the co-administered system, 5-FU-TCS NPs, and 5-FU-TCS NPs from the co-administered system proved the retention of 5-FU in the kidney, liver and spleen in the order of spleen<kidney<liver. These data are in correlation with reported studies.

The biodistribution data of 5-FU-NPs systems were comparable with the reported literature, where Yan et al. had proved the retention of 5-FU-NPs in different tissues as in the following order; liver>kidney>spleen after the intravenous administration of 30mg/Kg of 5-FU-succinyl chitosan nanoparticles (407-409). Here in our study also, the distribution pattern showed smaller fractions of 5-FU in each organ tested with the following order; kidney>liver>spleen>brain=heart=lungs. This data is in correlation with
histopathology results, where mild histopathological changes were observed in the kidney, liver and spleen samples of 5-FU-NPs treated animals (414-418).

- The biodistribution pattern of CRC-N, O-CMC NPs, CRC- N, O-CMC NPs from the co-administered system, CRC-TCS NPs, and CRC-TCS NPs from the co-administered system showed the retention of small amounts of CRC in all the organs tested. CRC being non-toxic, no significant pathological changes were observed.

- Overall, the combinatorial nanomedicine of 5-FU and CRC in N, O-CMC NPs and TCS NPs proved the synergistic anticancer effects against HT 29 cells under in vitro conditions and improved the bioavailability of the drugs under in vivo conditions. The in vitro results were well correlating with the reported literature, in which we could prove a significant enhancement in the in vitro anticancer effects of the combinatorial nanomedicine in comparison with the individual nanomedicine and bare drug treatment. The significant enhancement in anticancer effect arises from the CRC induced inhibition of COX-2 in HT 29 cells, which further improves the chemotherapeutic efficacy of 5-FU, which had been proven earlier.
FUTURE PERSPECTIVES

- The in vivo synergistic anticancer effects of the nanomedicine need to be studied in a suitable colon cancer xenograft mouse model. After successful evaluation in small animal model, this study can be extended to clinical trials.
  - Using HCT 116(423, 424), and HT 29 cell line (425), we can develop human colorectal carcinoma xenograft model in mice models[female Swiss nude mice (426) and female athymic BALB/C nude mice (39)].
- The current work would be more effective if it is actively targeted towards the colon cancer cells. Using monoclonal antibodies or some other specific targeting ligands which overexpress in colon cancer could be conjugated. Further the in vitro targeting needs to be proven, followed by the in vivo efficacy evaluation in colon cancer xenograft model.
  - For eg: Cetuximab, an IgG1 monoclonal antibody, specifically targets the epidermal growth factor receptor (EGFR) with high affinity. The EGFR is widely expressed by a range of tumours, including colorectal cancers (427-430).
- The efficacy of the current system could also be improved by encapsulating both drugs in a single nanoconstruct, which is being conjugated with targeting agents which are specific to colon cancer. Further the proof of concept in the in vitro conditions needs to be evaluated, followed by the efficacy evaluation in proper colon cancer xenograft model.
  - Developing a system with multiple drugs in a single nanoconstruct as in the case of core shell nanomedicine and subsequent evaluation will be more effective than multiple nanoformulations. For eg: Poly-L-lactide-co-glycolic acid (PLGA)-casein core shell systems were developed for the encapsulation of anti-cancer drug molecules, namely
paclitaxel (Ptx) and epigallocatechin gallate (EGCG) in one of the studies (414).

➢ In stage 2 and stage 3 colon cancers, the targeted combinatorial nanomedicine in combination with antiangiogenic agents would provide better therapeutic efficacy.

❖ Antiangiogenic agents target the angiogenic pathways in metastatic colorectal cancer.

❖ For eg: Bevacizumab is a humanized immunoglobulin (Ig)-G 1 monoclonal antibody directed against vascular endothelial growth factors and VEGF receptors (VEGF) (431).