COMBINATORIAL APPROACH OF CURCUMIN AND 5-FLUOROURACIL LOADED CHITOSAN DERIVATIVES-BASED NANOPARTICLES TOWARDS THE TREATMENT OF CARCINOMA OF COLON

Synopsis of the thesis submitted to the Amrita Vishwa Vidyapeetham University for the award of the degree of

DOCTOR OF PHILOSOPHY

Submitted by
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I Anitha A (KH.NS.D*NMS09004) hereby declare that this synopsis of the thesis titled “Combinatorial Approach of Curcumin and 5-Fluorouracil Loaded Chitosan Derivatives-Based Nanoparticles towards Treatment of Carcinoma of Colon” is a bonafide work done under the guidance of Dr. R. Jayakumar, Professor, Amrita Centre for Nanosciences and Molecular Medicine, Kochi and to the best of my knowledge and belief it contains no materials previously published or written by another person, no material which has been accepted for the award of any degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

Place: Kochi
Date: ____________________________

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Chapter 1: Introduction

Colorectal cancer is one of the major causes of cancer death worldwide and it affects men and women equally. It is one among the 40% of all cases of cancer being diagnosed yearly and third most leading cause of cancer death. The treatment modalities for colon cancer include surgery, radiofrequency ablation, cryosurgery, chemotherapy, radiation therapy and targeted therapy. Among which chemotherapy utilizes different drugs or drug combination to reduce the cancer cell growth. The FDA approved chemo drugs or drug combinations used in colon cancer include adrucil (5-fluorouracil 5-FU), avastin (bevacizumab), camptosar (irinotecan hydrochloride), capecitabine, cetuximab, eloxatin (oxaliplatin), erbitux (cetuximab) or drug combinations such as capox, folfiri, folfiri-bevacizumab, folfiri-cetuximab, folfox, zelox etc. In colon cancer, chemotherapy is given adjuvantly after surgery and the most common chemo drug used is 5-FU. Being a thymidylate synthase inhibitor, it inhibits the cancer cell growth through the arrest of cells in the S phase. The major drawback of 5-FU is its systemic toxicity results from its non-specificity, low plasma half life leading to the use of high doses and its inefficiency in chemo treatment results from cyclooxygenase 2 (COX-2) over expression in colon cancer. The effectiveness of 5-FU as a chemo drug can be improved by entrapping the drug in a polymer based carrier nanoparticle system, wherein the drug being protected, reduces the non-selective exposure and improves the plasma half life. In addition nanoparticles (NPs) have the ability of enhanced permeability and retention effect (EPR) which helps in the accumulation of drug loaded NPs in the tumour tissues in comparison with normal tissues. Besides the efficacy of 5-FU as a chemo drug can be improved by a combinatorial approach, where in 5-FU in combination with nontoxic COX-2 inhibitors such as curcumin (CRC) can be employed. There has been a reported study for the potential enhancement of 5-FU anticancer efficacy with CRC under in vitro and in vivo conditions through the inhibition of COX-2 gene/proteins.

Our research strategy is to explore the combinatorial anticancer effects of 5-FU with CRC for improving the efficacy of 5-FU. The improvement in chemo efficacy results from the CRC induced suppression of COX-2 expression, leads to improved effectiveness of 5-FU in conventional chemotherapy. In the current work,
nanoencapsulation technique has been employed to improve the bioavailability of both drugs and to reduce the non-selective exposure of 5-FU. The nanoencapsulation has been made using chitosan derivatives based NPs having different chemical functionality, $N, O$-carboxymethyl chitosan ($N, O$-CMC) and thiolated chitosan (TCS). Thus the system 1 (5-FU loaded $N, O$-CMC NPs / CRC-loaded $N, O$-CMC NPs) and system 2 (5-FU loaded TCS NPs/CRC loaded TCS NPs) were made separately and characterised. The \textit{in vitro} combinatorial anticancer effects were proved through different assays in colon cancer cells (HT29). The \textit{in vivo} pharmacokinetic results of system 1 and 2 in Swiss Albino mice model revealed the improved plasma half life of both drugs unlike their bare counter parts.

\textbf{Objectives of the study}

\textbf{The specific objectives of the study include:}

1. To develop and characterise $N, O$-carboxymethyl chitosan ($N, O$-CMC) and thiolated chitosan (TCS) from chitosan.
2. To develop and characterise $N, O$-CMC and TCS Nanoparticles.
3. To evaluate the \textit{in vitro} cytocompatibility and hemocompatibility of $N, O$-CMC and TCS Nanoparticles.
4. To develop and characterise 5-FU loaded $N, O$-CMC nanoparticles (5-FU-$N, O$-CMC NPs) and CRC loaded $N, O$-CMC nanoparticles (CRC-$N, O$-CMC NPs)
5. To evaluate the \textit{in vitro} drug release, combinatorial anticancer efficacy of drug loaded NPs ($N, O$-CMC system) towards colon cancer cells, \textit{in vivo} pharmacokinetics (PK) and biodistribution.
6. To develop and characterise 5-FU loaded thiolated chitosan nanoparticles (5-FU-TCS NPs) and curcumin loaded thiolated chitosan nanoparticles (CRC-TCS NPs)
7. To evaluate the \textit{in vitro} drug release, combinatorial anticancer efficacy of drug loaded NPs (TCS system) towards colon cancer cells and \textit{in vivo} pharmacokinetics (PK) and biodistribution studies in Swiss Albino Mice model.
Literature Review

COX-2 protein over expression in colon cancer leads to ineffective chemotherapy with 5-FU\(^1\). This problem can be solved by combining COX-2 inhibitors with chemodrugs.\(^1\) There has been reports showing the improvement in 5-FU efficacy with aspirin,\(^2\) celecoxib,\(^2\) geraniol\(^3\) and genistein.\(^3\) Among these, celecoxib is more effective in synergising the 5-FU efficacy in colon cancer but its side effects limits its potential. CRC is known to have COX-2 inhibiting effects\(^4\) and its anticancer potential towards many kinds of cancers were proven.\(^5\)-\(^10\) The idea of combinatorial anticancer effects of 5-FU with CRC against colon cancer was reported earlier through the inhibition of COX-2 gene/protein under *in vitro*\(^11,\) \(^12\) and *in vivo* conditions.\(^13\) The combinatorial approaches with CRC makes more important because of its wide anticancer potential and its safety in preclinical /clinical trials. Reports were suggesting the potential of CRC as a suitable replacement for genistein and geraniol and it can promotes 5-FU efficacy in various cancers including colon cancer.\(^14\) 5-FU triggers DNA mediated cell death in colon cancer by inhibiting the S phase in cell cycle.\(^15,\) \(^23\) Nanoencapsulation techniques being employed to enhance the plasma half life of both CRC\(^24,\) \(^26\) and 5-FU.\(^15,\) \(^18\) In addition, chitosan and its derivatives based nanoparticles were well exploited for drug delivery applications of different drugs.\(^17,\) \(^27,\) \(^28\)

Chapter 2: Materials and Methods

**Synthesis and Characterisation of *N, O*-CMC, *N, O*-CMC Nanoparticles and *In vitro* Evaluation**

*N, O*-CMC was developed from chitosan through carboxymethylation reaction using chloroacetic acid as the carboxymethylating agent. The resulting product was characterised using FT-IR and degree of substitution was determined. *N, O*-CMC NPs were obtained through ionic cross-linking reaction using pentasodium tripolyphosphate (TPP). The resulting NPs were characterised using FT-IR, DLS, SEM and AFM. The *in vitro* cytocompatibility (in colon cancer, HT 29 cells and normal cells, IEC6) and hemocompatibility was evaluated.
Synthesis, Characterisation and *In vitro* Evaluation of CRC-N, *O*-CMC NPs and 5-FU-*N*, *O*-CMC NPs

5-FU-*N*, *O*-CMC NPs and CRC-*N*, *O*-CMC NPs were developed through the ionic cross linking of TPP to the drug incorporated polymer solutions. Bovine serum albumin (BSA) was used as the stabiliser to increase the aqueous dispersibility. The resulting nanoparticle pellets were tested for its chemical composition (FT-IR), entrapment and loading efficiency, *in vitro* drug release, hemocompatibility, cellular uptake etc.

**Evaluation of *In vitro* Combinatorial Anticancer Effects of CRC-*N*, *O*-CMC NPs and 5-FU-*N*, *O*-CMC NPs**

The combinatorial anticancer effects of the drug loaded NPs were quantified through MTT, live dead, mitochondrial membrane potential and cell cycle analysis measurements using the individual NPs and bare drugs in combination as controls.

**Evaluation of *In vivo* Pharmacokinetics, Biodistribution and Histopathological Assessment of CRC-*N*, *O*-CMC NPs and 5-FU-*N*, *O*-CMC NPs**

The *in vivo* plasma concentration time profile of 5-FU-*N*, *O*-CMC NPs, CRC-*N*, *O*-CMC NPs and co-administered 5-FU-*N*, *O*-CMC NPs and CRC-*N*, *O*-CMC NPs were evaluated in Swiss Albino Mice using the bare drugs in combination and saline as controls (72 hours). The pharmacokinetic profile was evaluated by HPLC using the calibrated protocols up to 3 days. The organs of euthanized animals after 72 hours were analyzed for biodistribution (through HPLC) and histopathologically assessed by H and E staining.

**Synthesis and Characterisation of TCS, TCS NPs and *In vitro* Evaluation**

TCS was synthesised from chitosan using thioglycolic acid through EDC mediated reactions. The reaction mixture was dialysed for a week and lyophilised. The lyophilised product was characterised (FT-IR) and degree of thiolation was determined. TCS NPs were developed through TPP mediated cross linking. The
resulting NPs were characterised using FT-IR, DLS, SEM, and zeta potential. The *in vitro* cytocompatibility (HT29 and IEC6) and hemocompatibility was evaluated.

**Synthesis, Characterisation and *In vitro* Evaluation of CRC-TCS NPs and 5-FU-TCS NPs**

CRC/5-FU (individually) incorporated TCS solutions (CRC in ethanol, 1mg/ml for a ratio of 45ml 22.5 mg of TCS, 5mg of drug) were incubated with 0.1ml of 1% BSA solution followed by cross-linking with TPP (1%) for 30minutes. Drug loaded NPs was separated from the nanoparticle suspension by centrifuging the samples at 15000 rpm for 30 minutes. The resulting supernatant and pellet was collected and used for further characterization and studies [*in vitro* drug release, hemocompatibility, cellular uptake (rhodamine 123 labelled samples) etc].

**Evaluation of *In vitro* Combinatorial Anticancer Effects of CRC-TCS NPs and 5-FU-TCS NPs**

The combinatorial anticancer effects of the drug loaded NPs were quantified through MTT, live dead, mitochondrial membrane potential and cell cycle analysis measurements using the individual nanoparticles and bare drugs in combination as controls.

**Evaluation of *In vivo* Pharmacokinetics, Biodistribution and Histopathological Assessment of CRC-TCS NPs and 5-FU-TCS NPs**

The *in vivo* plasma concentration time profile of 5-FU-TCS NPs, CRC-TCS NPs and co-administered 5-FU-TCS NPs and CRC-TCS NPs were evaluated in Swiss Albino Mice using the bare drugs in combination and saline as controls. The pharmacokinetic profile was estimated through plasma using HPLC. The organs of euthanized animals after 72 hours were analyzed for biodistribution (HPLC) and histopathological assessment using H and E staining.
Chapter 3: Results and Discussion

*N, O*-CMC, TCS and its nanoparticles were synthesised, degree of carboxymethylation (57 ± 8%) and thiolation (60 ± 5%) was determined and characterised. The biocompatibility and hemocompatibility of the NPs were confirmed (Fig. 1). 5-FU-*N, O*-CMC NPs, CRC-*N, O*-CMC NPs, 5-FU-TCS NPs and CRC-TCS NPs were developed, and characterised (entrapment efficiency, loading efficiency and *in vitro* hemocompatibility were proved). The *in vitro* drug release profile for all the systems confirmed an initial burst release for the 5-FU system followed by a slow release for 4 days. The CRC NPs system showed an initial slow release followed by a sustained release profile up to 4 days. The *in vitro* combinatorial anticancer effects of 5-FU-*N, O*-CMC NPs, CRC-*N, O*-CMC NPs (system 1) and 5-FU-TCS NPs, CRC-TCS NPs (system 2) were proved through MTT, live dead, cell cycle analysis and mitochondrial membrane potential assays in HT 29 cells (for 48 hours, for a concentration of 20 µM of CRC/20 µM of 5-FU within the NPs). For a concentration of 20 µM of CRC and 20µM of 5-FU in the NPs, enhanced anticancer effects were proven in HT29 unlike their individual counter parts and bare drugs. In conclusion, 5-FU/CRC NPs in combination enhances the anticancer effects of 5-FU through the inhibition of COX-2 protein which has been reported previously.

![Fig. 1](image)

Fig. 1. (A) Schematic representation of *N, O*-CMC synthesis from chitosan, (B,C) SEM and AFM images of *N, O*-CMC NPs, (D) cytocompatibility of *N, O*-CMC NPs through MTT assay and (E, F) blood compatibility of *N, O*-CMC NPs through hemolysis and coagulation assays.
Table 1 Represents the characterisation data of 5-FU-N, O-CMC NPs, CRC-N, O-CMC NPs, 5-FU-TCS NPs and CRC-TCS NPs.

<table>
<thead>
<tr>
<th>Systems</th>
<th>System</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CRC-N-O-CMC NPs</td>
<td>100 ± 20</td>
<td>+23.4 ± 0.08</td>
<td>47.86 ± 1.34</td>
</tr>
<tr>
<td>2</td>
<td>5-FU-N-O-CMC NPs</td>
<td>150 ± 40</td>
<td>+33.7 ± 6.32</td>
<td>84.26 ± 1.46</td>
</tr>
<tr>
<td>3</td>
<td>5-FU-TCS NPs</td>
<td>150 ± 40</td>
<td>+48.2 ± 5.0</td>
<td>46.82 ± 3.20</td>
</tr>
<tr>
<td>4</td>
<td>CRC-TCS NPs</td>
<td>150 ± 20</td>
<td>+33.7 ± 3.0</td>
<td>85.55 ± 6.81</td>
</tr>
</tbody>
</table>

Fig. 2A Represents the cell death analysis through MTT measurements, where a, b, c, d, e, and f represents 5-FU, CRC, combination of CRC and 5-FU, CRC-N, O-CMC NPs, 5-FU-N, O-CMC NPs and the combination of both treated HT29 cells (48 hours). B, C, D, E, F, G and H represents the live dead assay results, where B, C, D, E and F represents the control HT 29 cells, cells treated with CRC, 5-FU, combination of CRC and 5-FU, CRC-N, O-CMC NPs, 5-FU-N, O-CMC NPs and the combination of both treated HT 29 cells, I and J represents the quantification of combinatorial anticancer effects through mitochondrial membrane potential (JC-1) and cell cycle analysis measurements (* indicates p< 0.05 compared to the samples).
Fig. 3. (A & C) the flow cytometric dot plots of quantification of combinatorial anticancer effects of CRC-TCS NPs and 5-FU-TCS NPs through mitochondrial membrane potential (JC-1) / cell cycle analysis measurements, where (a) control HT 29 cells, (b) treated with 5-FU, (c) treated with CRC, (d) treated with a combination of 5-FU and CRC, (e) treated with 5-FU-TCS NPs, (f) treated with CRC-TCS NPs, and (f) treated with a combination of CRC-TCS NPs and 5-FU-TCS NPs after 48 hours. (B & D) represents the graphical representation of mitochondrial membrane potential and cell cycle analysis measurements, where percentage cell death was plotted against samples. (E & F) represents the pharmacokinetic profile of 5-FU-TCS NPs and CRC-TCS NPs in Swiss Albino mice after 72 hours. (* indicates p< 0.05 compared to the samples).

The in vivo PK results of CRC-N, O-CMC NPs/5-FU-N, O-CMC NPs (system 1) and CRC-TCS NPs/5-FU-TCS NPs (system 2) individually and in combination
showed 28.6 ± 7.07 and 14.27 ± 4.00 µg/ml quantities respectively for 5-FU and CRC in plasma up to 72 hours. The plasma concentration of 5-FU from a block copolymer was reported earlier, and it showed a plasma concentration of 40-60 µg/ml of 5-FU up to 48 hours.29 The system was proved to be inhibiting the growth of colon cancer xenograft model.29 Even though our results of plasma concentration of 5-FU was slightly lower, the combination of CRC/5-FU in the NPs can reduce colon cancer cell growth through the COX-2 inhibiting activity of CRC, which can aid in improving the anticancer efficacy of 5-FU. Hence the current systems of 5-FU/CRC in TCS NPs/N, O-CMC NPs can produce combinatorial anticancer effects in colon cancer in which the efficacy of 5-FU as a chemo drug could be enhanced and the dose reduction can be achieved. For achieving this, the anticancer effects of the drug loaded NPs need to be checked in colon cancer model models.

Chapter 4: Conclusions and Future Perspectives
Nanoformulations of 5-FU/CRC was developed using two chitosan derivatives based nanoparticles with different chemical functionality ie., N, O-CMC and TCS to overcome the low bioavailability of CRC/5-FU. The in vitro hemocompatible nature of 5-FU-N, O-CMC NPs/CRC-N, O-CMC NPs, and 5-FU-TCS NPs/CRC-TCS NPs were proven. The in vitro combinatorial anticancer effects of 5-FU/CRC from the CRC-N, O-CMC NPs/5-FU-N, O-CMC NPs and CRC-TCS NPs/5-FU-TCS NPs were proved through different assays. The mechanism of enhanced anticancer effects could be due to the inhibition of COX-2 by CRC, resulting in the improved anticancer effects of 5-FU towards colon cancer as reported earlier.3,12,13 Over all, the entrapped 5-FU/CRC in N, O-CMC NPs and TCS NPs produced enhanced anticancer effects under in vitro, and improved bioavailability under in vivo conditions. The in vivo plasma concentration of 5-FU/CRC in the current experiment comes within the range where in vitro combinatorial anticancer effects were proved. Furthermore, in clinical application, the in vivo anticancer efficacy of the nanomedicine needed to be evaluated in colon cancer.
References


Publications in International Journals

Awards

1. **Best Poster Award** for the poster titled “Nanocarrier based on Carboxymethyl chitosan (N, O-CMC and O-CMC) for the delivery of curcumin to cancer cells” in Third Bangalore Nano International Conference (2010).

Conferences Attended

