
**UNIVERSITI PUTRA MALAYSIA**

# *SYNTHESIS AND CYTOTOXICITY EVALUATION OF SORAFENIB- AND 5-FLUOROURACIL-LOADED CHITOSAN, GRAPHENE-OXIDE AND FOLIC-ACID BASED NANOCARRIERS FOR LIVER AND COLON* CANCER

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**ITMA 2021 3**

# SYNTHESIS AND CYTOTOXICITY EVALUATION OF SORAFENIB- AND 5-FLUOROURACIL-LOADED CHITOSAN, GRAPHENE-OXIDE AND FOLIC-ACID BASED NANOCARRIERS FOR LIVER AND COLON CANCER

**By**

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**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia infulfilment of the requirement for the degree of Master of Science

**SYNTHESIS AND CYTOTOXICITY EVALUATION OF SORAFENIB- AND 5-FLUOROURACIL-LOADED CHITOSAN, GRAPHENE-OXIDE AND FOLIC-ACID BASED NANOCARRIERS FOR LIVER AND COLON CANCER**

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**May 2021**

**Chairman : Prof. Mohd Zobir Hussein**
**Institute : Advanced Technology**

Nanocarriers-based drug delivery systems have become the new option for treating cancer due to their negligible side effect. Sorafenib (SF) and 5-Fluorouracil (5FU) drugs have severe side effects on the human body. Therefore, new nanocarriers-based drug delivery systems should be implemented to load these drugs. In this study, SF and 5- FU-loaded chitosan nanocarriers with and without graphene oxide (GO) and folic acid (FA) were synthesized to evaluate the anticancer activity on human liver cancer (HepG2) and colon cancer (HT29) cells. All the nanocarriers were prepared by the ionotropic gelation method where drugs were entrapped with chitosan and chitosan/graphene-oxide composite via cross-linking with sodium tripolyphosphate (TPP). The nanocarriers were found uniform size with efficient drug loading and encapsulation. Chitosan nanoparticles (CS NPs) loaded with SF drug (SF-CS-SF NPs) was found 76 nm while folate conjugated SF loaded chitosan NPs (SF-CS-SF-FA NPs) was found 82 nm. Besides, SF and 5-FU loaded CS NPs (SF/5FU-CS-SF NPs) were found 78 nm and FA conjugated SF/5FU loaded CS NPs (SF/5FU-CS-SF-FA NPs) was found 142 nm. Moreover, the GO/CS composite based SF loaded (GO-CS-SF) was found 122 nm and folate conjugated GO/CS composite based SF loaded nanocomposite (GO-CS-SF-FA) was found 164 nm. All the nanoparticles' encapsulation efficiency was found to be 70-80% while nanocomposites encapsulation efficiency was found 80-90%. XRD and FTIR evaluation found the amorphous structure and the chemical bond formation of the nanocarriers, respectively. The in vitro release study showed the sustained release of the drugs from all the nanocarrier systems. The nanocomposites were found slightly slow release compared to nanoparticles. Overall, most of the drug (90%-100%) release was achieved within120 hours for all samples. The cytotoxicity study revealed better anticancer activity compared to the free drugs alone against human hepatocellular carcinoma (HepG2) and human colorectal carcinoma (HT29) cells. The IC50 value for pristine drugs is higher than nanocarriers. Moreover, all the nanocarriers have shown no toxicity to normal fibroblast human dermal fibroblast adult cells (HDFa). This is towards the new generation of drug delivery systems of tailor-made properties with better efficacy and accuracy.

Key words: Sorafenib, 5-Fluorouracil, Folic Acid, Chitosan Nanoparticles, Graphene oxide, nanoparticles, nanocomposite, drug delivery, therapeutic, HepG2, HT29 and HDFa cell lines.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra MalaysiaSebagai memenuhi keperluan untuk ijazah Master Sains

**SINTESIS DAN PENILAIAN KETOSIKAN SITO BAGI NANOPEMBAWA BERDASARKAN SORAFENIB- DAN 5-FLUOROURACIL-TERMUAT CHITOSAN, GRAFIN OKSIDA DAN ASID FOLIK UNTUK KANSER HATI DAN KOLON**

Oleh

**UMME RUMAN**

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Sistem penyampaian ubat berasaskan pembawa nano telah menjadi pilihan baru untuk merawat kanser kerana kesan sampingannya yang boleh diabaikan. Ubat-ubatan, Sorafenib (SF) dan 5- Fluorouracil (5FU) mempunyai kesan sampingan yang teruk pada tubuh manusia. Oleh itu, sistem penyampaian ubat berasaskan pembawa nano baru perlu dilaksanakan untuk memuatkan ubat-ubatan ini. Dalam kajian ini, SF dan 5-FU-dimuatkan di atas pembawa nanokitosan dengan/dan tanpa grafin okisda (GO) dan asid folik (FA) dan telah disintesis untuk menilai aktiviti antikansernya pada sel-sel kanser hati manusia (HepG2) dan kanser kolon (HT29). Kesemua pembawa nano telah disediakan dengan kaedah gelasi ionotropic, di mana ubat-ubatan telah terperangkap dengan komposit kitosan dan kitosan / grafin-oksida melalui hubungan silang dengan natrium tripolyphosphate (TPP). Penyampain nano yang telah disintesis didapati berukuran seragam dengan muatan dan enkapsulasi ubat yang cekap. Nanopartikel kitosan (CS NPs) yang dimuatkan dengan ubat SF (SF- CS-SF NPs) didapati bersaiz 76 nm sementara NPs kitosan SF konjugasi folat (NFs SF-CS- SF-FA) didapati bersaiz 82 nm. Selain itu, SF dan 5-FU dimuatkan CS NPs (SF / 5FU-CS-SFNPs) didapati bersaiz 78 nm dan FA konjugasi SF/5FU CS NPs dimuatkan (SF / 5FU-CS- SF-FA NPs) didapati bersaiz 142 nm. Lebih-lebih lagi, SF yang dimuatkan komposit GO/CS(GO-CS-SF) didapati bersaiz 122 nm dan komposit folat konjugasi GO/CS berasaskan SF dimuatkan (GO-CS-SF-FA) nanokomposit didapati bersaiz 164 nm. Semua kecekapan enkapsulasi nanopartikel didapati disekitar 70- 80% manakala kecekapan enkapsulasi nanokompositnya didapati disekitar 80-90%. Penilaian XRD dan FTIR, masing-masing mendapati struktur amorfos dan pembentukan ikatan kimia pembawa nano. Kajian pelepasan in vitro menunjukkan pelepasan ubat yang berterusan dari semua sistem pembawa nano. Bagi nanokomposit, didapati pelepasan sedikit perlahan berbanding dengan nanopartikel. Secara keseluruhan, majoriti pelepasan ubat (90 - 100%) dicapai dalam masa 120 jam untuk semua sampel. Kajian sitotoksisiti menunjukkan aktiviti antikanser yang lebih baik bagi nanokomposit berbanding dengan ubat bebas terhadap sel-sel karsinoma hepatoselular manusia (HepG2) dan sel-sel karsinoma kolorektal manusia (HT29). Nilai IC50 untuk ubat-ubatan asli adalah lebih tinggi daripada

pembawa nano. Tambahan lagi, semua pembawa nano tidak menunjukkan toksik kepada sel-sel fibroblas dewasa manusia biasa (HDFa). Ini adalah ke arah generasi baru sistem penyampaian ubat dengan ciri yang boleh dilaras dengan keberkesanan dan ketepatan yang lebih baik.

Kata kunci: Sorafenib, 5-fluorouracil, Asid folik, Nanopartikel kitosan, Grafin oksida, nanopartikel, nanokomposit, system penyampai, terapeutik, sel HepG2, HT29dan HDFa.

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I certify that a Thesis Examination Committee has met on 28 May 2021 conduct the final examination of UMME RUMAN on her thesis entitled "SYNTHESIS AND CYTOTOXICITY EVALUATION OF SORAFENIB- AND 5-FLUOROURACIL-LOADED CHITOSAN, GRAPHENE-OXIDE AND FOLIC-ACID BASED NANOCARRIERS FOR LIVER AND COLON CANCER'' in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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| | |
|---|---|
| DDS | Drug Delivery System |
| NPs | Nanoparticles |
| CS-NPs | Chitosan nanoparticles |
| CS | Chitosan |
| GO | Graphene Oxide |
| MDR | Multiple drug resistance |
| Rpm | Rounds per minute |
| SF | Sorafenib |
| 5FU | 5-Fluorouracil |
| FA | Folic Acid |
| STPP | Sodium Tripolyphosphate |
| PDI | Poly dispersity index |
| DMSO | Dimethyl sulfoxide |
| MTT | 3-94,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| PBS | Phosphate buffered saline |
| HCL | Hydrochloride |
| DLS | Dynamic light scattering |
| XRD | X-Ray Diffraction spectroscopy |
| FESEM | Field Emission Scanning Electron Microscopy |
| HRTEM | High Resolution Transmission electron microscopy |
| TGA/DTG | Thermo-Gravimetric and Differential Thermo-GravimetricAnalysis |
| RS | Raman spectroscopy |
| UV-Vis | Ultraviolet–visible spectroscopy |
| HPLC | High-performance liquid chromatography |
| EDX | Energy Dispersive X-ray |
| FTIR | Fourier Transform Infrared |
| EPR | Enhanced Permeability and Retention |
| HepG2 | Human liver hepatocellular carcinoma cells |
| HT 29 | Human colorectal carcinoma cells |
| HDFa | Human dermal fibroblast adult cells |
| $IC_{50}$ | Half maximal inhibitory concentration |
| RPMI-1640 | Roswell Park Memorial Institute-1640 |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

The term, “Nanotechnology” was first conceptualized by an American physicist name Richard Feynman back in 1959 [1]. Nanotechnology involves the discovery of novel materials and manufactures them for the development of nano systems, or nanostructured material for extensive use in the fields of science and research. This technology initializes the construction or reconstruction of nanomaterials at the molecular or atomic level. Nanomaterials exhibit great research and development potential in medical applications. Some of these applications include therapeutic, diagnosis, theranostic, biosensing, nanomedicine, and nanodrug delivery. Nanotechnology has transpired with therapeutics and theranostics nanocarrier-based drug delivery system (DDS) based on nanosized materials. The synthesis of new nanomaterials has led to the development of new drug delivery systems. These materials offer great excitement to scientists and researchers as they exhibit unique characteristics that are more superior to their bulk counterparts.

Nanomaterial-based DDS such as nanoparticles and nanocomposites can target the deadliest diseases like cancers, tuberculosis, etc. to deliver the therapeutics agents. Forthe sake of efficient drug transport, the nanostructured materials play a crucial role todeliver the drugs to the targeted sites. These nanocarriers DDS are effectively reducingthe drug dosage with controlled/sustained release of the drug, resulting in less or no side effects [2].

## 1.2 Nanomedicine

The term "Nanomedicine" was first reported by Drexler et al. in 1991 [3]. Integrationof nanotechnology towards the medical field has been considered as the implementation of nanomedicine. Nanomedicine is often exhibited as nanodrug delivery systems. The goal of nanomedicine is to develop sophisticated drug delivery strategies, effective drug delivery systems such as nanodrugs or nanodelivery systems[4]. The nanomedicine based nanodelivery systems (NDS) play an effective role to thedelivery of therapeutics and theranostics agents such as drug, gene, imaging agents, etc., to improve pharmacokinetic/pharmacodynamic profiles of therapeutics agents, enhance drug penetration and its biodistribution, optimize the efficacy of anti-cancer agents, target the specific disease site without affecting the normal cell or tissue [4-6].

## 1.3 Nanodrug Delivery Systems

Nanodrug delivery system (NDDS) usually refers to the nanomaterials or nanostructured materials-based delivery system to deliver therapeutic, diagnostic, or the combination of both, the so-called theranostic agents to the specific region of disease to overcome the toxicity of the agents [7]. The NDDS offers a potential opportunity to enable the stability, biodegradability, bioavailability, solubility, low toxicities of therapeutic agents [8]. NDDS gains attention in the last decade in the clinical phase most recently in the medical world. NDDS can transport and release drugs at the site of action over a long period of time by enhancing drug activity and preventing drug accumulation in normal cells, thus avoiding wastage of drugs, and killing of healthy cells. To treat cancer with low toxic systems is a big aspiration of the researchers. The NDDS encompasses the most important aspect in drug delivery by targeting the site-specific delivery of the drugs with unwanted side-effects. NDDSsometimes refers to a nanocarrier drug delivery system or the host of the therapeutics agent's delivery. In this work, chitosan and graphene oxide nanomaterials were used as the host to deliver Sorafenib and 5-Fluorouracil drugs to the liver and colon cancer cells. chitosan and graphene oxide both exhibited excellent properties such as biodegradability, bioavailability, non-toxic, anti-inflammatory, and so on which makethem worthy as a host for nanocarrier materials [9].

## 1.4 Problem Statement

The current conventional treatment of Sorafenib and 5-Flurouracil is usually associated with high toxicity, poor absorption in the tumor cell, low specificity, drug losses, damaging healthy organs or cells, non-specific distribution of drugs, unwanted distribution, multiple drug resistance (MDR), high clearance rate, drug loss before it reached the cancer cells, high clearance rate and tremendous side effects. Besides, theyare often cleared from the circulation before reaching the target site and thus do not accumulate in the tumor region. These drugs block the signaling pathways that can lead to some extent to disrupt normal cell functions. Even though they primarily inhibitcancer cell proliferation, but they also inhibit normal cell growth such as hair follicles,bone marrow and gastrointestinal tract cells in the body. This leads to a low rate of patient survival profile. Therefore, it is necessary to develop novel strategies and novel nanocarriers that will carry the drug molecules specific to the affected cancerous region in an adequate amount and duration within the therapeutic window [10-17].

Chitosan and graphene oxide are evaluated in this study as nanocarrier materials as there is not a lot of research has done with chitosan and graphene oxide to load Sorafenib and 5-Fluorouracil drugs. To develop the therapeutics novel nanodrug delivery systems using chitosan and graphene-based materials is a challenging task forthe current researchers to manufacture with efficient parameters and physio-chemical characteristics for better therapy, imaging, controlled release of drugs. For example, when larger size of nanocarriers is administered into the human blood vessels, they often are trapped by various biological compound such as protein, enzymes and otherdifferent organs and released therapeutics agents before it reached to the tumor cells.Besides, nanocarriers with very small size often escape the uptake by the targeted organs and eliminate from the body without proper release of therapeutic agents. As aresult, it is crucial for scientists

to optimize and formulate the chitosan and graphene based nanocarrier systems in the size of more than 50 nm and less than 200 nm to loadsignificant amount of therapeutics agents and deliver it effectively to the cancer cells.[18].

Sorafenib (SF) is a multi-kinase inhibitor, and it inhibits cell proliferation, angiogenesis, and threonine kinase activities in tumors [19]. However, like other anticancer drugs, the efficacy of SF is associated with high toxicity to normal healthytissues. Besides, the bioavailability of oral uptake of SF is low. Due to the poor aqueous solubility and low bioavailability (~8.43%), the clinical use of SF is limited for cancer treatment which is leading to the necessity of developing better formulationsof delivery system of SF to increase the antitumor efficacy [20]. On the other hand, 5-Fluorouracil (5FU) is the first-line drug for colon cancer. However, the low drug uptake, drug resistance, drug toxicity significantly limits the clinical efficiency of 5FU [21]. Studies have found that the anticancer efficacy of SF and 5FU has been improvedusing various types of nanocarriers, such as polymer nanoparticles, inorganic nanoparticles, micelles, liposomes, etc. [22-25]. Therefore, this work focused on the development of new formulation of nanocarrier systems with efficient loading of SF and 5FU for effective delivery or transport of the drugs to the cancer cells. Furthermore, the study focuses on the synthesis, characterization, and optimization of Sorafenib-loaded chitosan nanoparticles, Sorafenib and 5-Fluorouracil loaded dual drug-chitosan nanoparticles and Sorafenib-loaded chitosan graphene oxide nanoparticles. All the nanodelivery systems were conjugated with folic acid to synthesize the folate-conjugated nanocarrier delivery systems to test the efficacy. Nanodelivery systems were evaluated by the cytotoxicity study using HDFa, HepG2, and HT29 cell lines.

### 1.5 Hypothesis

The chitosan and graphene exhibit high surface area and high stability to hold the drugsas a result, the synthesized nanodelivery systems are anticipated to improve the delivery efficiency as well as enhance the accumulation of dose of Sorafenib and 5- Fluorouracil drugs in cancer region and thus anticancer action on cancer cells. Moreover, due to the properties of biodegradability and high drug loading ability, thechitosan and graphene oxide based nanocarriers could be a good option to deliver thedrugs to for liver and colon cancer and thus reducing the chances of unspecific drug delivery to the healthy tissues and delivering drugs only to the cancerous regions.

### 1.6 Scopes of Study

This research work is derived from the fact that most anticancer drugs in chemotherapyare severely toxic and harmful to normal human cells. As a result, this study is aimedto synthesize the nanocarriers based on chitosan nanoparticles as well as chitosan/graphene oxide nanoparticles with folic acid-coated and non-coated, to load Sorafenib and 5-Fluorouracil. The synthesized nanocarriers will be characterized by the X-Ray Diffraction (XRD), Field Emission Scanning Electron Microscopy (FESEM), High-Resolution Transmission Electron Microscopy (HRTEM), Thermogravimetric Analysis (TGA), Dynamic Light Scattering (DLS), Fourier Transform Inferred Spectroscopy

(FTIR), Energy Dispersive X-ray (EDX). The release studies and encapsulation/loading capacity was investigated by the High- Performance Liquid Chromatography (HPLC) and UV-visible spectroscopy. Moreover, the synthesized nanocarriers will be tested on the human liver (HepG2), colon (HT29) cancer, as well as normal human dermal fibroblast adult (HDFa) cell lines, to investigate their toxicity level and anticancer activity.

## 1.7 Objectives of Study

### 1.7.1 General Objectives

To synthesize and characterize Sorafenib-loaded chitosan nanoparticle, dual drugs Sorafenib and 5-Fluorouracil-loaded chitosan nanoparticle, and Sorafenib-loaded chitosan/graphene oxide nanocomposite. These nanocarriers were then functionalized with folic acid to obtain their folate-coated version of nanoparticles and nanocomposites. Finally, all the nanocarriers were characterized and evaluated on HDFa, HepG2 and HT29 cancer cell lines.

### 1.7.2 Specific objectives

The specific objectives of this study are as follows:

a) To prepare Sorafenib-loaded chitosan nanoparticle (SF-CS NPs), dual drugs Sorafenib and 5-Fluorouracil loaded chitosan nanoparticle (SF/5FU-CS NPs) and Sorafenib-loaded chitosan/graphene oxide nanocomposite (GO-CS-SF) by the ionic gelation method. Followed by coating SF-CS NPs, SF/5FU-CS NPs, and GO-CS-SF nanocomposites using folic acid (FA) to form folic acid coated Sorafenib loaded chitosan nanoparticles (SF- CS-FA NPs), folic acid coated Sorafenib and 5-Fluorouracil loaded chitosan nanoparticles (SF/5FU-CS-FA NPs) and folate functionalized Sorafenib loaded chitosan/graphene oxide nanocomposite (GO-CS-SF- FA).

b) To evaluate the size, shape, crystallinity, physiochemical properties, thermal stability of SF-CS, SF-CS-FA, SF/5FU-CS, SF/5FU-CS-FA, GO-CS-SF, GO-CS-SF-FA using the different analytical techniques.

c) To evaluate the in vitro release behavior of Sorafenib and 5-Fluorouracil from SF-CS, SF-CS-FA, SF/5FU-CS, SF/5FU-CS-FA nanoparticles and GO-CS-SF, GO-CS-SF-FA nanocomposites.

d) To evaluate the cytotoxicity of the SF-CS, SF-CS-FA, SF/5FU-CS, SF/5FU-CS-FA nanoparticles and GO-CS-SF, GO-CS-SF-FA nanocomposites on human hepatocellular carcinoma (HepG2) and human colorectal adenocarcinoma (HT29) cancer cell lines and human normal dermal fibroblast adult (HDFa) cell lines using the MTT assay.

## 1.8 Significance of Study

This current study aims to synthesize anticancer drug-loaded chitosan nanoparticles and chitosan/graphene oxide nanocomposite formulation to increase the efficacy of thedrug against liver and colon cancer. Chitosan nanoparticles and chitosan/graphene oxide nanocomposite were formulated to be used as nanocarriers for chemotherapeuticcancer drug delivery systems owing to increase the solubility, reduce the dose and toxicity and improve the bioavailability of the anticancer drugs. The final importanceof this study is to evaluate the inhibitory effect of the nanocarriers on human hepatocellular carcinoma (HepG2) cell lines and human colorectal adenocarcinoma cancer cell lines (HT29) by in vitro cytotoxicity studies.

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## LIST OF PUBLICATIONS

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**Ruman, U**., Buskaran, K., Pastorin, G., Masarudin, M. J., Fakurazi, S., & Hussein, M. Z. (2021). Synthesis and Characterization of Chitosan-Based Nanodelivery Systems to Enhance the Anticancer Effect of Sorafenib Drug inHepatocellular Carcinoma and Colorectal Adenocarcinoma Cells. Nanomaterials, 11(2), 497.

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