SYNTHESIS AND *IN-VITRO* TOXICITY EVALUATION OF ANTICANCER DRUGS-LOADED CHITOSAN NANOPARTICLES AS THERAPEUTIC NANOCARRIERS FOR THE TREATMENT OF HEPATOCELLULAR CARCINOMA

By

ALBALAWI FAHAD MOHAMMED

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chair : Prof. Mohd Zobir bin Hussein, PhD
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Hepatocellular carcinoma (HCC) is among the most common liver cancers globally with more than 600,000 new patients diagnosed annually. The limitations of chemotherapy in treating HCC include poor aqueous solubility, non-specific targeting of anticancer drugs, low retention of drugs in the tumor, and multi-drug resistance. The development of innovative intervention tools for early diagnosis and treatment has gained exceptional interest in HCC management. However, HCC is a multifactorial disease that requires a combination of treatment plans rather than a single therapeutic agent targeting only a single target. The complication of the disease, such as liver cirrhosis, limits surgical and therapeutic options due to liver malfunction might result in alteration of the safety profiles of systemic agents. Thus, multi-target inhibitors (MTIs) and multi-drug inhibitors (MDIs) that combine several drugs to inhibit numerous pathways are vital in treating HCC, but they may induce systemic toxicity due to liver malfunction. The concept of employing nanoparticles (NPs) in delivering multi-target inhibitors (MTIs) and multi-drug inhibitors (MDIs) has a strong potential in the therapeutic strategy and offers impressive outcomes to address HCC. Chitosan nanoparticles (ChNPs) have a great potential to be used as a drug delivery system in HCC therapy over conventional drug therapy. Furthermore, the anti-HCC drug-loaded ChNPs can lessen the dosage amount and duration of treatment and could resolve the problems of low and poor compliance, therefore, significantly reducing the side effects. In this work, encapsulated, single-loaded, and dual-loaded FDA-approved anti-HCC drugs (small molecule kinase inhibitors); cabozantinib (CBZ) and sorafenib (SF), and the antimetabolite drug, 5-fluorouracil (5FU) into chitosan NPs, were synthesized for better efficacy on HCC treatment with fewer drug side effects. These novel nanocarriers enhanced effective permeation through the cells, better stability in the bloodstream, and demonstrated controlled release capability of the encapsulated drugs, resulting in more potent multitarget inhibitors for HCC

treatment. In this study, the ionic gelation technique was used to synthesize chitosan NPs, loaded with MTIs and MDIs, via a crosslinking agent, sodium tripolyphosphate (TPP) with various Ch to TPP ratios (1:1.25, 1:2.5, 1:5, 1:10, 1:20). Subsequently, the impact of the amount of TPP on the reaction yield, particle size, entrapment efficiency, anticancer activities, and in-vitro drug release was explored. The increase in the TPP concentration led to a smaller particle size. The chitosan nanocarriers were found to be uniform in size with high drug loading and encapsulation efficiency. At the ratio of 1:2.5, ChNPs with single-loaded MTI were found to be in the range of 100 nm in their mean particle size distribution (PSD), compared to around 50 nm for dual drug-loaded ChNPs. The encapsulation efficiencies for single-loaded drugs are in the range of 40-50% compared to 50-70% for the dual-loaded. The XRD and FTIR of chitosan nanoparticles revealed an amorphous nature, which confirmed that the crystal structure of the drug was tapered. All the drugs from all the nanocarriers systems underwent a sustained release as evident in the in-vitro release study, as indicated by the TGA/DTG thermograms. Overall, the majority of the drugs show 90-100% release within the first 120 hours for all the samples. The cytotoxicity of these synthesized nanodelivery systems was evaluated by *In Vitro* study using normal human dermal fibroblast adult cells (HDFa) cells and human liver hepatocellular carcinoma (HepG2) cell lines. The nanocarriers system for the MTIs and MDIs showed low toxicity to the normal humandermal fibroblast adult cells (HDFa). The single- and dual-loaded drug systems exhibited anticancer effects, which were better achieved with MDIs compared to MTIs. Conclusively, CS/TPP concentration is one of the most important factors in optimizing the formulation for the development of anti-HCC nanocarriers. Dual drug-loaded CSNP systems are a novel and promising approach to enhancing therapeutic efficacy and reducing the deleterious effects of MDIs and MTIs. Findings from this work could lead to a new generation of nanodrug delivery systems of tailor-made multifunctional properties with better efficacy and accuracy.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

SINTESIS DAN PENILAIAN TOKSISITI *IN-VITRO* BAGI NANOPARTIKEL KITOSAN-TERMUAT UBAT ANTIKANSER SEBAGAI PEMBAWA NANO TERAPI UNTUK RAWATAN KARSINOMA HEPATOSELULER

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Karsinoma hepatoselular (HCC) adalah antara kanser hati yang paling biasa di seluruh dunia dengan lebih daripada 600,000 pesakit baharu didiagnosis setiap tahun. Kemoterapi yang terhad dalam merawat HCC termasuk keterlarutan akueus yang lemah, ubat antikanser yang bukan spesifik khusus, pengekalan ubat yang rendah dalam tumor dan rintangan pelbagai ubat. Pembangunan alat intervensi yang inovatif untuk diagnosis dan rawatan awal telah mendapat tarikan yang luar biasa dalam pengurusan HCC. Walaubagaimanapun, HCC ialah penyakit disebabkan oleh pelbagai faktor yang memerlukan gabungan pelan rawatan dan bukan hanya satu agen terapi yang menyasarkan hanya satu sasaran. Komplikasi penyakit seperti sirosis hati mengehadkan pilihan pembedahan dan terapi akibat kerosakan hati yang mungkin mengakibatkan perubahan profil keselamatan agen sistemik. Oleh itu, perencat berbilang sasaran (MTI) dan perencat berbilang ubat (MDI) yang menggabungkan beberapa ubat adalah penting dalam merawat HCC untuk menghalang banyak kesan. Namun begitu, gabungan ini boleh menyebabkan ketoksikan sistemik akibat daripada kerosakan hati. Konsep menggunakan zarah nano (NP) dalam menyampaikan perencat berbilang sasaran (MTI) dan perencat pelbagai ubat (MDI) mempunyai potensi yang kuat dalam strategi terapi dan menawarkan hasil yang mengagumkan untuk menangani HCC. Nanopartikel kitosan (ChNPs) mempunyai potensi besar untuk digunakan sebagai sistem penyampaian ubat dalam terapi HCC berbanding terapi ubat konvensional. Tambahan pula, ChNP yang sarat dengan ubat anti-HCC boleh mengurangkan jumlah dos dan tempoh rawatan serta boleh menyelesaikan masalah pematuhan yang rendah dan lemah. Oleh itu, ubat ini boleh mengurangkan kesan sampingan dengan ketara. Dalam bidang ini, ubat anti-HCC yang diluluskan oleh FDA (perencat kinase molekul kecil); cabozantinib (CBZ) dan sorafenib (SF), dan ubat antimetabolit,

5-fluorouracil (5FU) ke dalam NP kitosan, telah disintesis untuk keberkesanan yang lebih baik pada rawatan HCC dengan kesan sampingan ubat yang lebih sedikit. Pembawa nano novel ini meningkatkan resapan berkesan melalui sel, kestabilan yang lebih baik dalam aliran darah dan menunjukkan keupayaan pelepasan terkawal bagi ubat terkapsul yang menghasilkan perencat berbilang sasaran yang lebih kuat untuk rawatan HCC. Dalam kajian ini, teknik pengadukan ionik digunakan untuk mensintesis NP kitosan yang ditambah dengan MTI dan MDI melalui agen penghubung silang, natrium tripolifosfat (TPP) dengan pelbagai nisbah Ch kepada TPP (1:1.25, 1:2.5, 1:5, 1:10, 1:20). Seterusnya, kesan jumlah TPP ke atas hasil tindak balas, saiz zarah, kecekapan pemerangkapan, aktiviti antikanser dan pelepasan ubat in-vitro telah diterokai. Peningkatan kepekatan TPP membawa kepada saiz zarah yang lebih kecil. Pembawa nano kitosan didapati bersaiz seragam dengan penambahan ubat yang tinggi dan kecekapan pengkapsulan. Pada nisbah 1:2.5, ChNP dengan MTI tambahan tunggal didapati berada dalam julat 100 nm dalam taburan saiz zarah puratanya (PSD) berbanding dengan ChNP yang ditambah dengan dua ubat hampir 50 nm. Kecekapan enkapsulasi untuk ubat tambahan tunggal adalah dalam julat 40-50% berbanding 50-70% untuk ubat dwi-tambahan. XRD dan FTIR nanopartikel kitosan mendedahkan sifat amorf, yang mengesahkan bahawa struktur kristal ubat itu tirus. Semua ubat daripada semua sistem pembawa nano menjalani pelepasan berterusan seperti yang terbukti dalam kajian pelepasan in-vitro yang ditunjukkan oleh termogram TGA/DTG. Secara keseluruhan, majoriti ubat menunjukkan pelepasan 90-100% dalam tempoh 120 jam pertama untuk semua sampel. Sitotoksisiti sistem penghantaran nano yang disintesis ini dinilai oleh kajian In Vitro menggunakan sel-sel dewasa fibroblast dermal manusia (HDFa) normal dan sel-sel karsinoma hepatoselular hati manusia (HepG2). Sistem pembawa nano untuk MTI dan MDI menunjukkan ketoksikan yang rendah kepada sel dewasa fibroblast manusia biasa (HDFa). Sistem ubat tunggal dan dua tambahan mempamerkan kesan antikanser yang lebih baik dicapai dengan MDI berbanding MTI. Kesimpulannya, kepekatan CS/TPP adalah salah satu faktor terpenting dalam mengoptimumkan formulasi bagi pembangunan pembawa nano anti-HCC. Sistem CSNP yang sarat dengan dua ubat adalah pendekatan baru dan menjanjikan untuk meningkatkan keberkesanan terapi dan mengurangkan kesan buruk MDI dan MTI. Penemuan daripada kajian ini boleh membawa kepada generasi baharu dalam sistem penyampaian ubat nano dengan pelbagai fungsi khusus yang lebih berkesan dan tepat.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date: 8 February 2024

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
CBZ	Cabozantinib
CVD	Cardiovascular disease
ChNPs	Chitosan nanoparticles
cSrc	Tyrosine-protein kinase
DLS	Dynamic Light Scattering
EDX	Energy Dispersive X-ray
EGFR	Endothelial growth factor receptor
FDA	Food and Drug Administration
FESEM	Field Emission Scanning Electron Microscopy
FTIR	Fourier Transform Inferred Spectroscopy
GI	Gastrointestinal
HBV	hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	hepatitis C virus
HDFa	human dermal fibroblast adult cells
HepG2	human liver hepatocellular carcinoma
HICs	High-income countries
HRTEM	High-Resolution Transmission Electron Microscopy
MAPK	mitogen-activated protein
MTIs	Multi-target inhibitors
MDR	Multi-drug resistance
NAFLD	non-alcoholic fatty liver disease
Pgp	P-glycoprotein

PDGFR	Platelet-derived growth factor receptor
PGA	Polyglycosides
PLA	Polylactides
PNPs	Polymeric nanoparticles
SDIs	Single drug inhibitors
SF	Sorafenib
TGA	Thermogravimetric Analysis
TKI	tyrosine kinase inhibitor
TPP	sodium tripolyphosphate
VEGFR	Vascular endothelial growth factor receptor
XRD	X-Ray Diffraction

CHAPTER 1

INTRODUCTION

1.1 Background of research

Cancer has been the leading cause of death globally after cardiovascular disease although tremendous efforts have been devoted to improving the early diagnosis capacity and therapeutic strategies (Nagai & Kim, 2017). Lately, a few studies were conducted to assess the leading cause of mortality in high-, middle- and low-income countries and the findings demonstrated that cancer has overtaken cardiovascular disease (CVD) as the primary cause of death in high-income countries (HICs) (Mahase, 2019; The Lancet, 2019; Stringhini & Guessous, 2018). According to a report from the Prospective Urban and Rural Epidemiologic (PURE) study published in The Lancet, cancer is twice as many deaths (CVD) in HICs including Saudi Arabia, United Arab Emirates, Canada and Sweden.

As demonstrated in Figure 1, the GLOBOCAN, the GLOBOCAN database has projected that there will be an estimated 19.3 million new cancer cases and approximately 10 million deaths worldwide in the year 2021 (Sung et al., 2021). It was then forecasted the new cancer cases will hit 28.4 million by 2040.

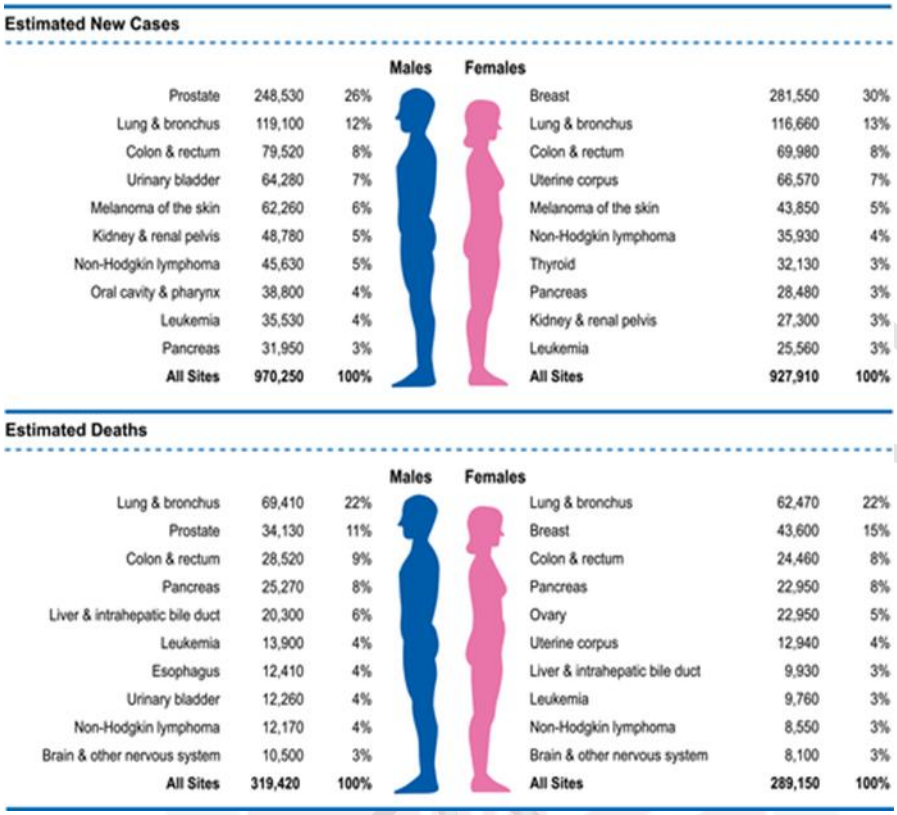

Figure 1.1: Estimated new cases and death in the year 2021 without including basal cell and squamous cell skin cancer and in situ carcinoma except for urinary bladder
(Source: Siegel, 2021)

Among types of cancer, it was reported that cancer of the liver was the fifth most common cancer in males while the ninth most common in females (Ndom, 2019). Although liver cancer was not the top 3 most common cancer in both genders, the fatality rate of liver cancer was ranked no. 3 with 830,000 deaths reported in 2020 (Trézéguet et al., 2021). Hepatocellular carcinoma (HCC) – was the most common liver cancer with more than 600,000 new diagnosed cases each year which is prominent in Asia and sub-Saharan Africa where chronic hepatitis B virus infections are endemic (Sung et al., 2021). On the contrary, the predominant risk factors in Western countries are alcohol, hepatitis C virus infection, and prevalence of non-alcoholic steatohepatitis. Underlying liver cirrhosis is a common underlying condition in HCC patients, which limits surgical and therapeutic options due to malfunctioning liver might result in alteration of safety profiles of systemic agents (Galle et al., 2018).

Surgery, radiation therapy and chemotherapy are the most common treatment choices for cancer. The selection of the treatment choices is relatively dependent on several factors including the size and location of the tumor. Besides, the stage

of cancer also plays a crucial role in deciding the treatment options. Chemotherapy was the main therapeutic approach for both localized and metastasized cancer. The primary strategy of conventional chemotherapy in destroying cancer cells is interfering with the DNA synthesis and mitosis process via chemical substances. Occasionally, chemotherapy was recommended to be combined with other forms of therapy such as surgery and radiation therapy to have better efficacy in cancer therapy.

Despite the promise of conventional chemotherapy in cancer treatment, it suffers several limitations including poor aqueous solubility, non-specific targeting of anticancer drugs, low retention of drugs in the tumor and multi-drug resistance (MDR) (Wei et al, 2021; Zhao et al., 2018). Chemotherapeutic drugs such as doxorubicin, paclitaxel cabozantinib, sorafenib and fluorouracil are hydrophobic and require solvents (alcohol and acid) to formulate the dosage form which frequently leads to severe toxicity. Besides, owing to its low selectivity properties, chemotherapeutic drugs also destroy other normal healthy cells in the body that naturally grow at a faster pace for instance hair, skin, blood cells and cells in the gastrointestinal (GI) tract (Senapati et al., 2018; Zhao et al., 2018). Additionally, in many instances, only a small amount of the administered drug reaches the tumor site which eventually leads to a relatively low therapeutic efficacy. MDR was the major blockade that limited the efficacy of conventional chemotherapy. MDR is the result of the overexpression of several proteins in the cell membrane such as P-glycoprotein (Pgp) which plays a role in transporting the chemotherapeutic drugs out of cells (Pomilio & Mercader, 2018). Therefore, eradication of cancer remains a major challenge owing to its heterogeneous nature and inability of delivering chemotherapeutic drugs to cancer cells and at the same time, minimizing the toxicity in normal healthy cells.

Nanotechnology is accepted as a window of new opportunities and solution for cancer care, and amazing treatment outcomes with its application has been published. Extensive reviews of advantages and limitations of nanotechnology for carcinogenesis treatment and their relevant factors and characteristics are available in literature (Friberg & Nyström, 2015; Kawasaki & Player, 2005; Wicki, Witzigmann, Balasubramanian, & Huwyler, 2015). It is recognized that nanotechnology manifests potential in substituting conventional cancer therapy owing to its unique advantages for specific delivery to target cells (Climent et al., 2018; Li, Yin, Cheng, & Lu, 2012). Target agents (antibodies, aptamer and peptides) functionalized on drug nanocarriers are commonly used to enhance the selectively targeting specific cell surface receptors which predominantly present on cancer cells; consequently, an adequate amount of the chemotherapeutic drug is transported to cancer cells and minimized the treatment impact on normal healthy cells. Hence, high drug dose is unnecessary to yield the similar or even better therapeutic effecf, which eventually brings down the treatment cost. Moreover, the ability remote control over the release of chemotherapeutic drug by responsive toward external (light, magnetic field and ultrasound) (Angelatos, Radt, & Caruso, 2005; Ge, Neofytou, Cahill, Beygui, & Zare, 2012; Rodzinski et al., 2016) or internal (pH, temperature) (Angelos et al., 2009; Du et al., 2009; J. Zhang & Misra, 2007) stimuli lessen the possibility of premature drug release during the circulation in the body. The advent of

nanotechnology in treating cancer has numerous positive outcomes as compared to conventional cancer therapy such as lengthen circulation time and therefore better bioavailability, improved solubility of chemotherapeutic drugs, slow-release of the drug and tailor-made properties such as multi-modal and multi-functional.

Several types of nanocarriers as shown in Figure 2 such as metallic nanoparticles (Baffou & Quidant, 2013; Ahmad et al., 2010; Kogan et al., 2005), polymer nanoparticles (Mukerjee & Vishwanatha, 2009; Tang et al., 2009; Tong & Cheng, 2007), silica nanoparticles (Argyo et al., 2014; Zhang et al., 2012; Lee et al., 2011; Tian et al., 2011), quantum dot (Delehanty et al., 2009; Medintz et al., 2005; Michalet et al., 2005), liposome (Allen & Cullis, 2013; Chang & Yeh, 2012; Harris et al., 2002; Batist et al., 2001) and dendrimer (Wolinsky & Grinstaff, 2008; Tomalia et al., 2007; Majoros et al., 2006; Majoros et al., 2005; Malik et al., 1999) were developed.

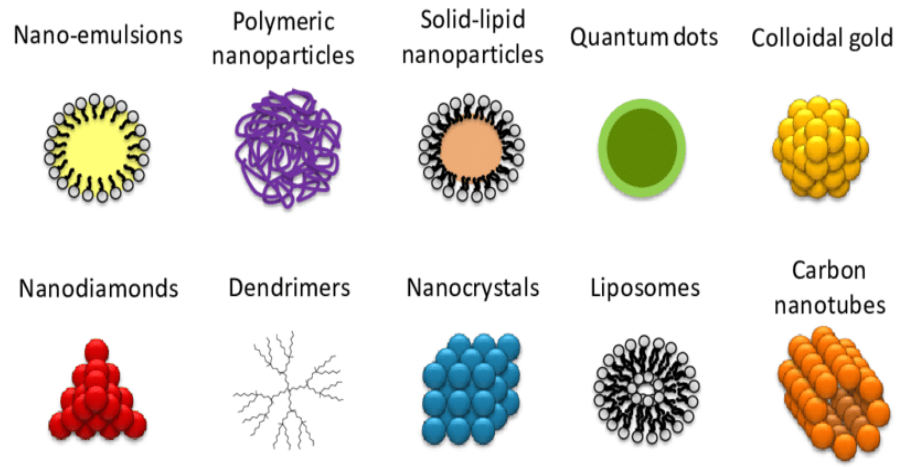

Figure 1.2: Schematic diagram representing the common type of nanomaterials used for nanomedicine
(Source: Fornaguera & Solans, 2017)

From the abovementioned nanocarriers, polymeric nanoparticles (PNPs) have been drawing increased attention from a few groups of researchers after the initial work of Langer and Folkman in 1976 (Langer & Folkman, 1976). PNPs are produced from a polymeric material with the colloidal organic compounds having a size range from 1 to 1000 nm. Based on their origin, polymers used in formulating PNPs can be subdivided into natural types and synthetic. Chitosan, gelatin, albumin and sodium alginate are examples of natural polymers that are obtained from either animals or plants to be used to synthesize PNPs (Niculescu & Grumezescu, 2021). On the other hand, the most common synthetic polymers used for PNPs preparation are polylactides (PLA), polyglycosides (PGA) and poly(N-vinylpyrrolidine). Furthermore, as illustrated in Figure 3, PNPs are synthesized either into nanospheres and nanocapsules structure depending on

their preparation method. Nanocapsules are systems in which the drug is confined to an aqueous/oily core surrounded by a unique, thin polymeric membrane with specific characteristics. Nanospheres, are matrix systems in which the drug is physically and uniformly dispersed, entrapped, or adsorbed.

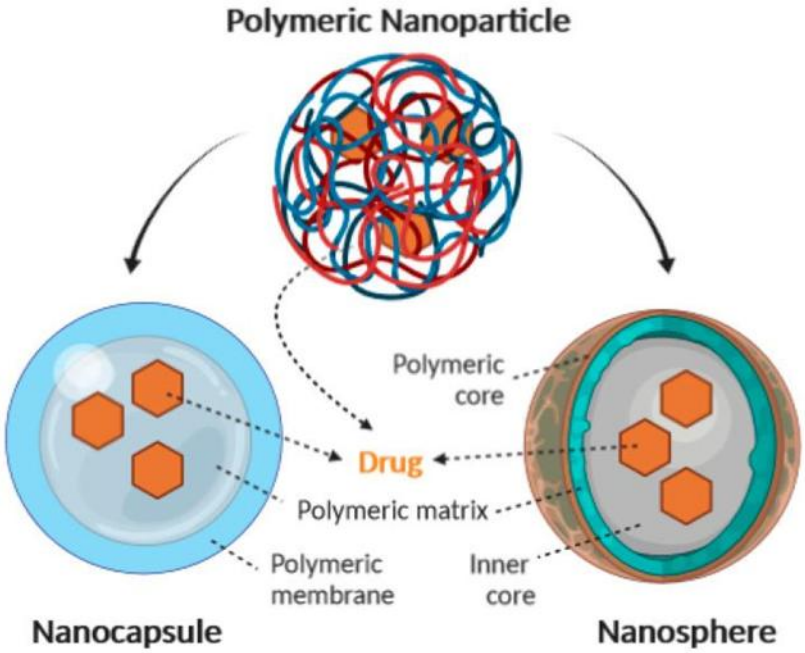

Figure 1.3: Schematic diagram represented types of PNPs based on their preparation method
(Source: Zielińska et al., 2020)

Owing to their advantageous properties such as good stability, low toxicity, biodegradability and hemocompatibility, PNPs are widely used for biomedical applications not limited to only delivering drugs, but also used as a diagnostic tool in the medical field (Sharifi-Rad et al., 2021; Sanna & Sechi, 2020). A more detailed type of PNPs and their biomedical applications were summarized in Figure 1.

Table 1.1: Types of PNPs used in biomedical applications

Particle class Materials	Particle class Materials	Application
Natural material/ derivative	Chitosan, dextran, gelatine, alginates, liposomes, starch	Drug delivery, gene delivery
Dendrimers	Branched polymers	Drug delivery
Fullerenes	Carbon-based carrier	Photodynamics drug deliver
Polymer carriers	Polylactic acid, poly(cyano)acrylates, polyethyleneimine, block copolymers drug delivery, gene delivery Polycaprolactone	Drug delivery, gene delivery
Ferrofluids	SPIONS, USPIONS	Imaging (MRI)
Quantum dots	Cd/Zn–selenides	Imaging, *In Vitro* diagnostics
Various	Silica–nanoparticles, mixtures of above	Gene delivery

SPIONS = Superparamagnetic iron oxide nanoparticles, USPIONS= ultra-small superparamagnetic iron-oxide nanoparticles

Various natural polymers such as chitosan have been widely employed in the medical field over the past decades profiting from their inexpensive cost production, renewable, biodegradability, biocompatibility, antimicrobial activity and relatively safe as it had been Food and Drug Administration (FDA) approved (Niculescu & Grumezescu, 2021). Biocompatibility is one of the most crucial requirements in selecting biomaterials for medical and pharmaceutical applications (William, 2020). Among the natural polymers, chitosan nanoparticles (ChNPs) have attracted tremendous attention as nanocarriers since they can be loaded with a wide range of natural and chemical substances including chemotherapeutic drugs, protein, and genes via simple chemical reactions. The presence of two distinct reactive functional groups (-NH2 group and -OH group) allows the conjugation of biological molecules. Additionally, the mucoadhesive properties of this polymer are the main factor that had drawn increasing interest from researchers as this property enable prolonged retention of the drug at the absorption site in which, making them suitable for oral and

intravenous administered nano-delivery systems (Charlie-Silva et al., 2020). ChNPs also play a role in protecting the loaded natural and chemical substances from enzymatic degradation which ensures the encapsulated substances can be delivered to the target site. Another remarkable advantage of ChNPs is they exert a continuous release of drug over an extended period, which is also known as sustained release, unlike liposomes and micelle which exhibit a burst-controlled release of drugs (Herdiana et al., 2021).

1.2 Problem statements

Liver cancer is a serious player that can be life-threatening. It's a multifactorial disease that cannot be combated with a single therapeutic agent which targets only a single target. Thus, multi-target inhibitors (MTIs) are vital in treating liver cancer to minimize the complications of the disease such as the development of chemotherapeutic drugs resistance. As illustrated in Figure 4, there are two major classes of MTIs, (a) single drug inhibitors (SDIs) that downregulate multiple kinases correspondingly while the other major group is (b) multi-drug inhibitors (MDIs) that aim more than one signalling pathways.

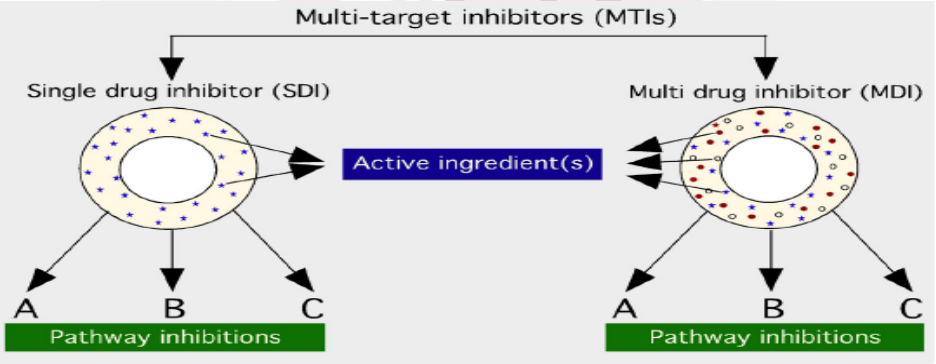

Figure 1.4: Schematic of MTI that affects more than one intracellular signalling pathway at the same time to halt the growth cancerous cells (Source: Gowda et al., 2013)

Small molecule kinase inhibitors for example cabozantinib (CBZ) and sorafenib (SF) are the most popular categories of single drug MTIs which are continuously evaluated as new therapeutic agents for cancer treatment. This is because these molecules deregulate kinase activity (Table 1) which is a vital mechanism that enables cancer cells to invade and controls normal cells proliferation and survival (Zhang et al., 2009).

Table 1.2: Single-agent MTIs currently undergoing preclinical and clinical use

Agent	Company	Indication	Targets
Sorafenib	Onyx/ Bayer	RCC, HCC	VEGFR, PDGFR, c-Kit, Raf
Nilotinib	Novartis	CML	Bcr-Abl, PDGFR, cSrc, c-Kit
Sunitinib	Pfizer	GIST, RCC	PDGFR, VEGFR, c-Kit, RET, FLT3
Crizotinib	Pfizer	NSCLC	EML4/ALK, HGFR
Motesanib	Amgen/ Takeda	Breast cancer	PDGFR, VEGFR, c-Kit
Vandetanib	Astra Zeneca	Thyroid, NSCLC	EGFR, VEGFR, RET
Lesaurtinib	Cephalon	AML	JAK2, FLT3, Trk
Cabozatinib	Exelixis	Thyroid, solid tumors	VEGFR, MET, c-Kit, FLT3, RET, TEK
Pazopanib	GlaxoSmithKline	RCC, sarcoma	VEGFR, PDGFR, c-Kit

(Source: Gowda et al., 2013)

Nearly 500 protein kinases were discovered in the human kinase map. Usually, more than one signalling pathway is affected in one tumour. Hence, kinase inhibitors commonly manufactured to target more than one oncogenic receptor such as vascular endothelial growth factor receptor (VEGFR), endothelial growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), or they manufactured to suppress the downstream intracellular pathways for example tyrosine-protein kinase (cSrc) and mitogen-activated protein (MAPK) pathways. Although tyrosine kinase inhibitor (TKI) therapy has evidently showed a numerous of advantages, it has its limitations as well as several adverse effects. Off-target activity is one of the major drawbacks of TKI therapy that causing a lot of adverse events, negatively influencing the patient's quality life and continuation of therapy (Bhullar et al., 2018). Furthermore, cancer tends to undergo continual mechanisms evolution that drive drug resistance leading to the low efficacy of TKI medications. Thus, other than single-agent MTIs, MDIs, synergistic drug combinations are also getting more and more crucial in cancer treatment. It was hypothesized that a ratio for optimal suppression of more than one kinase can readily be obtained via the combination of multiple agents.

The major challenge for MTIs is the safely delivering drugs and the accumulation of drugs primarily at the target site as the inhibition of multiple key signalling pathways has the tendency in causing systemic toxicity. Because of this, the application of nanotechnology for drug delivery is favoured to make sure that the MTIs assemble in the tumour vasculature and thus, enhancing the treatment outcome while reducing systemic adverse off-target effects. In recent years, the developments in nanomedicine and targeted cancer therapy brings a new insight into this field of therapeutic applications. Certain nanocarrier features offer several advantages over free drugs such as enhance drug efficacy, lessen the

off side of systemic adverse reactions, improve drug bioavailability, overcoming the biological barriers, enhance drug stability or allow for specific delivery of MTIs to the targeted cancer cells. Additionally, nanotechnology can bring new panoramas into MDIs, which can be highly efficient in connection with TKIs.

Herein, ChNPs loaded with chemotherapeutic drugs (Cabozantinib, Sorafenib and 5-Fluorouracil) were synthesized in this study. Chitosan, a substance derived from chitin, has been extensively studied and developed into ChNPs. These nanoparticles possess unique properties that make them particularly suitable for ocular and oral delivery applications. Controlled release self-nanoemulsifying drug delivery systems have emerged as a highly promising approach to address the challenges associated with the limited stability and bioavailability of numerous active pharmaceutical ingredients (APIs). Chitosan possesses the ability to effectively adhere to the mucus membrane, which is negatively charged. This characteristic leads to an extended duration of contact and improves the likelihood of cellular absorption. Very little research has been done on chitosan nanomaterial as MTI owing to the encapsulation of more than one chemotherapeutic drug together into ChNPs was challenging as they might not be compatible, which eventually caused complications. The efficient parameters and physio-chemical characteristics of nanocarriers have played a vital role in ensuring better therapy, imaging and controlled-release of drugs. For instance, administration of a larger size of nanocarriers via blood vessels often are trapped by various biological compounds not limited to protein, enzymes but also other organs and released chemotherapeutics agents before they reach the tumor cells. On the other hand, nanocarriers with very small sizes often escape the uptake by the targeted organs and eliminate them from the body without the proper release of therapeutic agents. As a result, it is crucial for scientists to optimize and formulate the chitosan-based nanocarrier systems with a size range between 50 nm to 200 nm, and loaded with an effective amount of chemotherapeutics agents for effective cancer treatment.

1.3 Hypothesis

The chitosan exhibits high surface area and high stability to hold the drugs as a result, the synthesized nanodelivery systems are anticipated to improve the delivery efficiency as well as enhance the accumulation of dose of chemotherapeutic drugs (CBZ, SF and 5- FU) in cancer region. Thus, it could enhance the anticancer action on cancer cells with minimal side effects on healthy cells. Moreover, due to the properties of biodegradability and high drug loading ability, chitosan-based nanocarriers could be a good option to deliver a chemotherapeutic drug to the liver cancer region and cut down the interactivities between drugs with normal healthy tissues during its circulation.

1.4 Scopes of Study

Despite significant achievements that have been made in treating cancers, resistance to chemotherapeutic drugs remains to be a major challenge and responsible for most relapses as well as the high mortality rate in cancer. Furthermore, systemic administration of MDIs and MTIs may potentially lead to systemic toxicity. As a result, this study is aimed to synthesize the nanocarriers based on ChNPs to load Cabozantinib, SF alone and in combination with 5-Fluorouracil. Also, to study the effect of four different concentrations (2.5, 5, 10, and 20 mg/mL) of crosslinking agent of sodium tripolyphosphate (TPP) on the yield, loading content, encapsulation efficiency, and the particle size in all systems. The synthesized ChNPs 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 profiles and encapsulation/loading efficacy were investigated by UV-VIS spectroscopy. Moreover, the toxicity and anticancer activity of the synthesized ChNPs will be examined via HepG2 cells (cancer cell line) and HDFa cells (normal cell line).

1.5 Significance of Study

This current study aims to synthesize anticancer drug-loaded ChNPs to increase the anticancer efficacy of the drug against liver cancer. ChNPs was formulated to be utilized as nanocarriers for chemotherapeutic drugs owing to their excellent solubility property a high dosage of the drug can be avoided to reduce the treatment impact on normal healthy cells, which eventually cut down the treatment cost. Furthermore, it is worth noting that the incorporation of ChNPs, along with dual drugs, has demonstrated a remarkable synergistic effect. This effect has resulted in a substantial enhancement in cell inhibition when compared to treatments that do not involve nanocarriers or only utilise a single drug.

1.6 Objective of Study

1.6.1 General Objective

The main objective of this work is to synthesize and characterize MTIs (CBZ-ChNPs and SF-ChNPs) and MDIs (CBZ/SF-ChNPs, CBZ/5-FU-ChNPs and SF/SF-ChNPs.

1.6.2 Specific Objectives

1. To prepare and characterize the five anti-cancer of chitosan-based drug delivery system - CBZ-ChNPs and SF-ChNPs, CBZ/SF-ChNPs, CBZ/5-FU-ChNPs and SF/SF-ChNPs using different analytical techniques.
2. To determine the optimum concentration of the crosslinking agent, (sodium tripolyphosphate, TPP) and analyze their loading content and encapsulation efficiency.
3. To examine the *In Vitro* drug release profiles of Cabozantinib, Sorafenib and Fluorouracil from their ChNPs.
4. To evaluate *In Vitro* cytotoxicity of the synthesized nanoparticles on HepG2 cells (cancer cell line) and HDFa cells (normal cell line) via the MTT assay.

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