HYDROPHOBICALLY MODIFIED PEGYLATED CHITOSAN DERIVATIVE: SYNTHESIS, CHARACTERISATION AND FORMULATION OF NANOPARTICLES

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2021

HYDROPHOBICALLY MODIFIED PEGYLATED CHITOSAN DERIVATIVE: SYNTHESIS, CHARACTERISATION AND FORMULATION OF NANOPARTICLES

by

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Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

May 2021

ACKNOWLEDGEMENT

First and foremost, I would like to owe my sincere acknowledgement to my supervisor, Dr. Erazuliana Abd Kadir for her precious guidance, support and patience throughout my study. It was a great opportunity to learn valuable knowledge under her supervision. I am thankful to my co-supervisor, Dr. Lim Vuanghao for his advice and support in my research project.

I would also like to express my gratitude to the staffs in the lab of Integrative Medicine Cluster and Oncology Cluster in Animal Research Centre and IPPT, USM for all types of help and technical assistance. I feel grateful to have my colleagues with their encouragement, guidance and help in my study.

A great appreciation to the USM Fellowship for providing the financial support to pursue my Master's degree and thanks to Universiti Sains Malaysia for providing Short Term Grant (304.CIPPT.6315098) for this project.

Lastly, I would like to express my gratitude to my parents who support and encourage me continuously and thank you for their understanding throughout these years.

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LIST OF SYMBOLS

%	Percentage
<	Less than
>	Greater than
±	Plus-minus sign
°	Degree
° C	Degree Celsius
µg/mL	Microgram per millilitre
µL	Microlitre
µM	Micromolar
µm	Micrometre
^{1}H	Proton
cm^{-1}	Reciprocal centimetre
cm^{2}	Centimetre square
g	Gravitational force
g	Gram
kDa	Kilodalton
kV	Kilovolt
L	Litre
M	Molarity
mg	Milligram
mg/mL	Milligram per millilitre
MHz	Megahertz
mL	Millilitre
mL/min	Millilitre per minute
mm	Millimetre

mV	Millivolt
rpm	Revolutions per minute
v/v	Volume per volume
w/v	Weight per volume
α	Alpha
β	Beta
δ	Delta

LIST OF ABBREVIATIONS

%CV	Percentage of coefficient of variation
%RE	Percentage of relative error
ANOVA	One-way analysis of variance
BCS	Biopharmaceutical Classification System
CD_3OD	Deuterated methanol
CMC	Critical micellar concentration
CO_2	Carbon dioxide
D_2O	Deuterated water
DE	Drug entrapment
DDS	Drug delivery system
dGC	Degraded glycol chitosan
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
EPR	Enhanced permeation retention
FBS	Fetal bovine serum
FTIR	Fourier transform infrared spectroscopy
GC	Glycol chitosan
GC-PEG	Glycol chitosan grafted with poly(ethylene) glycol
GIT	Gastrointestinal tract
Hh	Hedgehog
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
ITZ	Itraconazole
LOD	Limit of detection

LOQ	Limit of quantification
mPEG	Methoxypoly (ethylene glycol)
mPEG-ss	Methoxypoly (ethylene glycol) succinimidyl succinate
MPS	Mononuclear phagocytic system
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MWCO	Molecular weight cut-off
NEs	Nanoemulsions
NMR	Nuclear magnetic resonance
NP	Nanoparticle
O/W	Oil-in-water
P%	Level of palmitoylation
PBS	Phosphate buffer saline
PDI	Polydispersity
PEG%	Level of PEGylation
PGC-PEG	Palmitoylated glycol chitosan grafted with poly(ethylene) glycol
PMs	Polymeric micelles
PNS	Palmitic acid N-hydroxysuccinimide ester
PTX	Paclitaxel
QC	Quality control
RPMI	Roswell Park Memorial Institute Medium
RT	Room temperature
SD	Standard deviation
SGF	Stimulated gastric fluid
SIF	Stimulated intestinal fluid
TEM	Transmission electron microscope
UV-Vis	Ultraviolet Visible
W/O	Water-in-oil

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Appendix B ^{1}H-NMR spectrum of PGC-PEG

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BAHAN TERBITAN KITOSAN DENGAN PEG TERUBAHSUAI HIDROFOBIK: SINTESIS, PENCIRIAN DAN FORMULASI NANOPARTIKEL

ABSTRAK

Ubat bersifat hidrofobik menghadapi masalah seperti keterlarutan rendah, degradasi dalam persekitaran biologikal dan ketidaktepatan penyasaran ubat terhadap sel sasaran. Masalah ini mengakibatkan kekurangan kecekapan penyampaian drug dan merendahkan kesan terapi. Kajian tentang muatan drug dalam nanopartikel untuk meningkatkan kelarutan dan ketepatan penghantaran drug hidrofobik telah dijalankan untuk menambahbaikkan perubatan. Glikol kitosan (GC) merupakan bahan serba guna sebagai ‘nanocarrier’ memandangkan strukturnya yang boleh diubah kepada pelbagai terbitan, mempunyai sifat keterlarutan tinggi dalam julat pH yang besar, biokompatibel dan tidak bertoksik. Itraconazole (ITZ) boleh digunakan semula sebagai drug kemoterapi memandangkan ia menghalang pertumbuhan pelbagai jenis sel kanser. Tujuan kajian ini adalah membangunkan polimer GC yang dipalmitoylasi dan dicantumkan dengan PEG (PGC-PEG) dan memformulasikan polimer terubahsuai itu dengan ITZ kepada formulasi nanopartikel yang stabil. PGC-PEG dihasilkan melalui langkah-langkah berterusan melibatkan degradasi asid, PEGilasi dan palmitoylasi. Pencirian beberapa kumpulan polimer PGC-PEG mendapati polimer dengan 3.3% PEG dan 59% PNS dengan kepekatan misel kritikal (CMC) 0.063 mg/mL sesuai untuk diformulasi dengan ITZ. Formulasi PGC-PEG dengan ITZ (PGC-PEG-ITZ) menghasilkan homogen bercas positif iaitu berbentuk polimerik misel (PGC-PEG-ITZ-PM) dan nanoemulsi (PGC-PEG-ITZ-NE). PGC-PEG-ITZ-NE. Pada nisbah 1:10, PGC-PEG-ITZ-NE menunjukkan pemerangkapan drug yang lebih tinggi

(±80%) berbanding pemerangkapan oleh 1:10 PGC-PEG-ITZ-PM (±40%). Purata saiz partikel bagi 1:10 PGC-PEG-ITZ-PM dan PGC-PEG-ITZ-NE ialah 152-155 nm dan 575-590 nm masing-masing. Berdasarkan analisis mikroscopi elektron transmisi (TEM), nanopartikel dilihat berbentuk sfera. PGC-PEG-ITZ-NE bernisbah 1:10 dan PGC-PEG-ITZ-PM bernisbah 1:10 menunjukkan perlepasan ITZ terkumpul masing-masing sebanyak 93% and 89% dalam masa 72 jam. Kedua-dua formulasi menunjukkan tiada perubahan signifikan dalam pemerangkapan drug, saiz partikel dan kepoliserakan sepanjang 8 hari pada suhu bilik, malah menunjukkan kestabilan yang lebih baik dalam cecair gastrik terangsang (SGF) dan cecair usus terangsang (SIF) berbanding ITZ sahaja untuk tempoh 24 jam. Kedua-dua formulasi juga menunjukkan kesan sitotoksik *in vitro* terhadap sel kanser MCF-7 dengan PGC-PEG-NE (1.6 μM (24 jam) and 1.4 μM (48 jam)). Kesimpulannya, polimer PGC-PEG terubahsuai secara hidrofobik adalah ‘nanocarrier’ untuk formulasi nanopartikel dengan ITZ. Formulasi PGC-PEG-ITZ juga melindungi drug daripada degradasi dan menambahbaik aktiviti terapeutik ITZ terhadap sel kanser.

HYDROPHOBICALLY MODIFIED PEGYALTED CHITOSAN DERIVATIVE: SYNTHESIS, CHARACTERISATION AND FORMULATION OF NANOPARTICLES

ABSTRACT

Hydrophobic drugs encounter limitations such as poor solubility, vulnerable to degradation in biological environment and lack of selectivity towards targeted cells thus reduced the drug delivery and therapeutic efficacy. Drug-loaded nanoparticles (NPs) are widely studied for their potential to improve the conventional medication by increasing the drug solubility and targeting of the diseased sites. Glycol chitosan (GC) is a versatile material as a nanocarrier as its structure can be modified, soluble in wide range of pHs, biocompatible and non-toxic. Itraconazole (ITZ) can be repurposed into an anticancer drug as it is capable of inhibiting growth of several types of cancer cells. The aim of this study was to develop palmitoylated GC polymer grafted with poly(ethylene) glycol (PEG) (PGC-PEG) and formulate the polymer with ITZ into stable NPs formulations. PGC-PEG was synthesised in a stepwise manner involving acid degradation, PEGylation and palmitoylation. The characterisation of several batches of PGC-PEG revealed polymer with 3.3% PEGylation and 59% palmitoylation with critical micelle concentration (CMC) value of 0.063 mg/mL as suitable to be incorporated with ITZ. The incorporation of PGC-PEG with ITZ (PGC-PEG-ITZ) produced homogenous positively charged formulations of polymeric micelles (PGC-PEG-ITZ-PM) and nanoemulsions (PGC-PEG-ITZ-NE). At drug to polymer ratio of 1:10, PGC-PEG-ITZ-NE showed higher drug entrapment (±80%) compared to that of 1:10 PGC-PEG-ITZ-PM (±40%). The average particle size of 1:10 PGC-PEG-ITZ-PM and PGC-PEG-ITZ-NE was 152-155 nm and 575-590 nm, respectively. From

transmission electron microscope (TEM) analysis, the NPs were found in spherical shape. The 1:10 PGC-PEG-ITZ-NE and 1:10 PGC-PEG-ITZ-PM demonstrated cumulative ITZ release of 93% and 89%, respectively upon 72 hours period. The drug entrapment, particle size, and size polydispersity of both formulations showed no significant changes at room temperature up to 8 days, as well as better stability in stimulated gastric fluid (SGF) and stimulated intestinal fluid (SIF) compared to the naked ITZ for up to 24 hours period. Both formulations also showed *in vitro* cytotoxic effect against the MCF-7 cancer cell lines, with PGC-PEG-ITZ-NE (1.6 µM (24 hours) and 1.4 µM (48 hours)) showing lower IC_{50} compared to PGC-PEG-ITZ-PM values (2.8 µM (24 hours) and 2.3 µM (48 hours)). In conclusion, the hydrophobically modified PGC-PEG polymer was a suitable nanocarrier for nanoparticle (NPs) formulation of ITZ. The PGC-PEG-ITZ formulations also protected the drug from degradation and improved the therapeutic activity of ITZ against the cancerous cells.

CHAPTER 1

INTRODUCTION

1.1 Research background

Oral administration is the predominant route of administration due to its ease of administration, low cost and high patient compliance. When a drug is orally administrated, the drug has to be absorbed in the gastrointestinal (GIT) fluids before it reaches the targeted site. About 40% of the existing drugs in conventional drug delivery system (DDS) and 90% of the active ingredients in development pipeline are poorly aqueous soluble. Many new and existing drugs are categorised as Biopharmaceutical Classification System (BCS) class II and IV due to their poor solubility in aqueous solutions (Pouton, 2006).

The common issue faced in the conventional drug design is the limited solubility of drugs. The therapeutic efficacy of a drug depends upon its bioavailability which is correlated to its solubility, dissolution rate and gastrointestinal permeability. A drug with low water solubility is also being absorbed slowly in the gut (e.g. BCS class IV drugs) leading to inadequate therapeutic concentrations reaching the blood circulation to evoke pharmacological response (Kawabata et al., 2011).

In recent decades, nanotechnology has gained attention for its application in medical field, in particular for the cancer treatment. The use of NPs as nanocarriers has been a promising approach to improve the bioavailability of hydrophobic drugs and enhance the treatment efficacy by overcoming the biological barriers. Moreover, NPs can be designed into desired size with unique surface characteristics to maximise the drug delivered to the specific site, as well as having high loading capability and

stability in biological environment for better performance in drug delivery (Tran et al., 2020).

There are different types of NPs that have been used in the cancer, gene and protein therapeutics, such as liposomes, polymeric micelles (PMs), nanoemulsions (NEs), solid lipid NPs and gold NPs (Tran et al., 2020, Conti et al., 2006, Dadwal et al., 2018). Different types of NPs have their own characteristics and structures that suit the need in DDS. The use of polymers as NPs in DDS offers flexibility in designing the surface of the NPs for modulation of the physicochemical properties such as size and surface charge (Alexis et al., 2008). Carbohydrates and protein-based compounds are some of the natural polymers that can be degraded by enzymes into metabolised polysaccharides and peptides (Nair & Laurencin, 2007).

GC is a type of natural polymer derived from chitosan that can be utilised to form NP. GC backbone with free amine and hydroxyl group can be functionalised to have specific behaviour which helped in the formation of NPs (Lee et al., 2014). GC-based polymer is capable to self-assemble into amphiphilic NPs upon modification of the backbone structure to have hydrophobic and hydrophilic regions. The structure of the amphiphilic nanoparticle is commonly comprised of hydrophobic core and hydrophilic shell. Hydrophobic drug can be loaded into the hydrophobic core thus increasing the drug solubility and retention time in blood circulation (Kim et al., 2017).

Modifications of the NPs' core structure or surface could enhance the drug loading capability and stability of the NPs. Hydrophobic modifications of the polymer could improve the hydrophobic drug loading capacity into the NPs. Various hydrophobic materials have been used for hydrophobic modifications of GC which

include palmitic acid (Uchegbu et al., 2001), α-tocopherol (Duhem et al., 2012), 5β-cholanic acid (Kim et al., 2006, Kwon et al., 2003) and deoxycholic acid (Kim et al., 2005).

Polyethylene glycol (PEG) is approved by FDA for the use in the pharmaceuticals (Casettari et al., 2012). Studies have proven that PEG has the potential to be incorporated into NPs for a stable DDS. Grafting of PEG on the surface of the NPs could prolong the NP blood circulation time as PEG chain can reduce the interaction of the NPs with plasma protein (Parveen & Sahoo, 2011) and phagocytotic cells (Amoozgar & Yeo, 2012). Attachment of the PEG chain on the NPs also enhanced the drug release from the NP formulation (Zhang et al., 2015).

Itraconazole (ITZ), a common antifungal drug has the potential to be repurposed into an anticancer drug (Pace et al., 2016). ITZ exhibits anti-angiogenic activity (Chong et al., 2007), induces Hedgehog pathway and inhibits autophagic growth (Pantziarka et al., 2015, Ueda et al., 2017). The drug's chemotherapeutic efficacy has been tested in clinical trials and showed prominent anticancer effects on patients with prostate, lung and breast cancer (Pantziarka et al., 2015). ITZ has the promising potential to be repurposed into a chemotherapeutic agent as its pharmacokinetic and toxicological profiles are well-known. The drug is however highly hydrophobic and being less absorbed in the gut upon oral administration. This might prevent its use as a potent chemotherapeutic drug as high concentration level in blood circulation might be needed to meet the therapeutic requirement for cancer elimination. Formulation of the ITZ into NPs might improve absorption in the GIT

upon oral consumption thus increases its bioavailability and exposure to the cancerous target tissues.

The purpose of this project is to synthesise GC-based polymer involving hydrophobic modification and PEGylation. The novel PEGylated GC-based polymer (PGC-PEG) was formulated with ITZ into NP formulations (PGC-PEG-ITZ). The formulations were then characterised and subjected for the analysis of drug release profile, stability and cytotoxicity against breast cancer cell lines.

1.2 Objectives

The main objective of this study is to develop a novel NP formulation based on PGC-PEG polymer with the model hydrophobic drug of ITZ. The NP formulations were aimed to have nano-scale particle size and high drug loading, as well as possessing sustained drug release, stable upon storage and in biological environment, and capable of exerting cytotoxic effect against cancerous cells.

The specific objectives are as follows:

a) To synthesise PGC-PEG polymer using stepwise protocol of PEGylation and palmitoylation of GC
b) To characterise the physicochemical properties of PGC-PEG polymer using H-NMR, FTIR and UV-Vis spectrometer
c) To formulate PGC-PEG with ITZ into a stable PGC-PEG-ITZ NP formulation
d) To evaluate the drug release profile of PGC-PEG-ITZ via dialysis method
e) To determine the stability of PGC-PEG-ITZ upon storage at different temperatures and in biological fluids

f) To determine the *in vitro* cytotoxicity of PGC-PEG-ITZ on the breast cancer cells MCF-7

CHAPTER 2

LITERATURE REVIEW

2.1 Current issues with hydrophobic drugs

Combinatorial chemistry and high-throughput screening have shown that the number of hydrophobic drugs has significantly increased in drug discovery (Lipinski et al., 2001). Many new chemical entities (NCEs) are found to have poor aqueous solubility in recent years. It has been reported that 30- 40% of drugs are poorly water soluble and less than 10% of the drug candidates showed high permeability and high solubility on the WHO Essential Drug List (Williams et al., 2013).

The water solubility of a drug is a factor to determine its dissolution rate. The low dissolution rate results in the low bioavailability of orally administrated drugs, variations in pharmacokinetics and rapid metabolism (Kalepu & Nekkanti, 2015). The fact that most of the existing and new entities of drugs are categorised as either BCS Class II (low solubility and high permeability) or BCS Class IV (low solubility and low permeability) has created problems in drug research and development (Xu et al., 2013).

Poor GIT permeability of drugs affects the oral bioavailability as there is less drug dissolved in the GIT environment and being absorbed due to the less permeability through gut wall. The low pH environment and presence of different enzymes such as lipases, protease and pancreatin in the stomach and intestine, respectively, degrades the drugs before it reached the targeted sites (Homayun et al., 2019) thus could affect the efficacy of oral drug delivery.

The therapeutic efficacy of drugs depends on its physiochemical properties and the biological barriers the drug needs to encounter upon oral administration. Only 5-20% of chemotherapeutic drugs such as paclitaxel, docetaxel, doxorubicin and tamoxifen are bioavailable in the blood circulation for pharmacological response, which could be contributed by the low solubility and poor intestinal permeability, as well as the activity of P-glycoprotein (P-gp) efflux of the intestinal cells (Thanki et al., 2013). P-gp is the multidrug resistance protein which effluxes the therapeutic drugs out of the cells and reduces the accessibility of the drug to the targeted site (Silva et al., 2015).

2.2 Nanotechnology in drug delivery

The use of nanotechnology in medical field is quite common which involved applications in pharmaceuticals, molecular imaging, and cancer treatments. Nano-based drug delivery involved the use of nanoscale materials that can be made of different kind materials, such as natural or synthetic polymers, lipids or metals (Suri et al., 2007). NPs that can be loaded or bounded with drugs are also known as nanocarrier. Nanocarriers existed in various forms, such as PMs, liposomes, solid lipid NPs, polymeric NPs and NEs (Cho et al., 2008).

Nano-sized particles are developed to improve the solubility of hydrophobic drugs and to facilitate their efficacy upon oral delivery. NPs act as reservoir for the encapsulated hydrophobic drugs to protect them from degradation in GIT and also controlled the drug from being released rapidly into the blood circulation (Yoo et al., 2010). NPs increase the surface area and surface interactions on the targeted site thus enhances the rate of dissolution and controlled the bioavailability activity of the drugs (Merisko-Liversidge & Liversidge, 2008).

Application of NPs in DDS allows more effective therapeutic activity in the way of improving the solubility, bioavailability and controlled release of the drug (Patra et al., 2018). Surface modifications prolong the half-life of NPs by preventing the non-specific binding of the phagocytes to the NPs (Yetisgin & Cetinel, 2020). Some of the FDA-approved anticancer drugs that had been reformulated into nano-based formulation are currently available in the market which include the Abraxane® and Genexol-PM® for paclitaxel; and Doxil®, Myocet® and Lipo-Dox® for doxorubicin (von Roemeling et al., 2017, Bor et al., 2019).

2.3 Glycol chitosan (GC)

Chitosan-based NPs is one of the polysaccharide-based polymers that have been widely utilised to create NP-drug formulations. Chitosan is produced from chitin, the main component in the shell of crustaceans such as crabs, shrimps and lobsters. Chitosan is formed through the alkaline deacetylation or enzymatic degradation of chitin to form the structure of β-1,4-linked D-glucosamine (Figure 2.1) with random number of N-acetylglucosamine groups depending on the degree of acetylation.

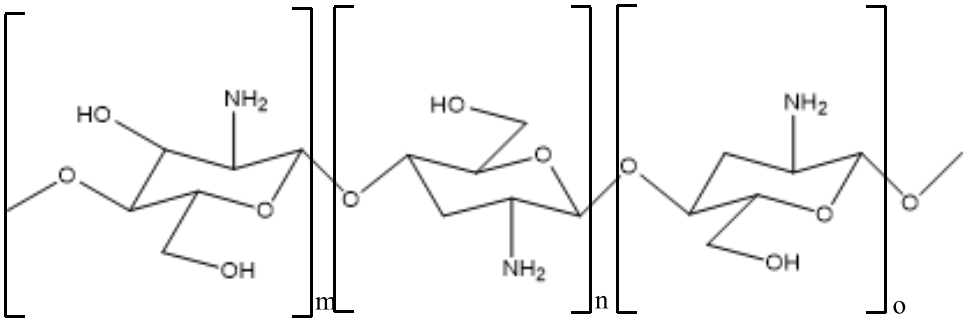

Figure 2.1: Chemical structure of chitosan.

It is a unique substance as it allows chemical modifications on the reactive amine group to form a wide range of chitosan derivatives. The chitosan derivatives are widely used in the formulation of targeted drug delivery system. Apart from its biocompatibility with the microenvironment in the human body, chitosan NPs are also non-toxic (Ould-Ouali et al., 2004), non-carcinogenic (Karlsson et al., 2018) and enzymatically biodegradable (Wang et al., 2011). Chitosan is also permeable through the columnar epithelial layers which aided the chitosan-based material to be absorbed through the gut wall thus enabling drug delivery into the targeted tumour cells (Vivek et al., 2013).

GC (Figure 2.2) is one of the chitosan derivatives that has hydrophilic ethylene glycol (EG) group on its sugar backbone (Ghaz-Jahanian et al., 2015). The EG group contributes to the steric stabilisation and good solubility of GC in broad range of pHs (Trapani et al., 2009). It is biodegradable, biocompatible and non-toxic towards the healthy tissues (Chooi et al., 2014). The free amine and hydroxyl groups at the GC backbone have been mostly utilised for further chemical modifications of the polymer to suit the need of the intended NPs (Kim et al., 2017). In recent years, more attention was directed towards the use of GC as a drug carrier or vehicle in drug delivery. Like chitosan, the use of GC as nanocarriers are also due to its biodegradability and low immunogenicity in human.

GC could be modified into self-assembled NPs with a hydrophobic core surrounded by hydrophilic shell in aqueous media. The hydrophobic core serves as the reservoir for hydrophobic drugs which help to enhance the drug solubility as well as delivery to the targeted sites. Studies reported that several designs of GC as nanocarrier

have been incorporated with different chemotherapeutic drugs such as paclitaxel (Kim et al., 2006, Saravanakumar et al., 2009), doxorubicin (Hyung Park et al., 2006, Hwang et al., 2008), docetaxel (Feng et al., 2019) and camptothecin (Min et al., 2008).

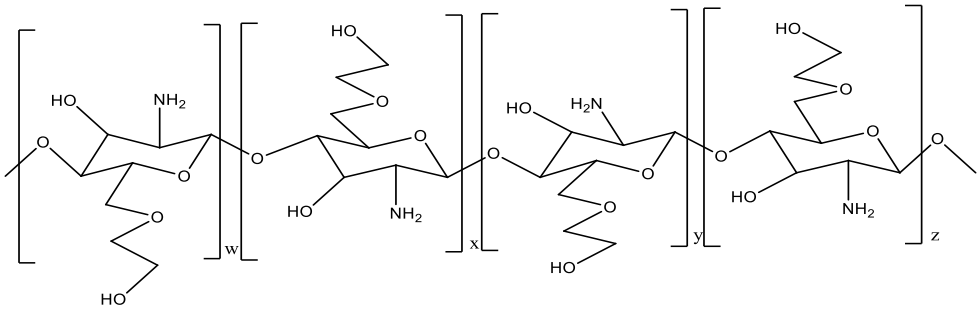

Figure 2.2: Chemical structure of glycol chitosan (GC).

2.4 Chemical modifications on the GC backbone

For the purpose of improving the delivery of hydrophobic drugs, the GC polymer had been modified by the attachment of hydrophobic moiety to the GC sugar backbones, particularly to the free amine groups. This leads to the formation of amphiphilic nanoparticles that are capable of self-assemble in aqueous solution. The amphiphiles, which can be PMs, tubules or vesicles, form a structure of outer hydrophilic shell and inner hydrophobic core. The presence of the hydrophobic moiety at the core of the amphiphiles attracts the hydrophobic drugs to reside at the inner part of the NPs structure. This allows the amphiphilic GC NPs to encapsulate and protect the hydrophobic drugs and later carrying the drug load to the intended sites.

GC polymer had been conjugated with different structures including 5β-cholanic acid (Kwon et al., 2003), deoxycholic acid (Kim et al., 2005), palmitic acid (Uchegbu et al., 2001) and tocopherol succinate (Duhem et al., 2012) to create an effective hydrophobic domain on the polymer chain. These attachment of the

hydrophobic moieties to the GC enables the self-assemble behaviour and forming amphiphilic NPs at concentration above their critical micelle concentration (CMC).

2.4.1 PEGylation

Poly(ethylene) glycol (PEG) is hydrophilic, water-soluble, biodegradable and non-toxic polymer that has the property of steric hindrance to avoid the uptake by the phagocytic immune cells (Cho et al., 2012). PEGylation is the process of conjugation of PEG to the structure of polymer. PEG attached to the nanoparticles is capable to prolong the circulation time of the NPs, by evasion from the host immune system (Turecek et al., 2016). Methoxy-poly(ethylene) glycol (mPEG) is a FDA-approved component which extensively used in the pharmaceutical formulations to prevent crosslinking of the polymers (Casettari et al., 2012).

NPs are prone to be eliminated from the blood circulation by the activity of mononuclear phagocyte system (MPS) or also known as reticuloendothelial system (RES). MPS is composed of cells such as monocytes and macrophages from the liver, spleen and lymph nodes which are involved in eliminating, clearing and degrading foreign substances in the blood circulation (Amoozgar & Yeo, 2012). Modification of the NP surface could prolong the life-span of NPs in the bloodstream (Suk et al., 2016). PEGylation on the surface of NPs neutralised the surface charges hence blocking the electrostatic and hydrophobic attractions of opsonin from binding to the surface of the NPs (Mozar & Chowdhury, 2018).

Most widely used method is by covalently attaching PEG chains to the particle surface to manipulate PEG's high hydrophilicity and neutral charge in reducing detection by the MPS. NPs modified with PEG had been reported to have the lowest

percentage of uptake by macrophages (Parveen & Sahoo, 2011). Gref et al. (2000) reported that PEG acted as “brush” for shielding the NPs from the MPS at PEG concentration above 5%.

Molecular weight and surface density of mPEG on the NPs are the key factors in altering the pharmacokinetics parameters such as absorption, distribution, metabolism, excretion and biodistribution of the NPs (Ait Bachir et al., 2018). Chen et al. (2014) reported that mPEG (2kDa) that was conjugated to chitosan NPs and loaded with methotrexate had an improved blood circulation time and targeting ability to the tumour tissues compared to folic acid (FA)-conjugated NPs. Yang et al. (2008) described that chitosan NPs grafted with high molecular weight and low surface density PEG had smaller and more compact NPs which might related to the avoidance of the NPs from the phagocytosis by the macrophages and unspecific erythrocytes interaction.

2.4.2 Palmitoylation

The attachment of the palmitic acid chains (palmitoylation) to the GC backbones was able to create hydrophobic moieties on the polymer structure (Uchegbu et al., 2001). The conjugation of the hydrophobic 16-carbon palmitic chains to the amino groups on the sugar chain of GC involved the reaction with palmitic acid N-hydroxysuccinimide (PNS). Palmitoylation of GC enables the polymer to self-assemble into NPs with high loading capacity to facilitate the transport of hydrophobic drugs through the biological barriers thus enhanced bioavailability of the drugs (TM & Lau, 2018).

2.5 Itraconazole (ITZ) as anticancer agent

Repurposing the Food and Drug Administrative (FDA)-approved drugs is one of the strategies to expedite the drug discovery process. Repurposing could cut the high cost in producing a new active pharmaceutical agent for human consumption by avoiding the lengthy years needed for clinical trials. Itraconazole (ITZ) has the promising potential to be repurposed into a chemotherapeutic agent as its pharmacokinetic and toxicological profiles are well-known. ITZ (Figure 2.3) is a broad-spectrum antifungal drug that was developed in the 1980s (Tsubamoto et al., 2017) for the treatment of fungal infections including aspergillosis, candidiasis, blastomycosis and cryptococcosis. The drug acts by inhibiting cytochrome-P450 dependent enzyme lanosterol 14-α-demethylase which resulted in the decrease of ergosterol synthesis (De Beule, 1996). Reduction in production of ergosterol resulted in the destruction of fungal membrane. This drug can be used for a long-term medication treatment of chronic fungal infections as it has low toxicity profile (Tsubamoto et al., 2017).

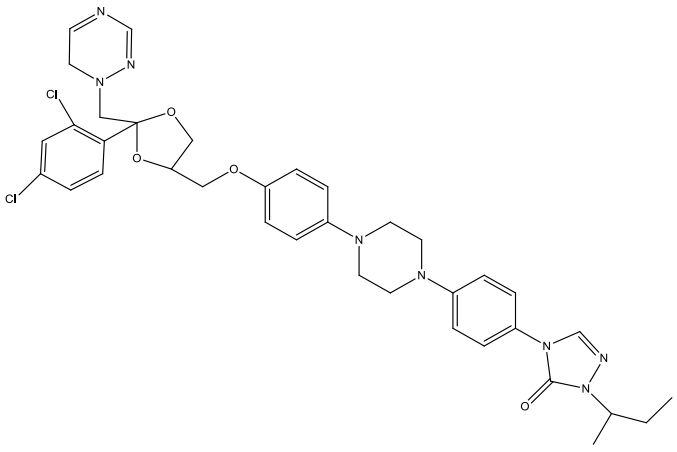

Figure 2.3: Chemical structure of itraconazole (ITZ).

In 2007, FDA approved ITZ as an anticancer chemotherapeutic agent and later in 2010 as a Hedgehog (Hh) signalling inhibitor (Pace et al., 2016, Tsubamoto et al., 2017). ITZ was found to suppress the growth of murine basal cell carcinoma via inhibition of the Hh pathway (Kim et al., 2010) and blocking the activity of breast cancer resistance protein (BCRP) (Gupta et al., 2007).

Hh pathway is always inactive in adult tissues and its improper activity could cause development of several types of tumours. In the inactive state (Figure 2.4a), smoothened receptor (SMO), a seven-pass transmembrane protein is suppressed by Patched1 (PTCH1), a twelve-pass transmembrane protein. Suppression of the SMO dephosphorylate glioma-associated oncogene homolog (GLI) and GLI forms a complex with kinesin protein (Kif7) and suppressor of fused (SUFU). Thus, GLI inhibits the transcription of the targeted genes including Bcl-2 (Wei et al., 2020), mammalian target of rapamycin (mTOR) signalling and vascular endothelial growth factor (VEGF) (Li et al., 2019).

When Hh ligands increased and overexpressed due to external environment or certain factors, the Hh pathway will be activated (Figure 2.4b). The ligands attached to the PTCH1 activated the SMO. Phosphorylation of GLI dissociated from the SUFU and activated the signalling cascade of the GLI. GLI localized into nucleus and it binds with DNA and regulates the gene transcription (Pounds et al., 2017). Uncontrolled activation of Hh pathway can lead to the development of cancer in human body.

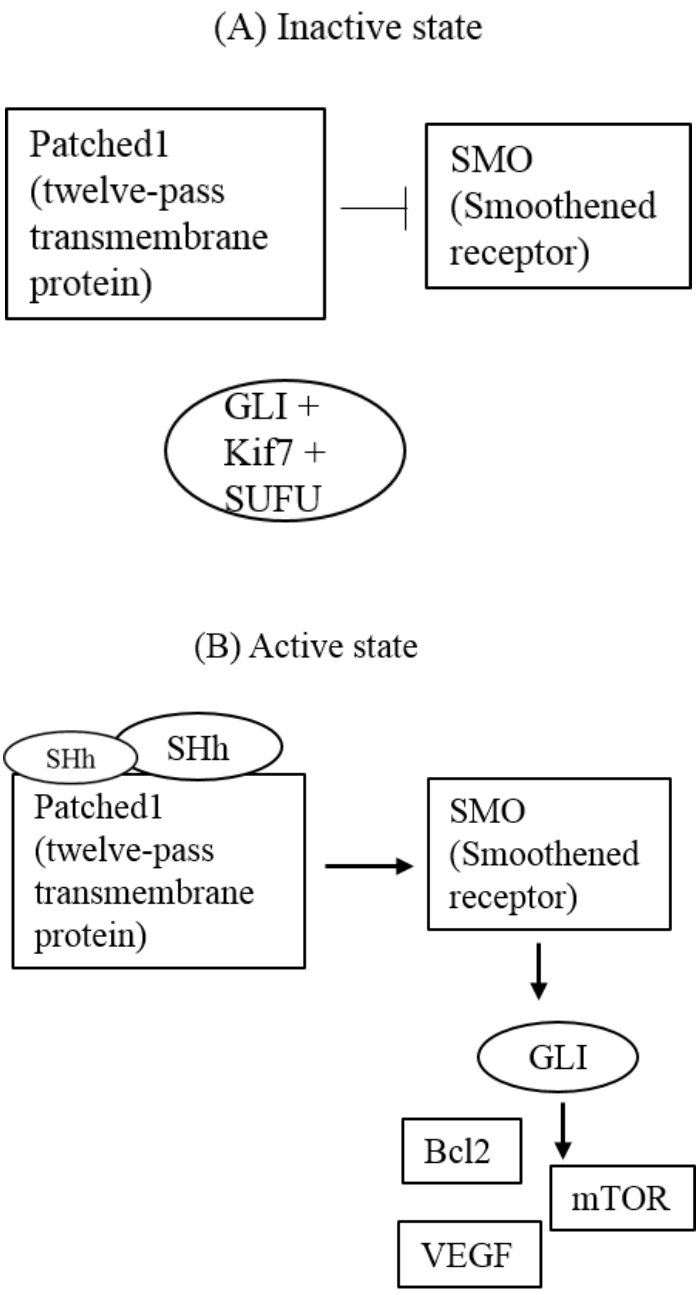

Figure 2.4: Hedgehog pathway in (a) inactive state and (b) active state.

Studies reported that ITZ induced apoptosis, autophagy (Liu et al., 2014), cell cycle arrest and inhibition of angiogenesis (Head et al., 2017, Chong et al., 2007). All these reported properties had been involved in inhibiting the growth of different types of cancer such as colorectal cancer (Popova & Buczacki, 2018), basal cell carcinoma (Kim et al., 2018), breast cancer (Wang et al., 2017) and gastric cancer (Hu et al.,

2017). Clinical data also showed that ITZ possesses an anticancer effect on basal cell carcinoma (Kim et al., 2014), prostate cancer (Antonarakis et al., 2013), triple-negative breast cancer (Tsubamoto et al., 2014a) and non-small cell lung cancer (Liang et al., 2017). Various anticancer mechanisms of ITZ have been studied thoroughly in both *in vitro* and *in vivo* models, as well as in clinical trials. Table 2.1 summarises the anticancer properties of ITZ.

ITZ is a highly lipophilic drug with a log P value of 5.66 and pK_a value of 3.7 which can only be solubilised in water at low pH values (Buchanan et al., 2007). It has poor aqueous solubility which is approximately 1 ng/mL at pH 7 and 4 ng/mL at pH 1. Low dissolution rate of ITZ affects the GIT absorption process which in turn reduces the therapeutic effect of the drug (Domínguez-Gil Hurlé et al., 2006). Formulation of ITZ into NPs might enhance absorption and bioavailability of ITZ in the GIT upon oral consumption.

Table 2.1: Properties of ITZ reported in the literature.

Activity	Mechanism of Action	Experiment Model	Publication
Inhibition of Hedgehog pathway	Reduces accumulation of SMO; inhibits transcription factor protein GLI; inhibit proliferation; induces G0/G1 cell cycle arrest	Breast cancer cell, basal cell carcinoma cell, gastric cancer cell	Tsubamoto et al., (2014a); Hara et al., (2016); Wahid et al., (2016); Hu et al., (2017); Wang et al., (2017)
Inhibition of angiogenesis	Inhibits vascular endothelial growth factor (VEGF) signalling	HUVEC, non-small cell lung cancer cell, prostate cancer cell	Chong et al., (2007); Aftab et al., (2011); Antonarakis et al., (2013) Rudin et al., (2013); Head et al., (2015); Liang et al., (2017); Alhakamy & Md., (2019)
Autophagy	Inhibits mammalian target of rapamycin (mTOR) and WNT/β-catenin pathway	HUVEC, epithelial ovarian cancer cell, cervical cancer cell	Chong et al., (2007); Tsubamoto et al., (2014b); Tsubamoto el al., (2014c); Pace et al., (2016); Choi et al., (2017); Liang et al., (2017); Ueda et al., (2017)
Multi drug resistance reversal	Prevents ATP-binding cassette transports, reduces the function of drug efflux protein such as P-glycoprotein and breast cancer resistance protein	Breast cancer cell	Gupta et al., (2007)

2.6 Formulation of ITZ

NPs formulations have been developed to enhance the efficacy of hydrophobic DDS. The NP formulations of hydrophobic drugs bring several advantages over the conventional DDS. With NPs, bioavailability of the hydrophobic drugs could be improved whilst the small size of NPs could reduce the chance of elimination by MPS. Small size of NPs also helps in penetration of the NPs through the biological membrane of the tumours thus increasing accumulation of the NPs around the cancer cell. The targeting feature of the NPs could also help in lowering the cytotoxicity towards healthy cells as the cells only act on the diseased cells.

2.6.1 PMs

PMs in DDS was first introduced by H. Ringsdorf in 1984 through the development of doxorubicin conjugated copolymer micelles (Kálal et al., 1978), followed by Kataoka's group in the early 1990s (Yokoyama et al., 1992) for the development of PMs formulation with doxorubicin, a hydrophobic anticancer drug. PMs are commonly known to have the structure of hydrophilic corona and hydrophobic core in the aqueous environment. Hydrophobic core entraps hydrophobic drugs, maintains the drug stability and controls the drug release profile (Gothwal et al., 2016), whereas hydrophilic core prevents PMs from the recognition of MPS hence improves the blood circulation time (Kedar et al., 2010).

Hydrophilic corona can be coated with polymers such as poly (N-vinyl pyrrolidone), poly(N-isopropylacrylamide), PEG and poly (ethylene oxide) (PEO) (Biswas et al., 2016, Kedar et al., 2010). PEG with a molecular weight of 2- 15 kDa is commonly used as the hydrophilic segment of PMs for the purpose of drug delivery

(Zhang et al., 2014). PEGylated PMs have long blood circulation time, thus increases the chance of PMs accumulation at the tumour target site (Lee & Yeo, 2015). The hydrophobic core of PMs can be contributed by polymers such as poly (ι-lactic acid) (PLA), poly(e-caprolactone) (PCL) and poly(beta-benzyl-ι-aspartate) (PBLA) (Kore et al., 2014).

Critical micellar concentration (CMC) is the concentration of polymer above which micelle formation is favoured (Gaucher et al., 2010). At the CMC, micelles started to form and minimised the contact with water molecules. The increased hydrophobic chain length resulted in lower CMC value while maintaining constant segment of hydrophilic part (Xu et al., 2013, Zhou et al., 2016a).

2.6.2 NEs

NEs which are also known as miniemulsions or submicron emulsions are dispersion of two immiscible liquids in the form of either oil-in-water (O/W) or water-in-oil (W/O) emulsions. The mean droplet size of NEs can be ranged from 100 to 500 nm (Singh et al., 2017). NEs commonly consist of oils, surfactants and aqueous phase. Different kinds of oil can be used in the NEs formulation including vegetables oils, mineral oils and triglycerides (Gonçalves et al., 2018). The oil phase can be selected based on the drug solubility in the oil for the development of the NEs (Qadir et al., 2016). Surfactants added in the formulation of NEs help to reduce the surface tensions between the oil and water (Lovelyn & Attama, 2011), as well as to prevent droplet aggregations (Singh et al., 2017). Surfactants such as polyoxyethylene sorbitan monolaurate (Tween 20, 40, 60 and 80) (Wang et al., 2008), polyoxyethylene-660-hydroxystearate (Solutol HS-15) (Scheller et al., 2014) and sorbitan laurate (Jadhav et al., 2015) are widely used for the NE formulations.

The attractive features of NEs as a robust nanocarrier including increase drug loading capacity and stability, enhanced bioavailability and protection, as well as controlled release of compounds. NEs provide protection for the hydrophobic drugs from hydrolysis and degradation when encapsulated in the oil phase. Hydrophobic compounds can be remained in the gastrointestinal tract for a longer time. In turn, NEs can enhance the absorption and efficacy of the hydrophobic compounds (Tayeb & Sainsbury, 2018).

As reported by Thakkar et al. (2015), the bioavailability of ITZ has been improved by the NEs DDS. The small-sized NE droplets have large surface area for higher dissolution rate of the drug, thus increased the drug absorption rate and improved the bioavailability of the drug. The NE enabled less amount of drug needed to reach the therapeutic drug concentration, thus minimising the side effects associated with the drug.

CHAPTER 3

METHODOLOGY

3.1 Materials

The chemical reagents, cell culture media and equipment utilised in this study with their manufacturers are listed in Table 3.1-3.3.

3.1.1 Chemicals

Table 3.1: List of chemicals and reagents used in the study.

Item	Manufacturer
Glycol chitosan	Sigma Aldrich, Saint Louis, MO, USA
Methoxypoly (ethylene glycol) succinimidyl succinate	
Palmitic acid N-hydroxysuccinimide ester	
Olive oil	
Corn oil	
Soybean oil	
Rapeseed oil from Brassica rapa	
Absolute ethanol, 99.8%, denatured	HmbG, Selangor, Malaysia
Hydrochloric acid, 37%, AR grade	
Glacial acetic acid	
Hexane, AR grade	
Potassium dihydrogen phosphate (KH_2PO_4)	R&M Chemicals, Essex, UK

Di-sodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$)	
Sodium bicarbonate	Bio Basic Canada Inc
Itraconazole	Acros Organic, China
Acetonitrile, HPLC grade	Fisher Scientific Korea Ltd, Korea
Methanol, HPLC grade	
Seamless cellulose dialysis tube (MWCO 3.5kDa, 5kDa)	

3.1.2 Cell culture reagents

Table 3.2: List of cell culture reagents used in the study.

Item	**Manufacturer**
Dimethyl sulfoxide (DMSO), ≥99.5%	R&M Chemicals, Essex, UK
Roswell Park Memorial Institute 1640 (RPMI)	Nacalai Tesque, Kyoto, Japan
Penicillin-streptomycin solution	Sigma Aldrich, Saint Louis, USA
3- (4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide (MTT)	
Fetal bovine serum (FBS)	Thermo Fisher Scientific, USA
Trypsin-EDTA (0.25% EDTA)	

3.1.3 Equipment

Table 3.3: List of equipment used in the study.

Item	Model	Manufacturer
Freeze dryer	Alpha 1-2 LD Plus	Martin Christ, Germany
Fourier transform infrared (FTIR) spectroscopy	Nexus 670	Thermo Nicolet, USA
Nuclear magnetic resonance (NMR) spectroscopy	Bruker AC 400	Bruker, Germany
Zetasizer	Nano ZS	Malvern Instruments, Worcestershine, USA
Transmission electron microscopy (TEM)	Libra 120	Carl Zeiss, Oberkochen, Germany
UV-Vis spectrophotometer	Varian Cary	Varian Australia Pty Ltd, Australia
pH meter	pH 1500 Cyberscan	Eutech Instruments, Singapore
Water bath with shaker	WNB 22	Memmert, Schwabach, Germany
Centrifuge	Heraeus Pico 17	Thermo Fisher Scientific, Singapore
Ultrasonic homogeniser	150V/T	Biologics, Inc, Virginia, USA
Weighing balance	AUW220D	Shimadzu, Tokyo, Japan

High Performance Liquid Chromatography (HPLC)	Agilent Technologies 1200 series	Santa Clara, CA, USA
Inverted microscope	Olympus CKX41	Olympus, Tokyo, Japan
Carbon dioxide (CO_2) incubator	Haraeus BB15	Thermo Fisher Scientific, Singapore
UV-Vis absorbance microplate reader	ELx808 Absorbance Reader	Biotek, Vermont, USA

3.2 Methods

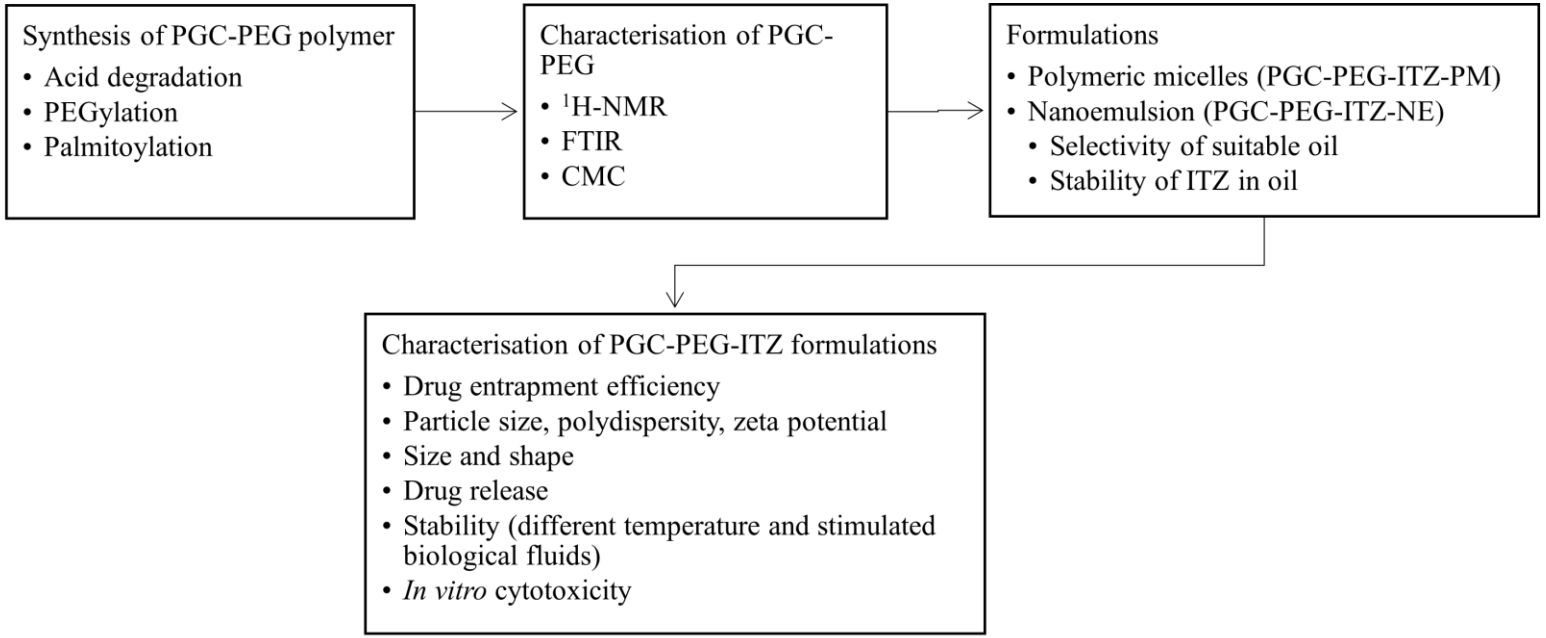

Figure 3.1: Overall flowchart of the research.