

Fabrication, Characterization and
Evaluation of β -elemene
Nanoparticles for
Anti-cancer Treatment

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Declaration

Unless otherwise acknowledged, the work presented in this thesis is my own. No part has been submitted for any other degree at The University of Nottingham or any other institution.

This MRes dissertation was conducted under supervision of:

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Prof. David Needem provided guidance at the beginning of the project.

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Abstract

Cancer is one of the leading causes of death worldwide, causing about 9.6 million deaths in 2018. Systemic chemotherapy is a widely used treatment against cancer. However, it is difficult for chemotherapeutic to reach the specific tumour tissues, which can result in some side effects in normal healthy tissues. With the development of nanotechnology in medical area, it becomes possible to deliver drugs to target region by using nanoparticles. Low-density lipoprotein is an excellent example of nanocarrier system for hydrophobic materials. According to some studies, LDL uptake by cancer cells are higher than normal cells.

β -elemene, extracted from the traditional Chinese medicinal herb Curcuma, has been used to effectively treat certain types of tumours in China. Due to the moderate anti-cancer activity and poor solubility in water of β -elemene, efficient delivery of β -elemene into tumour cells is a major challenge.

Herein, the objective of this project is to design, fabricate, characterize, optimize and evaluate a new targeting drug delivery system of β -elemene for cancer treatment.

Fabrication method used in this project is rapid solvent exchange method, which is rapid injection of β -elemene and POPC at 833 $\mu\text{L/s}$ in water under stirring (500 RPM). This method involved rapid injection using an automatic displacement syringe and stirring using magnetic stirrer.

POPC coated β -elemene NPs with diameters of ~ 27 nm were fabricated by using rapid solvent exchange method. The average hydrodynamic diameter and polydispersity index of NP was measured by using DLS and TEM. To improve the quality of NPs (in the form of more stable and smaller size), fabrication method was optimized. Furthermore, the cytotoxicity of POPC coated NPs was compared with pure β -elemene and uncoated β -elemene NPs. POPC coated NPs showed activity against breast cancer cell lines *in vitro* where the following IC_{50} values were obtained at $154\mu\text{M}$.

It was observed that the cellular uptake of nanoparticles depends on the time of incubation and the concentration of nanoparticles in the medium. The uptake of nanoparticles was rapid with confocal microscope demonstrating their localization mostly in the lysosome.

In summary, a novel POPC coated β -elemene NPs has been designed and fabricated demonstrating appropriate characteristics for intratumoral drug delivery.

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Table of Contents

Abstract.....	3
Acknowledgements	4
Table of Contents	5
List of Abbreviations.....	8
List of Figures	9
List of Tables	12
1 Introduction.....	13
1.1 Cancer.....	13
1.1.1 Cancer statistics	14
1.1.2 Treatment of cancer	14
1.2 Nature derived product among anti-cancer drugs.....	15
1.2.1 Natural source drug	15
1.2.2 β -elemene and its market prospect.....	17
1.2.3 Mechanism of β -elemene	17
1.2.4 Current situation of β -elemene	18
1.3 Nanomedicines for cancer.....	18
1.3.1 Liposome.....	19
1.3.2 Micelle	20
1.3.3 Dendrimers	20
1.3.4 Polymer-drug conjugates.....	21
1.3.5 Nanoparticles (NPs)	22
1.3.6 Nanocarriers on the market.....	22
1.4 LDL like nanoparticles.....	24
1.4.1 Low-Density Lipoprotein (LDL).....	24
1.4.2 LDL as an anti-cancer drug carrier	25
1.4.3 LDL like nanoparticles	26
1.4.4 Nanoparticle analysis.....	27
1.4.5 Theoretical background	30

1.5	<i>In vitro</i> activity of drug loaded nanoparticles in cancer cells	35
1.5.1	Cell culture	35
1.5.2	Cytotoxicity assay	35
1.5.3	Cell uptake	37
1.6	Objectives of this project.....	37
2	Materials and Methods.....	38
2.1	Materials.....	38
2.1.1	Materials used for fabrication of nanoparticles.....	38
2.1.2	Materials used for cell culture.....	38
2.2	Methods	39
2.2.1	Fabrication of nanoparticles	39
2.2.2	Characterization and Optimization of Nanoparticles	40
2.2.3	Cell lines used and cell culture:.....	41
2.2.4	<i>In vitro</i> activity of β -elemene loaded nanoparticles in cancer cells.....	42
3	Results.....	46
3.1	Design and Fabrication	46
3.1.1	Aims and objectives	46
3.1.2	β -elemene nanoparticles	46
3.2	Characterisation and optimization	51
3.2.1	Aims and Objectives	51
3.2.2	TEM data	51
3.2.3	Optimization of β -elemene nanoparticle	52
3.3	Evaluation	56
3.3.1	Aims and objectives	56
3.3.2	MTT assay	56
3.3.3	Flow cytometry	57
3.3.4	Confocal microscope.....	58
4	Discussion.....	61
4.1	β -elemene nanoparticles.....	61
4.1.1	Fabrication of β -elemene nanoparticles.....	61
4.1.2	Optimization and stability of β -elemene NPs	62
4.2	Evaluation of POPC coated β -elemene nanoparticles	62

5	<i>Conclusions and future perspectives</i>	<i>64</i>
5.1	Conclusions.....	64
5.2	Future work	65
	<i>Reference</i>	<i>66</i>
	<i>Appendices.....</i>	<i>72</i>

List of Abbreviations

AML	Acute Myeloid Leukaemia
CFDA	China Food and Drug Administration
DLS	Dynamic Light Scattering
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
EDL	Electrostatic Double Layer
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FCM	Flow Cytometry
HPMA	N-(2-Hydroxypropyl) methacrylamide
IC ₅₀	The Half Maximal Inhibitory Concentration
LDL	Low-Density Lipoprotein
MCF-7	Michigan Cancer Foundation-7
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nm	Nanometre
nM	Nano Molar
NLCs	Nanostructured Lipid Carriers
NP	Nanoparticle
PBS	Phosphate Buffer Serum
PCL	Polycaprolactone
PDI	Polydispersity Index
PEG	Polyethylene Glycol
PI	Propidium Iodide
PLA	Poly (L-lactide acid)
PLGA	Poly Lactide-co-Glycolic Acid
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PPO	Poly (propylene oxide)
P-S	Penicillin-streptomycin
RT	Room Temperature
SEDDSs	Self-emulsion Drug Delivery Systems
SHM	Staggered Herringbone Microfluidic
SLNs	Solid Lipid Nanoparticles
TEM	Transmission Electronic Microscopy
VEGF	Vascular Endothelial Growth Factor
VDW	Van der Waal
Vs.	Versus

List of Figures

Figure 1-1 Schematic representations of Basic liposomes[25]	19
Figure 1-2 The schematic diagram of a typical micelle[30]	20
Figure 1-3 Schematic representation of dendrimer structure Branching structure are drawn using green colour while ligand (red circle) and core (pink structures) are marked on the scheme.....	21
Figure 1-4 Schematic representation of polymer-drug conjugates Blue curvy line symbolizes polymer to which drug (Red) and targeting moieties in green are marked on the scheme.....	21
Figure 1-5 Schematic representation of NP structure Blue ring present lipid coat while drug (red hexagon) and targeting moieties (green triangles) are marked on the scheme.	22
Figure 1-6 Schematic representation of the LDL. LDL particle consist of cholesteryl ester, free cholesterol and triglycerides core and phospholipids monolayer shell attached by apoB-100.[48].....	25
Figure 1-7 Schematic of the microfluidic mixing device (Figure adapted from Zhigaltsev et al. 2012) Lipids dissolved in ethanol and aqueous buffer are pumped into two inlets separately. Nanoparticles are fabricated by millisecond mixing of ethanol stream and aqueous stream. The mixing channel structures were 200 μm wide and 79 μm high, and the dimensions of herringbone were 50 μm $\times$ 79 μm	27
Figure 1-8 Schematic representation of the principle behind DLS.	29
Figure 1-9 Free energy diagram of Classical Nucleation Theory	32
Figure 1-10 The diagram of interaction energy vs. separation distance	33
Figure 2-1 Diagram of solvent exchange method system. This system consists of the automatic displacement syringe and glass vial, which contains water and a magnetic flea is placed on the magnetic stirrer.	39
Figure 3-1 β-elemene nanoparticles in 10% ethanol-water (a)Particle size data obtained for β -elemene nanoparticles in 10% ethanol-water were plotted against varying final β -elemene nanoparticles concentrations. (b) Corresponding PDI values of avg. hydrodynamic diameters were plotted against varying final β -elemene nanoparticles concentrations. Data were obtained in triplicate. Error bars indicates std. deviation of the three individual values. β -elemene nanoparticles were prepared using solvent exchange method for mixing ratio of 1:9 at 500 RPM.	47

Figure 3-2 β -elemene and POPC in ethanol-water mixture using different volume % of ethanol (a) Particle size data of POPC coated β -elemene nanoparticles obtained from the DLS in the form of average hydrodynamic diameter were plotted against different injection ratio (x-axis). (b) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against different injection ratio (x-axis). Measurements for samples with 1:4 mixing ratio were carried out in 20% ethanol-water while for mixing ratio of 1:9, 1:19, 1:49 and 1:99, measurements were carried out in 10%, 5%, 2% and 1% ethanol-water separately. 48

Figure 3-3 Pure β -elemene nanoparticles and POPC coated β -elemene nanoparticles in 10% ethanol-water (a) Particle size data obtained for pure β -elemene NPs (shown as blue squares) and POPC coated β -elemene nanoparticles (shown as orange circles) prepared by using solvent exchange method technique and measured using DLS in 10% ethanol-water are plotted against final β -elemene nanoparticle concentrations (as shown on X-axis). (b) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters of POPC coated β -elemene nanoparticles (shown as black circles) against varying final β -elemene nanoparticles concentrations (as shown on X-axis). Data were obtained in triplicate. Error bars indicates std. deviation of the three individual values. 50

Figure 3-4 TEM image and image analysis results from POPC coated β -elemene nanoparticles (0.15 mM) in 10% ethanol-water (a) The TEM image obtained with Tecnai T12 Biotwin TEM. 13 μ L of a sample containing POPC coated β -elemene nanoparticles (0.15 mM) in water, was deposited on a carbon film. (b) Frequency distribution of the measured 324 particles in the form of histogram.....51

Figure 3-5 POPC coated nanoparticles of β -elemene and POPC at varying compositions (a) Avg. hydrodynamic diameters of POPC coated nanoparticles (shown on Y-axis) obtained from the cumulant mode of DLS were plotted against varying ratio of β -elemene to POPC (shown on X-axis). (b) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against varying ratio of β -elemene to POPC (as shown on X-axis). Data were obtained in triplicate. Error bars indicates std. deviation of the three individual values. 53

Figure 3-6 Stability of β -elemene nanoparticles in water. (a1) Particle size data of POPC coated NPs of 50% drug and 50% POPC were plotted against days. (a2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. (b1) Particle size data of POPC coated NPs of 40% drug and 60% POPC were plotted against

days. (b2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. (c1) Particle size data of POPC coated NPs of 30% drug and 70% POPC were plotted against days. (c2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. (d1) Particle size data of POPC coated NPs of 20% drug and 80% POPC were plotted against days. (d2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. Data were obtained in triplicate. Error bars indicates std. deviation of the three individual values.....54

Figure 3-7 Stability of β -elemene nanoparticles in water stored at 4 °C. (a1) Particle size data of POPC coated NPs of 50% drug and 50% POPC were plotted against days. (a2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. (b1) Particle size data of POPC coated NPs of 40% drug and 60% POPC were plotted against days. (b2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. Error bars indicate std. deviation of the three individual values. 55

Figure 3-8 Cell growth curves generated from 72 h MTT assay data for MCF-7 cells (a) Growth inhibition data (shown on Y-axis) for POPC coated nanoparticles is plotted against β -elemene concentration (shown on X-axis). (b) Growth inhibition data (shown on Y-axis) for pure β -elemene nanoparticles is plotted against β -elemene concentration (shown on X-axis). (c) Growth inhibition data (shown on Y-axis) for pure β -elemene is plotted against β -elemene concentration (shown on X-axis). Error bars indicate std. deviation of values. Absorbance at 490 nm (optical density – OD490) was read using an Envision 2104 Multilabel Reader.....56

Figure 3-9 Cell viability data obtained from Flow cytometer. Cells were incubated with different concentration of POPC coated β -elemene nanoparticles for 72 hours and stained with propidium iodide.....57

Figure 3-10 Cell uptake images consisting of cells (green) and nanoparticles (red) were obtained from Confocal microscope. (a) Image obtained at the time point of 5 minutes. (b) Image obtained at the time point of 10 minutes. (c) Image obtained at the time point of 15 minutes. (d) Image obtained at the time point of 20 minutes. (e) Image obtained at the time point of 25 minutes. (f) Image obtained at the time point of 30 minutes. (g) Image obtained at the time point of 35 minutes. (h) Image obtained at the time point of 40 minutes. (I) Image obtained at the time point of 60 minutes. (J) Image obtained at the time point of 120 minutes. (a) Image obtained at the time point of 240 minutes. 60

List of Tables

Table 1-1 Examples of marketed nanomedicines for cancer treatment	23
Table 2-1 Properties of β -elemene[107]	38
Table 2-2 Different injection ratio of ethanol to water.....	40
Table 2-3 Compositions ethanolic solutions containing β -elemene and POPC.....	41
Table 3-1 % Intensity distribution of 0.001 mM β -elemene NPs	48
Table 3-2 IC ₅₀ data obtained from MTT assay	57

1 Introduction

Nature sourced drugs occupy a large percentage in anti-cancer drugs. However, nature sourced drugs usually cannot target tumour site specifically, which means they have serious side effects. How to deliver drugs to tumour site effectively becomes a hot topic in the world. This thesis provides detailed insights into fabricating 20-30nm nanoparticles of anticancer drug β -elemene and lipids by using rapid solvent exchange method. The design of this kind of nanoparticles is motivated by the design of natural Low-Density Lipoprotein (LDL) (discussed late in section 1.4). Cholesteryl ester core of LDL is replaced with hydrophobic drug and nanoparticles are prepared in the same size range with LDL[1]. Hence, in this introductory chapter the general characteristics of cancer and its treatment are briefly described with further focus on chemotherapy in cancer therapy. Meanwhile, anti-cancer drug β -elemene which is commercially available or under research is reported. Furthermore, the function principle of nanoparticles on cells are reviewed.

In this chapter, an overall background and objectives for the work carried out in this thesis are described.

1.1 Cancer

Normal cells can receive signals from body, telling them when to grow, divide and die. However, sometimes these may go wrong, and cell become abnormal. The abnormal cell may keep dividing to make more and more abnormal cells. These can form a lump, called a tumour. But not all tumours are cancer. A tumour that is not cancer (a benign tumour) may grow, but it cannot spread to anywhere else in the body. It usually only causes problems if it grows and presses on nearby organs. A tumour that is cancer (a malignant tumour) can grow into nearby tissue. Sometimes cancer cells spread from where the cancer started (the primary site) to other parts of the body. They can travel around the body in the blood or through lymph fluid which is part of the lymphatic system. When these cancer cells reach another part of the body, they may grow and form another tumour. This is called a secondary cancer or a metastasis.

1.1.1 Cancer statistics

Over the years, cancer continue to threaten human health. According to cancer research UK, there were approximately 359,960 new cases of cancer in 2015 in the UK, which is around 986 people every day. 163,444 of patients died from cancer in the UK in 2016. 1 in 2 people in the UK born after 1960 will be diagnosed with some form of cancer during their lifetime[2]. Macmillan cancer support organization reported that there are an estimated 2.5 million people living with cancer in the UK in 2015 which has increased by over a million people since 2010, rising to 4 million by 2030[3]. Besides, there were an estimated 18 million cancer cases around the world in 2018 according to American institute for cancer research[4]. Breast, prostate, lung and bowel cancers together accounted for over half (53%) of all new cancer cases in the UK in 2015, while lung, breast, colorectal and prostate cancers have been the four most common causes of cancer death in 2018 and accounting for about 42.7% cancer cases today. 50% of people diagnosed with cancer in England and Wales survive their disease for ten years or more (2010-11). Cancer survival is improving and has doubled in the last 40 years in the UK. In the early 1970's the median survival time after diagnosis was one year, by 2007 it was six years and by 2011 it was ten years[2]. According to Macmillan cancer support organization, the number of people who have survived five or more years since diagnosis has increased by over 260,000 (or 21%) in the five years to 2015. Meanwhile, the number of cancer survivors in the UK is projected to increase by approximately one million per decade from 2010 to 2040; resulting in four million people living with cancer in 2030[3]. As described above, developing new ways to treat cancer is an urgent topic for researchers to improve cancer survival rate. Therefore, some cancer treatment will be talked about in the chapter followed.

1.1.2 Treatment of cancer

Due to the complexity of cancer, its location and the stage it has reached make a difference. Therefore, cancer patients take different types of treatment depending on the type of tumour they suffer from. Nowadays, cancer patients can have various treatment options, including chemotherapy, surgery, radiation, hormonal therapy, immunotherapy, gene therapy. Some people just take one of the treatments above, but most people have a combination of treatments.

Surgery is a technology consisting of a physical intervention on tissues. Surgery, which is one of the oldest techniques, is most commonly used to remove the tumour and some of the

nearby healthy tissue. In some cases, surgery is just performed to ease symptoms in cancers that otherwise cause pressure on vital organs which can be painful for patients and result in poor functioning of other organs. Surgery can be done as an open incision or laparoscopy.

There are ways of performing surgery that do not involve incisions and they include:

cryosurgery, lasers, hyperthermia, and photodynamic therapy[5].

Sometimes, the surgeon cannot remove the entire tumour, or removing it might cause too much damage to the body. In these cases, surgery is used to remove as much of the tumour as possible. Other treatments, such as radiation therapy or chemotherapy, may be used after surgery to continue treating the cancer. Radiotherapy uses high-energy radiation such as X-rays, gamma rays, and charged particles that kill cancer cells[6, 7]. Radiotherapy is used for eliminating tumours, preventing recurrence or shrinking tumours before surgery.

Chemotherapy uses drugs that stop the growth of cancer cells or kills them. It is widely used for many cancer types in order to treat or prevent recurrence. As well as killing cancer cells, chemotherapy can damage some healthy cells in the body, such as blood cells, skin cells and cells in the stomach. This can cause a range of unpleasant side effects. As the work in this thesis is relevant to the improvement of chemotherapeutics, a detailed review on chemotherapeutic marketed drug delivery and ones that are under research is provided in this chapter.

1.2 Nature derived product among anti-cancer drugs

1.2.1 Natural source drug

The role of natural products as a source for remedies has been recognized since ancient times. Natural derived products have been the source of most of the active ingredients of medicines. Despite major scientific and technological progress in combinatorial chemistry, drugs derived from natural product still make an enormous contribution to drug discovery today. Many pharmaceutical agents have been discovered by screening natural products from plants, animals, marine organisms and microorganisms. An analysis of the number of chemotherapeutic agents and their sources indicates that over 60% of approved drugs are derived from natural compounds[8].

Over the past decade, screening and identifying novel compounds for their biomedical applications has become an upcoming area of research. The use of plant and microbial secondary metabolites has aided in doubling of our life span in the 20th century[9]. They have reduced pain and suffering, and revolutionized medicine by enabling the transplantation of organs. Since their chemical diversity is based on biological and geographical diversity,

the entire globe is explored for bioprospecting by researchers. Researchers have had easy access to terrestrial life from which most of the pharmaceutically successful natural products originate. However, the ocean hosts a vast repertoire of life forms brimming with natural products of potential pharmaceutical importance. Marine bioprospecting is a relatively new phenomenon; thus, marine life is a relatively unexplored area of opportunity. New methods are being developed to grow the so-called 'unculturable' microbes from both the soil and the sea. Most biologically active natural products are secondary metabolites with complex structures. In some cases, the natural product itself can be used, but in others, derivatives made chemically or biologically are the molecules used in medicine. Biosynthetic pathways are often genetically manipulated to yield the desired product. With the advent of combinatorial biosynthesis, thousands of new derivatives can now be made by this biological technique, which is complementary to combinatorial chemistry[10].

Natural source drug occupies a large percentage of anti-cancer drug. One of the most famous natural source drugs in anti-cancer area is paclitaxel whose brand name is Taxol. Paclitaxel is used for the treatment of lung, breast, ovarian, bladder, prostate, oesophageal, melanoma, as well as other types of solid tumour cancers. It was approved for medical use in 1993 by FDA. Contact inhibition, which was lost by cancerous cells, can result in normal cells stop dividing when they get in touch with other cells. Rapid dividing cells can be killed by Paclitaxel effectively[11]. Unfortunately, many chemotherapeutics including paclitaxel do not have the ability of distinguishing the different characteristics between the cancerous cells and the normal cells. Chemotherapeutics affect blood cells, the cells in the mouth, stomach and bowel, and the hair follicles mostly. Therefore, paclitaxel and many chemotherapeutics can cause a lot of side effects. For example, paclitaxel may cause patients' white and red blood cells and platelets temporarily decrease which is called low blood counts. Cancer patients may suffer from infection, anaemia and/or bleeding because of this side effect. Besides, paclitaxel can also lead to hair loss, arthralgias and myalgias (pain in the joints and muscles), peripheral neuropathy (numbness and tingling of the hands and feet), nausea and vomiting, diarrhoea, mouth sores and hypersensitivity reaction[12].

As a summary, a new kind of chemotherapeutic which has fewer side effects and tumour targets is needed.

1.2.2 β -elemene and its market prospect

Elemenes, which include α -, β -, γ -, and δ -elemene, are a group of natural chemical sesquiterpenoid compounds extracted from the traditional Chinese medicinal plant *Curcuma* WenYuJin[13]. Elemenes contribute to the floral aromas of some plants and are used as pheromones by some insects[14]. According from Traditional Chinese Medicine, elemene can regulate several molecular targets and exert promising anticancer effects. The IUPAC name and molecular formula of β -elemene is (1S,2S,4R)-1-ethenyl-1-methyl-2,4-bis(prop-1-en-2-yl) cyclohexane and $C_{15}H_{24}$ separately. The molecular weight of β -elemene is 204.35 g/mol. Because of β -elemene's prevalence in a variety of medicinal plants, scientific interest was attracted by this compound. Experiments performed *in vitro* show that β -elemene has anti-proliferative effects toward some cancer cell types, indicating the possibility of its use in chemotherapy. β -elemene has the highest antitumor activity compared with other elemenes structure isomers[15].

Recently, β -elemene has attracted great interest because of its potent anti-proliferation effects toward some types of cancer cells. Based on some small-scale clinical trials with low quality in China, β -elemene has been approved by the State Food and Drug Administration of China for anti-cancer treatment[13]. Up-to-now, β -Elemene has been clinically used to treat leukaemia and carcinomas in brain, breast, liver and other tissues.

1.2.3 Mechanism of β -elemene

In recent years, the study of mechanism of action of β -elemene has made great progress. According to recent studies, β -elemene possesses anti-proliferative effect on cancer cells by creating an apoptotic trigger. Interestingly, it also induces protective autophagy in some cancerous cell lines and is less cytotoxic compared to other widely accepted chemotherapeutic agents. This provides an edge with the perception of limited toxicity to normal cells[15]. The most well studied mechanism by which anticancer agents induce cell death in cancer cells is by the induction of apoptosis[16]. DNA fragmentation and activation of caspases are the major hallmarks of apoptosis[17]. Apoptosis has been strongly induced in cancerous cells by elemene. β -elemene exerts its effects by modulating multiple molecular targets. It modulates CDKs to arrest the cell cycle and alters various proteins such as survivin, caspase, and the Bcl-2 family proteins to induce cell apoptosis. In addition, β -elemene targets the Wnt/ β -catenin, Notch, PI3K/Akt/mTOR, and MAPK/ERK signalling pathways and regulates transcription factors such as STAT3. Furthermore, β -elemene regulates several key molecules of tumour angiogenesis and metastasis, such as VEGF and

matrix metalloproteinase, in addition to modulating the expression of ncRNAs that contribute to anticancer activities[13].

1.2.4 Current situation of β -elemene

However, the clinical application of elemene has been limited by its poor solubility in water, poor stability, and low bioavailability. Therefore, studying a new drug delivery system for elemene has important significance[13]. Currently, elemene liposome injection and oral emulsion have been approved by the China Food and Drug Administration (CFDA) to treat various cancers, and these have achieved a synergistic effect with chemotherapy in the clinic[18]. Chemotherapy combined with elemene injection significantly improved survival and tumour response, as well as reduced toxicity. Currently, novel delivery systems, including solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), long-circulating liposomes, active targeting SLNs, multidrug-loaded liposomes and micro emulsions, self-emulsion drug delivery systems (SEDDSs), and active targeting micro emulsions, have been actively developed and these have contributed too many advancements for elemene. β -elemene has moderate antitumor activity and is mainly used as an adjunctive drug to enhance the efficacy, reduce the toxicity of chemoradiotherapy, and reverse drug resistance. Because of the moderate anti-cancer activity and poor solubility in water, researchers have synthesized a variety of β -elemene derivatives and studied their antitumor activities[13]. This thesis will talk about a novel way to improve β -elemene solubility in water and its tumour targeting ability.

1.3 Nanomedicines for cancer

Chemotherapy is the main treatment for tumour recurrence and for the late stage tumours. However, it is difficult for chemotherapeutic to reach the specific tumour tissues, which can result in some side effects in normal healthy tissues[19]. The development of drug delivery has focused on reducing the undesirable side effects of chemotherapy in the past half a century.

With the development of science, it becomes possible to deliver drugs to target region by using nanoparticles[20]. As an application of nanotechnology in medical area, nanomedicine could potentially maximize bioavailability both at specific region in the body and over a period of time[21, 22]. This means nanoscale devices are less invasive and have less side effect.

For this project, nanomedicines developed for drug delivery in cancer are of interest.

It all started with the concept of the “magic bullet” where Paul Erlich proposed that a drug that could be directed into the cells of interest circumventing healthy tissue[23]. Indeed, the first nanocarrier was a liposomal lamellar structure developed in 1964 and 15 years later was used for drug delivery paving the way for various nanocarriers for targeted cancer drug delivery[24, 25]. Specifically, this was followed by further development of nanocarriers for drug delivery such as polymer conjugates, micelles, nanoparticles, nanoparticles, nanoparticles and dendrimers. Anticancer drugs are often highly metabolised to more polar conjugates and hence are then rapidly excreted by the kidney giving short life. However, nanocarriers have afforded longer drug half-life due to prolonged drug-nanocarrier circulation times; followed by targeted intracellular delivery and/or tumour targeting. Also, in some instances nanocarriers have increased drug stability during storage[26, 27].

Similar to previously described systems, ligands can be attached to achieve active targeting. Despite high density, monodispersity and a well-defined number of ligands on the surface, dendrimers, due to expensive synthesis and sometimes toxicity, they have not been progressed to a commercial formulation for delivering anticancer drugs.

Some different kinds of nanocarriers are discussed as followed.

1.3.1 Liposome

Liposome was discovered by Bangham and co-workers about 50 years ago by accident.

Liposomes are microscopic vesicles consist of an aqueous volume and a bilayer of lipids. The size of liposome ranges from approximately 30 nm to a few micrometres[28].

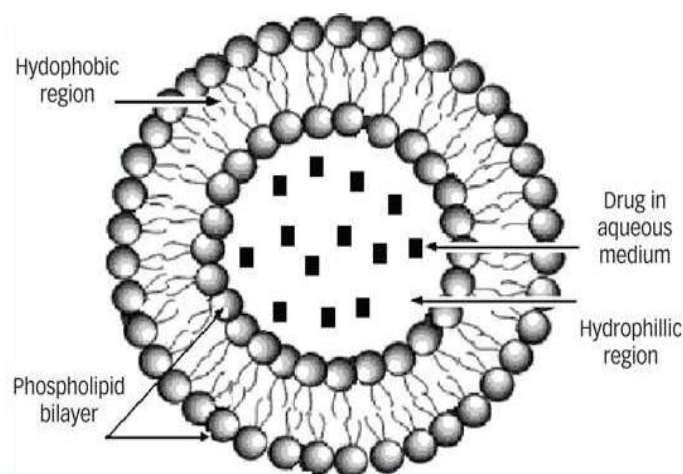


Figure 1-1 Schematic representations of Basic liposomes[29]

Since liposomes are formed from amphipathic lipids, they can be used as a non-toxic vehicle to carry both hydrophilic and hydrophobic drugs. Besides, Liposomes can provide controlled hydration, sustained release and targeted drug delivery. Liposomes can be administered

through modifying with different ligands and direct interaction between cells and drugs[30]. Liposomes also have some drawbacks like low solubility, less stability and short half time. Liposome was the first nanocarrier to be developed with sound market prospect[31].

1.3.2 Micelle

Micelle refers to self-assembled aggregates of amphiphilic molecules. These amphiphilic molecules are usually called amphiphiles. When the concentration of amphiphiles reach or exceed the critical micelle concentration, amphiphiles assemble into micelles[32]. A typical micelle has a hydrophobic core region surrounded by a hydrophilic outer shell (oil-in-water micelle). In a typical micelle, nonpolar molecules are solubilized with the micelle core while polar molecules are absorbed on the micelle surface[33].

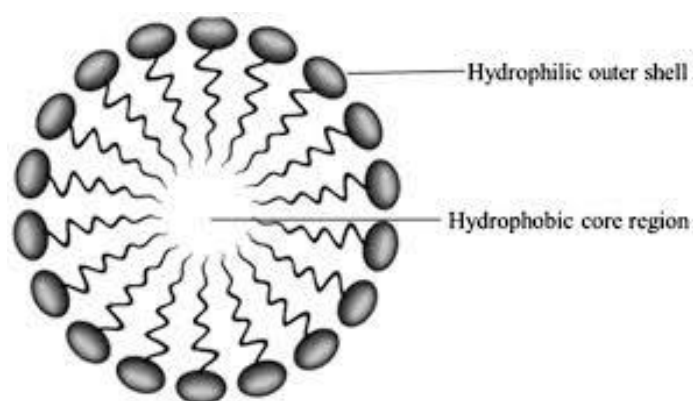


Figure 1-2 The schematic diagram of a typical micelle[34]

Polymeric micelle stands out for the solubilization and targeted delivery of poorly soluble drugs to various pathological sites in the body[33]. The most investigated polymers are PEG, poly(propylene oxide) (PPO), PEG with poly(L-lactide) (PLA), poly- ϵ -caprolactone (PCL) and PEG conjugated to lipids instead to another polymer[35]. A drug can be chemically conjugated, physically encapsulated or both methods can be used in the same micellar formulation[36]. Although demicellisation in biological media is reported[37], micelles have progressed to the clinical trials.

1.3.3 Dendrimers

Dendrimers are defined branched molecules[38]. It looks like a tree which bring forth branches from the core and bear fruit of ligand. Dendrimers have the potential to be applied in several areas especially in drug delivery system by conjugating other chemical compound to the surface of dendrimer.

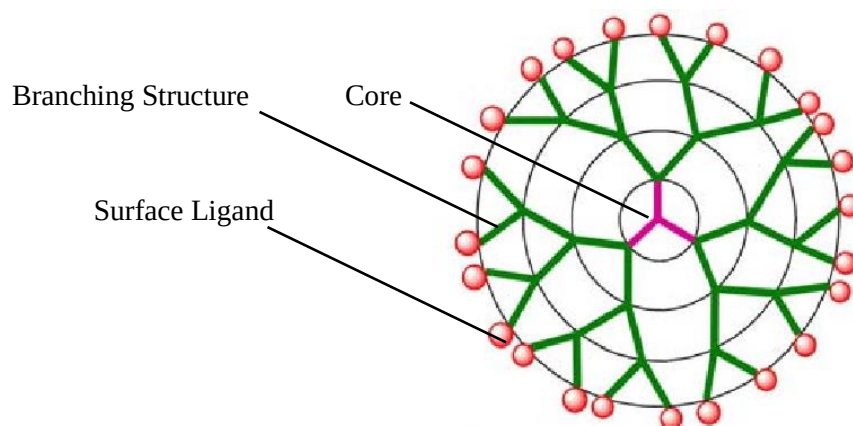


Figure 1-3 Schematic representation of dendrimer structure Branching structure are drawn using green colour while ligand (red circle) and core (pink structures) are marked on the scheme.

Dendrimers could become an appropriate drug delivery vehicle because of the encapsulation ability, water solubility and large number of ligands. By encapsulating hydrophobic compounds and attaching targeting molecules to the dendrimer, undesirable side effect of anticancer drugs can be reduced. A successful dendrimer scaffold designed for the delivery of drugs is the Poly Ethyl Glycinamide (PEE-G), which is shown to have high aqueous solubility, stability and low inherent toxicity [39].

1.3.4 Polymer-drug conjugates

Polymer-drug conjugates are drug delivery system in which drug molecules are bonded to polymer molecules. This kind of drug delivery system is designed to target and release drug at the tumour site, minimizing systemic toxicity[40]. The most successful polymers being tested now are poly lactide-co-glycolic acid (PLGA), N-(2-hydroxypropyl) methacrylamide (HPMA) and polyethylene glycol (PEG)[41-43].

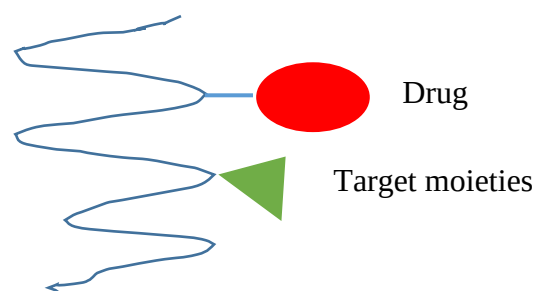


Figure 1-4 Schematic representation of polymer-drug conjugates Blue curvy line symbolizes polymer to which drug (Red) and targeting moieties in green are marked on the scheme.

1.3.5 Nanoparticles (NPs)

Nanoparticles are referred to nanoobjects of which all external dimensions are in the nanoscale and the dimensions do not differ significantly (typically by less than 3 times). The size of a nanoparticle is usually between 1 and 100 nanometres[44]. The formulation studied and adapted in this project is 'phospholipid-coated nanoparticle (motivated by LDL particle, as discussed next) having a hydrophobic core, and suspended in aqueous solution'.

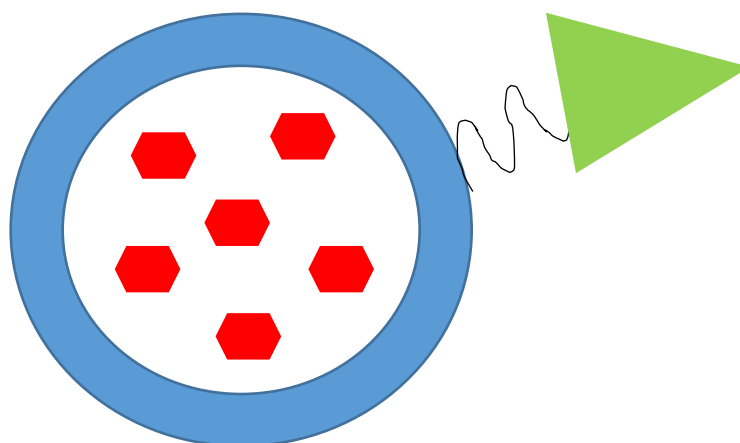


Figure 1-5 Schematic representation of NP structure Blue ring present lipid coat while drug (red hexagon) and targeting moieties (green triangles) are marked on the scheme.

Because of its tiny size and high surface area to volume ratio, nanoparticle sometimes possess unexpected properties which are different from the properties of bulk material. Nanoparticles are also able to pass through cell membranes in organisms which make them become potential vehicles to carry drugs[45].

1.3.6 Nanocarriers on the market

Over ten nanomedicine formulations for cancer have been commercialized in the last 20 years (Table 1-1). The first approved Nano formulation for cancer treatment was Doxil[®], which is shown to be well tolerated in humans and exhibited reduced side effects especially drastically lower cardiotoxicity. Because of involving drug loading in the intraliposomal aqueous phase and coating with polyethylene glycol (PEG) bilayer, the safety and efficacy were improved, leading to Food and Drug Administration (FDA) approval of Doxil[®] in 1995. Another liposomal formulation Myocet[®] is approved in Europe for metastatic breast cancer after showing an improved therapeutic index, fewer side effects and the same efficacy as DOX alone. Additionally, daunorubicin citrate liposome formulation - DaunoXome[®] was approved as a first-line cytotoxic therapy for the treatment of advanced, HIV-associated

Kaposi's sarcoma as it was shown to be as effective as the three-drug regimen (adriamycin, bleomycin and vincristine), but with a reduced incidence of side effects. Other nanomedicines include Marquibo[®] - vincristine sulphate liposomal injection for treatment of leukaemia and Onyvide[®] - PEGylated liposome of irinotecan approved for post-gemcitabine metastatic pancreatic cancer treatment. Mifamurtide (Mepact[®]), an adjuvant liposomal formulation containing muramyl tripeptide phosphatidyl ethanolamine, activates macrophages, monocytes and other immune cells of the reticuloendothelial system increasing production of cytokines and chemokines. These activated cells then attack the cancer cells. This formulation is approved in Europe, Mexico, South Korea, Switzerland, and Israel for non-metastatic osteosarcoma.

Table 1-1 Examples of marketed nanomedicines for cancer treatment

Name	Cancer type	Status	Reference
Doxil [®]	HIV-related Kaposi sarcoma, ovarian cancer, multiple myeloma	Approved by FDA	40
Myocet [®]	Metastatic breast cancer	Approved in Europe	41
DaunoXome [®]	HIV- associated Kaposi's sarcoma	Approved by FDA	42
Marquibo [®]	Leukemia	Approved by FDA	43
Onyvide [®]	Post- gemcitabine metastatic pancreatic cancer	Approved by FDA	44
Nab-paclitaxel (Abraxane [®])	Breast, lung and pancreatic cancer	Approved by FDA	47
NanoTherm [®]	Glioblastoma	Approved in Europe	49
Oncaspar [®]	Lymphoblastic leukemia	Approved in Europe	50

There are also a few commercialised polymer-conjugate formulations. For instance, Oncaspar[®] is PEG-L-asparaginase formulation marketed for acute lymphoblastic leukaemia or Zinostatin, a conjugate of styrene-maleic acid (SMA) and an antitumour protein neocarzinostatin (NCS) SMANCS, approved for hepatocellular carcinoma in Japan.

In 2010, iron-oxide NPs (NanoTherm[®]) received European regulatory approval for treatment of glioblastoma by hyperthermia. These small (15 nm) particles agglomerate following direct injection into the tumour and activation by a magnetic field produces heat. Depending on the temperature reached and length of treatment, the tumour cells are either directly destroyed or sensitized before chemotherapy or radiation.

1.4 LDL like nanoparticles

LDL is the abbreviation of low-density lipoprotein which is one of the five major groups of lipoproteins. The main function of LDL is delivering cholesterol to the cells. LDL have many impacts on the body, but this section will talk about its structure and its application in combating cancer.

1.4.1 Low-Density Lipoprotein (LDL)

LDL is an excellent example of nanocarrier system for hydrophobic materials. A single LDL particle consist of 2000-3000 lipid molecules and one single copy of Apo B-100. LDL can be divided as two components which are highly hydrophobic core consisting of cholesteryl esters (1536 cholesteryl ester)[46] and amphipathic shell. This core also carries varying numbers of triglycerides and free unesterified cholesterol ($<1/3$) and is surrounded by a shell of phospholipids monolayer and unesterified cholesterol, as well as the single copy of Apo B-100[47, 48]. The Phospholipids orient with the polar ends outward and the lipophilic fatty acid chains inwards. The apoB-100, again, wraps around the surface of the LDL particle. Due to the Apo B-100 on the surface, LDL particles can be targeted to LDL receptor. LDL particles are approximately 22 nm to 27.5 nm in diameter[49]. Mora et al.[50] measured LDL size on frozen plasma specimens (-70°C) by proton NMR spectroscopy. The diameters of LDL particles are approximately 21.1 (0.8) for women and 20.6 (0.7) for men. According to a study consist of 1172 men and 1168 women in cycle 3 of the Framingham Offspring, LDL with different density have different diameters (21.8 nm to 27.5 nm). The mass of LDL particles is about 3 million Daltons[51]. Different LDL particles can contain a variable number of cholesterols, triglycerides, phospholipids and other molecules, the mass and size of LDL change in a certain extent.

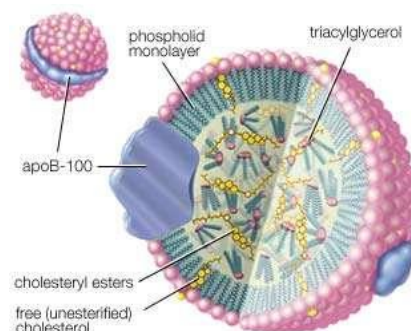


Figure 1-6 Schematic representation of the LDL. LDL particle consist of cholesteryl ester, free cholesterol and triglycerides core and phospholipids monolayer shell attached by apoB-100.[52]

Understanding the structure and size of LDL particles is very important in this project. This project is aiming to fabricate nanoparticles with hydrophobic drug core and lipid shell whose structure is like the LDL.

1.4.2 LDL as an anti-cancer drug carrier

The main cause of death resulted from cancer is that cancer cells tend to spread, or metastasize, throughout the body. Compared with normal cells, cancer cells lose the ability of contact inhibition and keep dividing. Like all dividing cells, cancer cells need a lot of cholesterol because cholesterol is essential in forming mammalian cell membranes. There are two ways for cells to obtain cholesterol. One is producing by themselves, and the other one is catching them from LDL. Cells can use one of the ways to get cholesterol while they can use both ways as well[53]. It is well known that cancer cells are rapid growing cells, so they need a lot of cholesterol. This means cancer cells will require more LDL than normal cells.

According to some studies, LDL uptake by cancer cells are higher than normal cells while the concentration of LDL in cancer patients' blood is lower than healthy people[54]. There is some other evidence about this. LDL is taken up 3-100× more by human acute myeloid leukaemia (AML) cells than normal cells[55]. LDL was absorbed 4-25× more by human AML cells than normal white blood cells[56]. LDL is also yearned by some human solid tumours like epidermoid cervical cancer EC-50 cells, which take 50× more LDL in than normal gynaecologic tissue cells[54]. Besides, gastric carcinoma cells and parotid adenoma cells take up more LDL than normal cells[57]. It is noteworthy that a lot of brain tumour take up 3 times LDL compared with normal brain tissue. medulloblastoma, malignant meningioma and oligodendroglioma are three typical examples among these brain

tumour[58]. Some other tumours like glioma V-251MG[59], squamous lung tumour[60], G2 hepatoma[61, 62] and choriocarcinoma[63] also have a high-level uptake of LDL.

Except human tumours, researches also study mouse some tumours. LDL is taken up several times more by mouse tumour cells like MS-2 Fibrosarcoma cells[64], MAC 13 tumour cells[65] than normal cells. Numerous malignancies are known to over-express LDL receptor, which means cancer cells can take up more LDLs than non-cancerous. However, some tumours like epithelioid carcinoma A-431[66], guinea pig leukemic lymphocytes[67], and cervical cancer EC-168[68] do not internalize abundant LDL although they have large amounts of LDL receptors.

Because of the overexpress of LDL receptors, it was previously reported that LDL itself was used as a drug carrier[54]. It is reported by Vitols et al. that lung cancer tissue takes up 1.5-3 times more LDL particles than surrounding tissue in 7 out of 9 patients with primary lung cancer[69]. However, a number of factors hinder the use of native lipoproteins as a drug delivery vehicle[69]. These native LDLs can aggregate naturally during storage and it is hard to isolate LDL from the fresh donor plasma in significant quantities[70]. Besides, loading of therapeutic drugs[71] and issues about safety[72] are another two limitations. To overcome these difficulties, reconstituted LDL was reported as an alternative approach. Reconstituted LDL uses purified ApoB-100 to stabilize drug [73-76]. Unfortunately, it is difficult to isolate ApoB-100 protein due to its large size and the tendency to aggregate. Therefore, it is difficult for reconstituted LDL to be adapted to industry[77].

In summary, this project involves preparation of a synthetic LDL-like nanoparticles and cell trails of cytotoxicity and cell uptake.

1.4.3 LDL like nanoparticles

In this project, rapid solvent exchange method[78, 79] is used to fabricate nanoparticles. Rapid solvent exchange method was initially used for liposomes fabrication[80, 81]. It was used for advanced microfluidic platforms later[82]. The method used in this project is motivated by the technique reported by Zhigaltsev et al.[83]

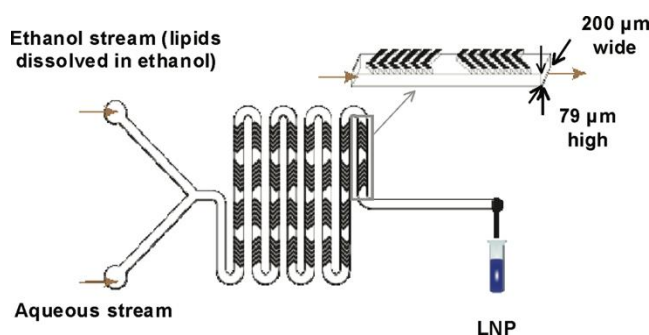


Figure 1-7 Schematic of the microfluidic mixing device (Figure adapted from Zhigaltsev et al. 2012) Lipids dissolved in ethanol and aqueous buffer are pumped into two inlets separately. Nanoparticles are fabricated by millisecond mixing of ethanol stream and aqueous stream. The mixing channel structures were 200 μm wide and 79 μm high, and the dimensions of herringbone were 50 μm $\times$ 79 μm .

By using a staggered herringbone microfluidic mixing device (SHM), Zhigaltsev et al. fabricate nanoparticles with limit size. In this “bottom-up” synthesis system, nanoparticles are fabricated by rapid mixing lipids dissolved in ethanol and aqueous buffer. Three test ethanol stream, 1-palmitoyl-2-oleoyl PC (POPC), mixture of POPC and cholesterol and mixture of POPC with triglyceride triolein, are pumped into one inlet by using syringe pump. 20 nm nanoparticles, consisting of POPC monolayer coated triglyceride, are formed by using SHM device with high mixing rates. Assuming a core of hydrophobic triolein (molecular weight of 885.4 g/mol and density of 0.91 g/ml) surrounded by POPC monolayer (area per molecule of 0.7 nm²[84], thickness of 2 nm), it is calculated that limit size particles of ~20 nm diameter can be formed when the ratio of POPC to triolein is 60 to 40 (mol/mol). Zhigaltsev et al. think particle size can be influenced by the rapid increase in the polarity of the medium, which is affected by the rate of mixing and the ratio of aqueous to ethanol volumes. By increasing the ratio of the aqueous flow rate to the ethanol flow rate, the “limit size” particles were formed. These sizes were considered as the smallest possible sizes that this kind of material could achieve to form a stable monolayer structure. The results are similar to the rapid precipitations where, if mixing rate is much faster than particle growth rate, solution supersaturation can be afforded to initiate nucleation to produce smallest size nanoparticles[85].

Motivated from their design, β -elemene is considered as a replacement for triolein to fabricate nanoparticles. This project is aimed to fabricate a kind of LDL like nanoparticles that consist of β -elemene cores surrounded by POPC monolayer.

The system used in this project is a little bit different from SHM. The mainly difference is no junction compared with Y junction used by Zhigaltsev et al.[83]

1.4.4 Nanoparticle analysis

After fabricating nanoparticles, it is important to know the accurate size of the formulated nanoparticles. The most commonly reported technologies used to measure particle size are dynamic light scattering, transmission electron microscopy, scanning electron microscopy,

field flow fractionation and atomic force microscopy[86]. We have used two techniques in this thesis, which are dynamic light scattering (DLS) and TEM.

1.4.4.1 Dynamic Light Scattering (DLS)

DLS, sometimes referred to as Photon Correlation Spectroscopy or Quasi-Elastic Light Scattering, is a kind of most commonly used technique in the labs working with nanoparticles. DLS is usually used to measure the size of particle in the form of hydrodynamic diameter along with the polydispersity dispersed in a liquid.

Brownian Motion is the theory behind DLS. Small particles move rapidly at random in the solvent due to the bombardment by the solvent molecules that surround them. If particle become larger, they will move slower. The velocity of the Brownian motion is defined by a parameter called translational diffusion coefficient. The software calculates the size of a particle from the translational diffusion coefficient by using the Stokes-Einstein equation;

Equation 1-1

$$d(H) = \frac{kT}{3\pi\eta D}$$

Where,

d(H) = hydrodynamic diameter

D = translational diffusion coefficient

k = Boltzmann's constant

T = absolute temperature

η = viscosity

What should be noted here is the diameter that is obtained by this technique is actual the diameter of a sphere which has the same translational diffusion coefficient as the particle we measured. Many factors can influence particle translational diffusion coefficient, including the size of the particle, surface structure of a particle and ionic strength of medium. Even small changes happen which can affect the diffusion speed, then the hydrodynamic size will change.

According to Rayleigh Scattering, if the particles are smaller than $d=\lambda/10$, then the scattering from a particle illuminated by a vertically polarized laser will be essentially isotropic. The Rayleigh approximation tells us that the amount of scattering is inversely proportional to the fourth power of the wavelength or sixth power of the particle diameter.

When a plane electromagnetic wave, which has a fixed wavelength, amplitude, frequency, polarization and wave factor, is incident on the sample, a speckle pattern is observed where the position of each speckle is seen to be in constant motion. Dark spaces (where scattered light phase additions are mutually destructive and cancel each other out) and bright blobs of light (where the light scattered from particles arrives with the same phase and interfere constructively to form a bright patch) can be observed constantly by the detector. The intensity fluctuates and the rate of this depend on the size of the particles.

The software uses various algorithms to obtain size from correlation function. Two fundamental different approaches are often taken by the software. One of them is fitting single exponential to the correlation function to obtain mean size, which is called z-average diameter, and an estimate of the width of the distribution (polydispersity index). Another is obtaining the distribution of particle sizes by fitting a multiple exponential to the correlation function.

The schematic principle behind particle size determination showed below.

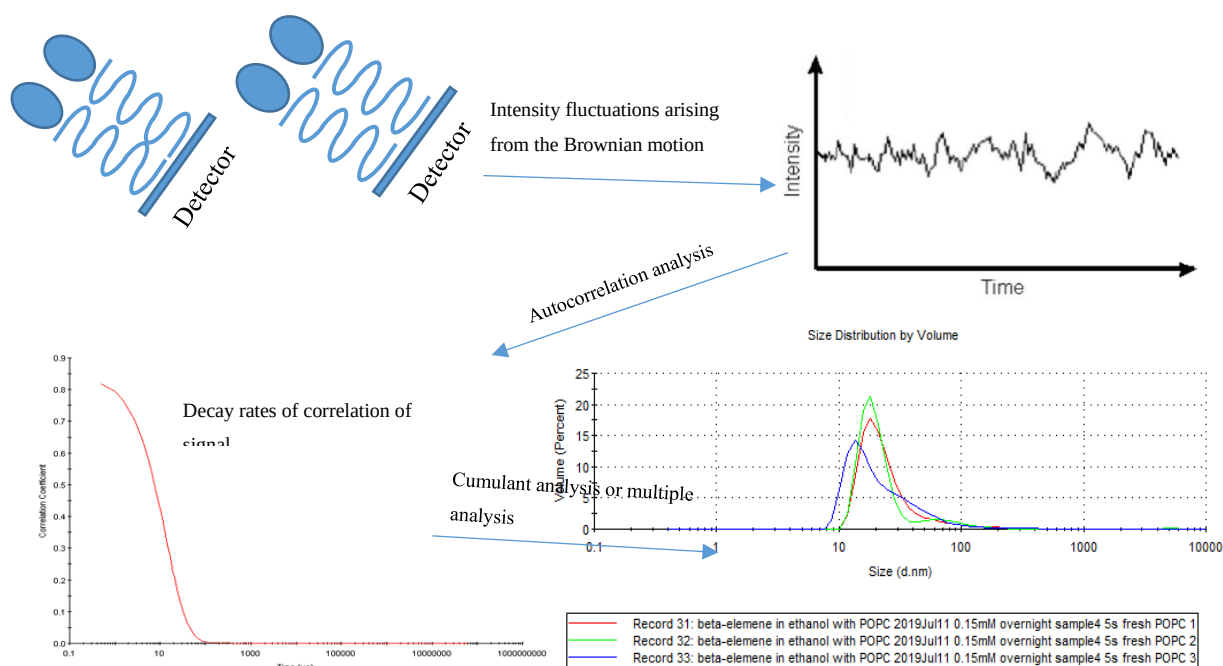


Figure 1-8 Schematic representation of the principle behind DLS.

1.4.4.2 Transmission Electron Microscopy (TEM)

TEM is a microscopy technique used widely in many areas. It is not a novel technique. Max Knoll and Ernst Ruska demonstrate the first TEM in 1931. Ruska won a Nobel Prize in

physics in 1986 for the development of transmission electron microscopy. The instrument used in this project is Tecnai T12 Biotwin TEM.

TEM, using electrons instead of light, operates on the same basic principles as the light microscope. When TEM operates, A beam of electrons is transmitted through a specimen, the information contained in the electron waves exiting from the sample is used to form an image. The projector lenses allow for the correct positioning of this electron wave distribution onto the viewing system. Compared with light microscopes, TEM is able to image at a significant higher resolution. Because of the small de Broglie wavelength of electrons, TEM have the ability of capturing fine detail-even as small as a single column of atoms.

Preparing TEM samples could be a complex procedure[87]. Specimens should be thinner than 100 nanometres for a conventional TEM.

TEM is a very useful technique in this project. According to the **section 1.5.1**, the particle diameter that is obtained by DLS is actual the diameter of a sphere which has the same translational diffusion coefficient as the particle we measured. The structure of nanoparticles in this project can be observed by using TEM. Besides, TEM can also be used to measure the particle size and size dispersion, which can be used to make comparison with data gotten from DLS.

1.4.5 Theoretical background

1.4.5.1 *Classical nucleation Theory*

In this project, “nucleation” is the theory behind the progress of the particles precipitation which involves solvent exchange method[88]. Firstly, nucleation process is described in this section.

When a system strikes up a non-equilibrium metastable state, being a supersaturated condition, which stands for a local minimum of the free energy, the process of nucleation takes place. The system is stable to small fluctuations because of the local minimum free energy. However, with an increase in stochastic (microscopic) density fluctuations, the random collisions of the dissolved molecules of the solute in the solution lead to the formation of the clusters of a new phase which are called as ‘Nuclei’. The global minimum of the free energy possessed by the bulk system could be represented by these nuclei. Besides, the transformation of the phase takes place through the energy barrier which represents the local maximum free energy state[89].

Random collisions, leading to the aggregation of homogeneously distributed molecules towards the formation of clusters or “embryos”, can explain the concept of nucleus formation in a supersaturated solution consisting of these molecules. It can be regarded as the critical nucleus when the cluster or embryo reaches critical size. If this critical nucleus can be assumed as a sphere, the radius of it can be predicted on the basis of classical nucleation theory[90].

Many parameters can affect the critical free energy for precipitation, but Horn et al.[91], Lince et al.[92] and Thanh et al.[93] thought the most significant parameters are the surface tension and supersaturation to their respective powers. Assuming nucleus as “sphere”, the interior of the nucleus is in the bulk new-phase state and the surface tension is the same as a planar surface of the coexisting new and mother phases[94]. With this hypothesis, the free energy of homogeneous nucleation (ΔG) for a spherical particle (nucleus) of radius (r), the surface free energy per unit area (γ), and the free energy per unit volume of bulk crystal (ΔG_v), is

Equation 1-2

$$\Delta G = 4\pi r^2 \gamma + \frac{4}{3} \pi r^3 \Delta G_v$$

As the surface energy is always positive and the bulk free energy is always negative, the maximum free energy through which a cluster will pass to form a stable nucleus can be obtained by differentiating ΔG with respect to r and setting it to zero ($d\Delta G/dr = 0$). The critical free energy ΔG_{crit} is

Equation 1-3

$$\Delta G_{crit} = \frac{16\pi\gamma^3}{3\Delta G_v^2} = \frac{4}{3} \pi \gamma r_{crit}^2$$

The free energy per unit volume of bulk crystal is expressed as

Equation 1-4

$$\Delta G_v = - \frac{k_B T \ln(S)}{V_m}$$

Where k_B is Boltzmann’s constant (which is defined to be exactly 1.380649×10^{-23} J/K)[95], T is temperature and V_m is the molar volume of the precipitating material and S is the degree of supersaturation. The degree of supersaturation is the supersaturation value (C_{ss}) divided by the saturation value (C_s).

The critical radius of the nucleus is

Equation 1-5

$$r_{crit} = \frac{2\gamma V_m}{k_B T \ln(S)}$$

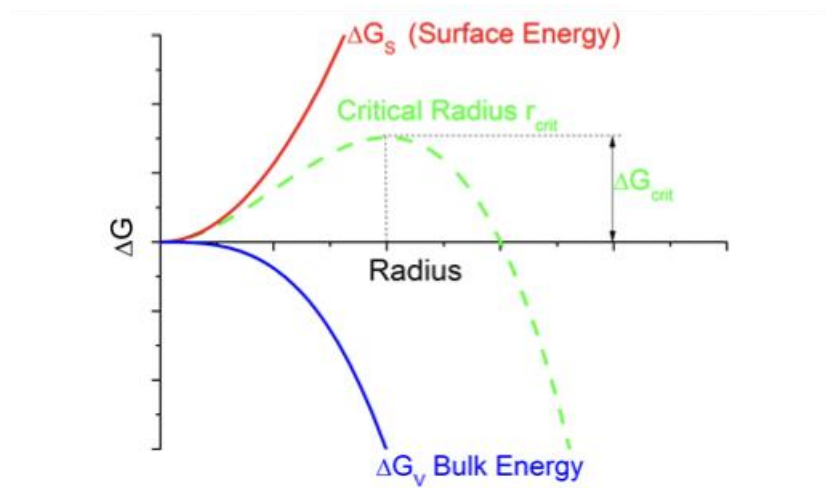


Figure 1-9 Free energy diagram of Classical Nucleation Theory

This diagram illustrates a metastable state thermodynamically that any particles with radius larger than critical size will grow constantly while particles with radius smaller than critical size will be unstable.

It can be said that the critical nucleation radius is proportional to the surface tension and inversely proportional to the natural log of degree of supersaturation. For hydrophobic materials like triolein, the degree of supersaturation could be in the range of 1 to 1.1[96]. Therefore, the higher value of S for hydrophobic materials could be achieved only through higher rate of mixing.

1.4.5.2 DLVO Theory

This theory assumes that van der Waals (VDW) attractive and repulsive electrostatic double layer (EDL) forces are independent and dominate aggregation of particles. The total interaction is determined by the sum of these two forces.

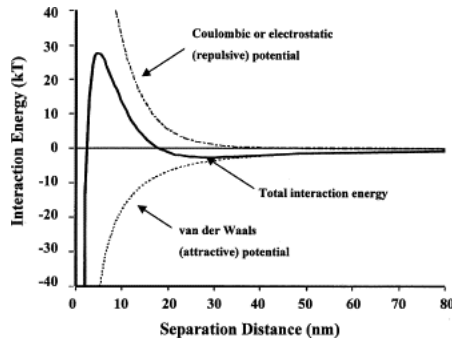


Figure 1-10 The diagram of interaction energy vs. separation distance

As shown in diagram, solid black line represents the resultant interaction energy between two particles, which is the sum of attractive van der Waals energy and repulsive electrical double layer interaction energy. When two particles move away from each other, Van der Waals attraction decreases. Meanwhile, the electrostatic repulsion decreases and the interference between their electrical double layers decreases as these two particles move apart from each other. The diagram illustrates different resultant interaction energy at different distance.

At closer distance, the highest energy of interaction, which represents by the primary minimum, results in irreversible attachment of the particles. When the distance become larger, the interaction energy goes through a maximum energy barrier and is dominated by the attractive energy, which is recognized as secondary minimum.

Assuming two spheres with radius of r_1 and r_2 stay away from each other, the van der Waals interaction can be expressed as

Equation 1-6

$$E_{vdw} = - \frac{A}{6D} \frac{r_1 r_2}{(r_1 + r_2)}$$

In the equation, D represents the distance between two spheres and A represents Hamaker constant. The Hamaker constant can be calculated as

Equation 1-7

$$A = \pi^2 C \rho_1 \rho_2$$

In this equation, C is a interaction energy constant which is decided by van der Waals attraction and the molecule's property[97].

The repulsive electrical double layer interaction energy between two spheres of radius r is expressed as

Equation 1-8

$$E_{EDL} = \frac{64\pi k_B T r \rho_\infty \gamma^2}{\kappa^2} e^{-\kappa D}$$

Where,

k_B = Boltzmann constant

T = temperature

ρ_∞ = the number density of ion in the bulk solution

γ = the reduced surface potential

κ = Debye length

Then, the interaction energy between two particles can be expressed as

Equation 1-9

$$E_{Total} = E_{vdW} + E_{EDL}$$

1.4.5.3 Ostwald Ripening

Ostwald ripening is a phenomenon which describes the change of an in homogeneous structure over time[98]. Ostwald ripening describes that particles with different sizes can grow when their solubility changes. Molecules on the surface of a particle are less stable than the ones in the interior. Thus, molecules on the surface of small particles tend to detach from the particle because the system tries to reduce its overall energy. When these molecules detached from small particles diffuse into the solution, the concentration of molecules increases in the solution. After supersaturation of this kind of molecules, they tend to condense on the surface of larger particles. Due to the more energetically favoured of larger particles compared with smaller particles, thermodynamically driven spontaneous process occurs. Therefore, large particles become larger and small particles become smaller. In other words, aggregation happens and the average size of this kind of particles increase.

The growth rate can be expressed as below when attachment and detachment of molecules is slower than diffusion.

Equation 1-10

$$\bar{r}^2 = \frac{64\gamma c_\infty v^2 k_s}{81R_g T} t$$

Where,

$\bar{r}$ = average radius of all the particles

γ = particle surface tension

c_∞ = solubility of the particle material

v = molar volume of the particle material

k_s = reaction rate constant of attachment with units of length per time

R_g = ideal gas constant

T = absolute temperature

t = time

As described above, the Ostwald Ripening is expected to be present in the process of fabricating nanoparticles. To reduce the effect of this kind of ripening, the rate of injection needs to be as fast as possible (833 $\mu\text{L/s}$ in this project).

1.5 *In vitro* activity of drug loaded nanoparticles in cancer cells

The ultimate goal of anticancer nanoparticles is delivering cytotoxic drug to the site of tumours and reduce toxicity to normal cells. In other words, nanoparticles are aimed to target site of disease. Therefore, *in vitro* activity of drug loaded nanoparticles is essential to be researched.

1.5.1 Cell culture

Cell culture is defined as culture cells under controlled conditions *in vitro*. These conditions generally consist of suitable vessel, medium or substrate which supplies essential nutrients, gases which usually contains CO_2 and O_2 , growth factors and suitable physio-chemical environment like pH, osmotic pressure and temperature. Cell culture conditions can vary for each cell type.

1.5.2 Cytotoxicity assay

Meanwhile, as a kind of anticancer drugs, this thesis will present some other techniques used for determining cytotoxicity and cell uptake. According to measurement types of end points, there are 4 cytotoxicity assays: Dye exclusion, Colorimetric assay (MTT assay, MTS assay, XTT assay, WST-1 assay, WST-8 assay, LDH assay, SRB assay, NRU assay and crystal violet assay), Fluorometric assay (AlamarBlue assay and CFDA-AM assay) and Luminometric assay[99]. In this thesis, MTT assay and Flow cytometry are used to measure cytotoxicity and cell viability. Brief background, principle, advantages and disadvantages of these methods are presented.

1.5.2.1 MTT assay

As mentioned before, MTT assay is a kind of colorimetric assay for assessing cytotoxicity and cell viability. The IUPAC name of MTT is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. This yellow tetrazole can be reduced to purple formazan in living cells[100]. NAD(P)H-dependent cellular oxidoreductase enzymes in mitochondria can

reduce the tetrazolium dye MTT to its insoluble formazan[101]. This purple formazan can dissolve in solubilization solution, which is usually either dimethyl sulfoxide, an acidified ethanol solution or a solution of detergent sodium dodecyl sulphate in diluted hydrochloric acid and form purple solution. A spectrophotometer is used late to measure the absorbance of this solution at a wavelength between 500 and 600 nm.

Reduction of MTT is assumed to be dependent on NAD(P)H-dependent oxidoreductase enzymes largely in the cytosolic compartment of the cell[102]. In other words, reduction of MTT depends on cellular metabolic activity. Rapidly dividing cells can reduce high rate of MTT.

MTT assay is a widely used colorimetric assay in the world. This method is easy to use and has a high reproducibility[103, 104]. Because of the cytotoxicity of MTT formazan, cells with MTT formazan needles will float in the plate wells. Therefore, it is difficult to remove cell culture media from wells, giving significant well-to-well error. To reduce error caused by background interference, additional control experiments should be conducted[105].

1.5.2.2 Flow Cytometry

Flow cytometry is a technology which enables the rapid, sequential analysis of single cells through excitation of cells with a light source (usually a laser) and the subsequent collection of emitted light (scatter and fluorescence). As a technology with high precision, Flow cytometry can be used in a broad range of research applications including: cell counting, cell sorting, protein engineering detection, cell characteristics and function determination, Biomarker detection, microorganisms detection...[106]

Flow cytometer is consisting of 5 main components: flow cell, measuring system, detector, amplification system, analytical computer. In a flow cytometer, samples (usually stained cells in suspension) pass through the laser beam in “single file” after being aligned in the flow cell[107]. Light scatter information is collected from each cell light. For almost all flow cytometers is collected off a blue (488nm) laser and the wavelength of scattered light is the same as the laser. The fluorophore emits light at the wavelength that is the fluorescent light. Besides, presence of fluorescence light indicates binding of fluorophore to cell and the intensity of fluorescent light is proportional to the amount of fluorophore bound to the cell. If several fluorophores are used, light emitted from combined multiple fluorophores will cause overlap. In this situation, filters and mirrors can be used to separate different wavelengths of lights.

Compared with fluorescent microscopy, Flow cytometry can measure a large number of cells and is easy to acquire total fluorescence of cells. Besides, a modern flow cytometer is able to offer high-throughput, automatic quantification of detailed parameters on a cell-by-cell basis. Modern flow cytometers are usually equipped with multiple lasers and fluorescence detectors to identify more precisely. Some Flow cytometer in the market even have 10 laser and 30 fluorescence detectors.

1.5.3 Cell uptake

There are different entry pathways for drugs, molecules and nanoparticles to enter the cell membrane. Cells can take up LDL by clathrin-dependent endocytosis. As this thesis talked about before, this project intends to fabricate a kind of LDL like nanoparticles. Therefore, cell uptake of nanoparticles is of interest in this project. When nanoparticles meet with a cell, they can enter the cell mainly through endocytosis[108].

In this project, Confocal Microscopy is used to observe cell uptake of nanoparticles. Confocal microscopy is an imaging technology which use a spatial pinhole to block out-of-focus light in image formation to increase optical resolution and contrast of a micrograph[109]. Confocal microscopy is used in this project to observe cell uptake by staining cells with LysoTracker Green and staining nanoparticles with Dil C18.

Confocal microscope is invented to overcome some limitations of conventional widefield fluorescence[110].

1.6 Objectives of this project

1. To design and fabricate POPC coated β -elemene nanoparticles.
2. To characterize particle size and polydispersity index of POPC coated β -elemene nanoparticles.
3. To determine the influence of injection ratio on the size and PDI of β -elemene nanoparticles.
4. To optimize solvent exchange method and evaluate the physical stability of NPs.
5. To compare the cytotoxicity of POPC coated β -elemene NPs with pure β -elemene and uncoated β -elemene NPs.
6. To observe cell uptake of POPC coated β -elemene NPs using confocal microscope.

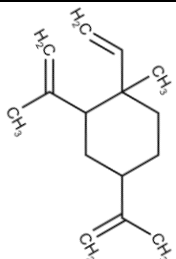
2 Materials and Methods

2.1 Materials

2.1.1 Materials used for fabrication of nanoparticles

β -elemene was obtained from Sigma Aldrich. POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) was purchased from Avanti polar lipids, Inc. Ethanol and all solvents were obtained from Sigma Aldrich unless otherwise stated. Purified water used was obtained from Mili Q system adapted with 0.22 μ m membrane filter.

Table 2-1 Properties of β -elemene[111]

Name	β -elemene
Structure	
Chemical Formula	C ₁₅ H ₂₄
Average Molecule Weight	204.35
Density	0.862 g/cm ³
Index of Refraction	1.501
Water Solubility	0.004 g/L (19.57 nM)
Log P	5.4

2.1.2 Materials used for cell culture

Human carcinoma cell lines (MCF-7) were all sourced from the American Type Culture Collection (ATCC).

Reagents:

All routine cell culture materials, phosphate buffer serum (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue, 20% SDS solution, dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) were purchased from Sigma-Aldrich. Foetal bovine serum (FBS), Dil Stain (1,1'-Diocetadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate ('Dil'; DiI_{C18}(3))) and propidium iodide were

purchased from Thermo Fisher Scientific. LysoTrackerTM Green DND-26 was purchased from Cell Signaling Technology, Inc.

2.2 Methods

2.2.1 Fabrication of nanoparticles

In this section, organic solution preparation and direct injection with stirring were discussed. Below is a brief introduction of preparing organic solution and fabricating nanoparticles. Before experiment, 10 mM ethanolic stock solution of β -elemene (11.8 μ L in 5 mL ethanol) was prepared and stored in 4 $^{\circ}$ C refrigerator. In this experiment, 2 mL ethanolic stock solution of β -elemene was diluted to 5, 1, 0.5, 0.375, 0.25 and 0.1 mM to get a series concentration of organic solutions. An automatic displacement syringe (eVol[®] XR), as mentioned by Pablo Hervella[112], was involved to inject organic phase into distilled water contained in a 10.5-mL glass vial as shown in **Figure 2-1** to fabricate β -elemene nanoparticles. The glass vial was kept on a magnetic stirrer (at a speed of 500 RPM). Organic solutions of β -elemene at concentrations of 10, 5, 1, 0.5, 0.375, 0.25 and 0.1 mM were injected into the distilled water at the speed of 0.833 mL/s by pressing the dispense button on the autopipette. After injection, the vial was removed from the stirrer immediately and particle size was measured by using a DLS.

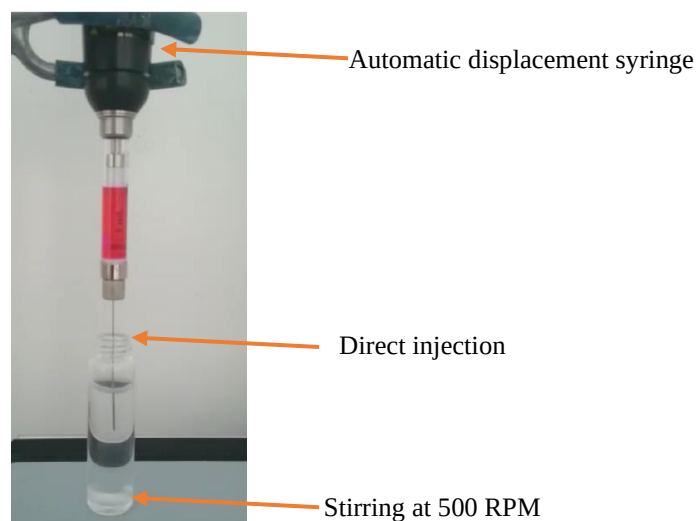


Figure 2-1 *Diagram of solvent exchange method system. This system consists of the automatic displacement syringe and glass vial, which contains water and a magnetic flea is placed on the magnetic stirrer.*

After obtaining the data of β -elemene nanoparticles without POPC, injection ratio is of interest in this project. The Different ratios of ethanol to water are described in the table below.

Table 2-2 Different injection ratio of ethanol to water

Initial Concentration of β -elemene-POPC (mM)	Volume of Ethanolic Solution (mL)	Volume of Water (mL)	Ethanolic Solution: Water Ratio	Final Concentration of β -elemene-POPC (mM)
0.5	2	8	0.25	0.1
1	1	9	0.11	0.1
2	0.5	9.5	0.05	0.1
5	0.2	9.8	0.02	0.1
10	0.1	9.9	0.01	0.1

Once injection ratio is determined, POPC coated β -elemene nanoparticle could be fabricated with the selected volume ratio. The desired volume of POPC, ready-made solutions from Avanti Polar Lipids, Inc., was transferred to a 20 mL glass vial, dried in the Termovap Sample Concentrator and dissolved in 1 mM ethanolic solution of β -elemene to achieve the premix solution. The mol ratio of β -elemene to POPC, which need to be further tested, used here is 1:1.

As mentioned before, this premix solution was directly injected into a glass vial (10.5 mL) contained distilled water on a magnetic stirrer (at a speed of 500 rpm) by using automatic displacement syringe to obtain POPC coated β -elemene nanoparticles.

2.2.2 Characterization and Optimization of Nanoparticles

2.2.2.1 Particle Size Analysis

The Zetasizer Nano ZS (Malvern Panalytical Ltd) dynamic light scattering (DLS) analyser, equipped with a 4mW He-Ne laser operating at a wavelength of 633nm, was used to measure particle size. The instrument was prewarmed at least 30 minutes before measurement. The samples, prepared by using solvent exchange method, were measured by using disposable cuvettes at the temperature of 25 °C. The solvent properties, such as refractive index, viscosity and absorption, were set previously.

Transmission electron microscopy was used to observe nanoparticle structure and size dispersion. This study was carried out at the Nanoscale and Microscale Research Centre (nmRC), University of Nottingham, Nottingham. The sample was prepared by depositing 13

μL of the nanoparticle sample on a lacey carbon grid for 5 minutes followed by blotting. After blotting, samples were then transferred to holder. These samples were then investigated with Tecnai T12 Biotwin TEM which runs at an accelerating voltage of 100kV. Images were captured using a Soft Imaging System Megaview II camera system. The TEM images were analysed by using image analysis software Image J (Fiji).

2.2.2.2 Optimization of nanoparticles

To research the influence of different ratio of β-elemene to POPC, some additional experiments were carried out. The respective compositions of β-elemene-POPC premix solution are described in the table below.

Table 2-3 Compositions ethanolic solutions containing β-elemene and POPC

Core	Coating	β-elemene: POPC Ratio
β-elemene (mM)	POPC (mM)	
1	0.25	4
1	0.43	2.33
1	0.67	1.5
1	1	1
1	1.5	0.67
1	2.33	0.43
1	4	0.25

2.2.2.3 Stability of the nanoparticles

Colloidal stability of β-elemene loaded NPs was assessed upon storage at room temperature (RT) or in the refrigerator (4 °C) for up to 3 weeks. Particles were measured at time point of 0 d, 1 d, 2 d, 5 d, and 20 d by using DLS. Hydrodynamic particle diameter, obtained from DLS, were collected to analyse the stability of nanoparticles.

2.2.2.4 Statistical analysis

Unless it is stated differently, all experiments were performed in triplicate (n=3±s.d.), where n is number of independent replicates. Statistical evaluation of data was performed using one-way analysis of variance (ANOVA) comparison using GraphPad prism 7.01 GraphPad Software, UK.

2.2.3 Cell lines used and cell culture:

Breast cancer cell line:

MCF-7

This project use breast cancer cell line MCF-7. MCF-7, abbreviation of Michigan Cancer Foundation-7, is a breast cancer cell line established by Dr. Soule and co-workers in 1973[113]. It was isolated from a 69-year-old Caucasian woman[114]. After establishing this cell lines, MCF-7 was commonly used and proved to be a suitable model cell line for breast cancer investigation all over the world[115]. As a non-invasive and poorly aggressive cell line[116], MCF-7 is commonly considered to have low metastatic potential[117]. MCF-7 cell line consists of large number of individual phenotypes, which is differ in gene expression profile, signal pathway and receptor expression. Under appropriate selective conditions, the small sub-lines existing in the parental line can be expanded. According to the variant examined, Extensive aneuploidy are presented with significant variations in chromosome numbers between 60 and 140 by MCF-7 cells. A fraction of stem cells able to generate clonal variability contained by MCF-7 cells could explain the heterogeneity of this cell line and as a model for breast tumour heterogeneity.

General cell culture:

Cells were serially sub-cultured in 75 cm² tissue culture flasks (Costar) incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cells were grown in RPMI 1640 medium supplemented with 10% v/v FBS, 1% P-S and 1% L-clusterin. Once cells had reached approximately 75% -85% confluence they were subcultured. Medium was aspirated and tissue culture flasks were washed with 1.5 mL trypsin-EDTA. Then, 3.5 mL trypsin-EDTA was added to detach cells. After centrifugation at 800 rpm, cells were re-suspended in approximately 7 mL fresh medium, and 1.5 to 2 ml suspension was transferred to a new flask containing 12 ml fresh, complete medium. Cultures at approximately 70% confluence were used for the experiments.

******It was ensured that cells are mycoplasma-free using Alonzo test. Additionally, bulked up stocks of cells were preserved in liquid nitrogen in 1 ml aliquots stored in cryovials. This was all done in order to ensure minimal genotypic/phenotypic drift.

2.2.4 *In vitro* activity of β -elemene loaded nanoparticles in cancer cells

Nanocarriers have the ultimate goal to deliver a cytotoxic drug to the site of the disease and minimize toxicity elsewhere in the body. Many nanocarriers successfully deliver drug to the tumour and elicits antitumor activity greater than the drug alone (see **section 1.3.7**).

2.2.4.1 *Cytotoxicity assays*

2.2.4.1.1 MTT assay

The MTT assay was used for mitochondrial activity evaluation, a surrogate marker for viable cell number. Briefly, cells were seeded into two 96-well plates at a density of 5×10^3 cells/well in 100 μ L of culture medium. This allows the initial optical density (OD) to be determined. NPs were prepared previously using solvent exchange method.

After 20 h incubation at 37 $^{\circ}$ C, cells were treated with drug alone (β -elemene), NPs without drug (POPC), β -elemene only nanoparticles and POPC coated β -elemene NPs starting with β -elemene concentration of 300 μ M followed by serial dilutions where each one was 2 times lower than previous. Eight wells were used for each condition. Control cells were treated just with medium. Following 72 h incubation time, 25 μ L of 5 mg/ml MTT solutions were added to each well without removal of the medium. Cells were incubated for a further 2 h at 37 $^{\circ}$ C to allow formazan production. The aqueous medium was then aspirated, and the formazan product solubilized in lysing buffer (100 μ L/well). Absorbance at 490 nm (optical density – OD490) was read using an Envision 2104 Multilabel Reader.

2.2.4.1.2 Flow Cytometry

The instrument used to observe cell viability in this project is Beckman Coulter FC500, which is a 5 colours analyser. Cells were seeded into two 6-well plates at a density of 2.5×10^4 cells/mL in 2 mL of culture medium, which allows the initial OD to be determined. After 20 h incubation at 37 $^{\circ}$ C, cells were treated with POPC coated β -elemene nanoparticles starting with β -elemene concentration of 300 μ M followed by serial dilutions where each one was 2 times lower than previous. Control cells were treated just with medium. Following 72 h incubation time, cells in each well were detached by 1ml trypsin-EDTA. After centrifugation at 800 rpm, cells were re-suspended in 500 μ L PBS and transferred to FACS tubes. 25 μ L of 50 μ g/mL PI was added to tubes of cells. Analyse cells immediately by using Beckman Coulter FC500.

Propidium Iodide (PI), being used in this project, cannot cross the plasma membrane so do not stain live cells. In the process of necrosis, the plasma membrane is compromised, and the dye can enter and stain the cells identifying them as necrotic. However, in the progress of apoptosis membrane integrity preserved and the dye cannot enter and stain the cells identifying them as apoptotic. When operating a Flow cytometer, PI can be used to distinguish necrosis and apoptosis after culturing cells with nanoparticles for 72 hours. After measurement, all information on every parameter is recorded as an FCS file or FCS compliant LMD files. Sometimes, colour compensation needs to be done when overlap happens. A portion of signal need to be removed to calculate a fluorochrome as a percentage

to measure itself[118]. Kaluza, a kind of useful software for flow cytometry, can be used to analyse information from FCS Files in detail.

2.2.4.1.3 Data Analysis

Estimated concentrations of each test agent were calculated that could inhibit cell proliferation by 50% (IC₅₀). The OD₄₉₀ value for the IC₅₀ was calculated using the following formula.

Equation 2-1

$$Percent = \frac{A_s - A_B}{A_C - A_B} \times 100\%$$

Where,

Percent = the percentage of viable cells

A_s = absorbance of samples

A_B = absorbance of blank solution

A_C = absorbance of control cells

This equation was used to obtain the percentage of viable cells. Then, data were typed in the GraphPad Prism 8 and graphs can be obtained from the software.

Data obtained from Flow cytometer can be analysed by using Kaluza, an analysis software from Beckman Coulter, Inc.

2.2.4.2 Cell uptake

In the experiment of observing cell uptake, nanoparticles need to be stained firstly. After fabrication of nanoparticles at a concentration of 0.15 µM, particles sizes were measured by using DLS. Then, 50 µL 1 mg/mL Dil stain stock solution was added into the vials, which contains 5ml nanoparticles solution, to stain nanoparticles with a colour of red. Particles sizes were measured after staining. An Amicon Ultra-15 tube, containing a filter with 3kDa cut off, was used to carry nanoparticles solution. After centrifugation at 2500 g for at least 45 minutes, nanoparticles were concentrated because they cannot pass through the filter. 250 µL nanoparticles solution was left finally. Particles sizes were measured after diluting nanoparticles 1:20 into solvent (10% ethanol-water).

Cells were seeded into glass base dish at a density of 2.5×10⁴ cells/mL in 2 mL of culture medium in the first day. After overnight incubation at 37 °C, medium was aspirated, and cells were washed with fresh medium for 2-3 times. Then, 1mL fresh growth medium was added and LysoTracker Green was diluted 1:10000 directly into medium for a working concentration of 100 Nano mol.

Growth medium containing dye in the glass base dish were removed and cells were washed with fresh growth medium 2-3 times. After that, 1 mL fresh growth medium were added to the glass base dish. Nanoparticles, already stained with Dil stain, were diluted 1:20 directly into normal growth medium for a working concentration of 0.15 Micro mol. The sample was observed using Confocal Microscope. The software took photos every 5 minutes for up to 40 minutes.

Another experiment consists of 6 samples. Sample 1 was analysed immediately after staining by using confocal microscope to get the image of cells. Growth medium containing dye in the glass base dish of sample 2-6 were removed and cells were washed with fresh growth medium 2-3 times. After that, 1 mL fresh growth medium were added to the glass base dish. Nanoparticles, already stained with Dil stain, were diluted 1:20 directly into normal growth medium for a working concentration of 0.15 Micro mol. Sample 2 was analysed immediately after staining by using confocal microscope while sample 3-6 was incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ for 0.5, 1, 2, 4 hours separately. Finally, after incubation, samples were analysed by using confocal microscope.

3 Results

3.1 Design and Fabrication

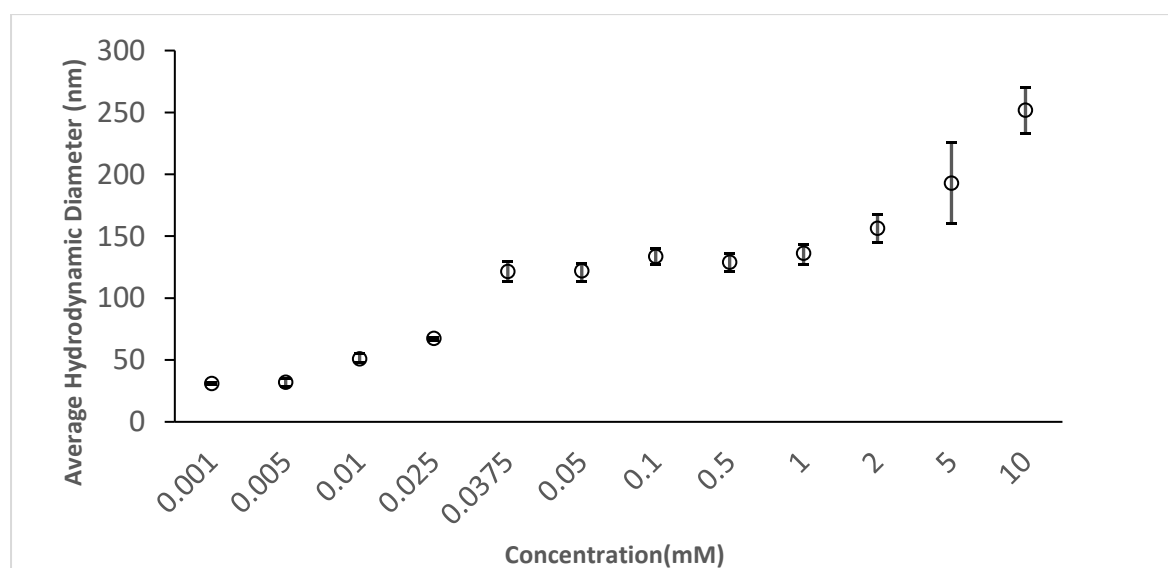
3.1.1 Aims and objectives

1. To design and fabricate POPC coated β -elemene nanoparticles.
2. To measure particle size and polydispersity index of POPC coated β -elemene nanoparticles with DLS.

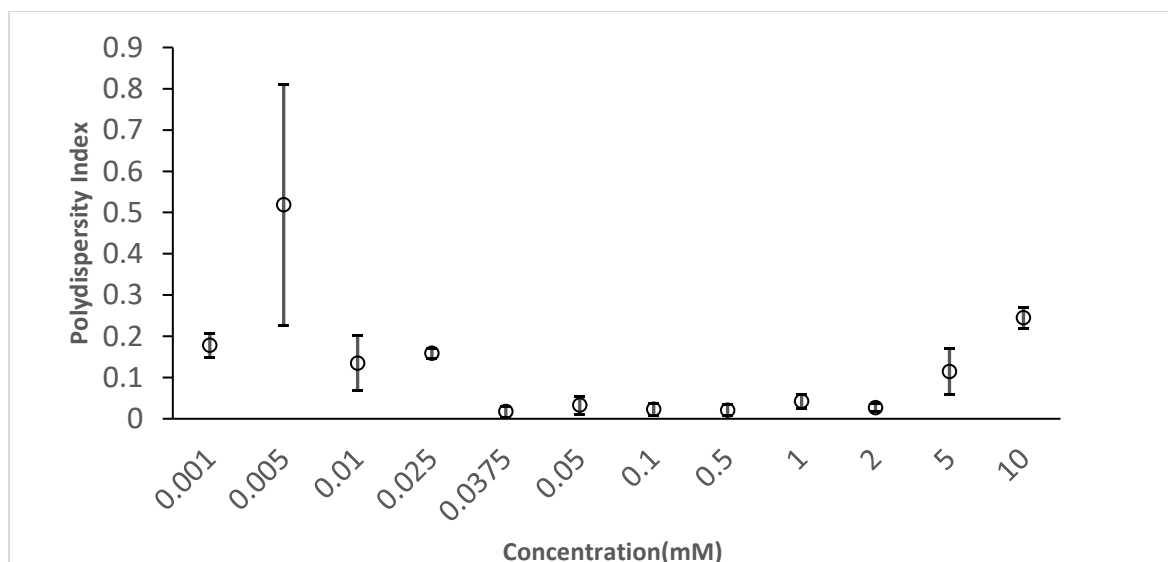
3.1.2 β -elemene nanoparticles

Motivated from the fabrication of triolein nanoparticles, the uncoated β -elemene nanoparticles were studied initially to explore the smallest possible size that can be fabricated using solvent exchange method. Particle size were measured by using DLS and TEM while polydispersity index was measured by using DLS. After knowing the initial size of pure β -elemene nanoparticles, POPC was introduced to fabricate POPC coated β -elemene nanoparticles. Results are presented in the following section.

3.1.2.1 Pure β -elemene nanoparticles



(a)



(b)

Figure 3-1 β -elemene nanoparticles in 10% ethanol-water (a) Particle size data obtained for β -elemene nanoparticles in 10% ethanol-water were plotted against varying final β -elemene nanoparticles concentrations. (b) Corresponding PDI values of avg. hydrodynamic diameters were plotted against varying final β -elemene nanoparticles concentrations. Data were obtained in triplicate. Error bars indicates std. deviation of the three individual values. β -elemene nanoparticles were prepared using solvent exchange method for mixing ratio of 1:9 at 500 RPM.

The Figure above illustrates that the average hydrodynamic diameters of β -elemene NPs prepared without lipids increase with the increase of β -elemene concentration. As is shown in **Figure 3-1**, Average PDI value for 0.005 mM were 0.519, indicating a possible low number density. For 0.001 mM final β -elemene concentration, there are two peaks in the graph of intensity PSD (shown in **Table 3-1**), indicating two size ranges of particles were fabricated. According to the particle size data for 0.001 mM final β -elemene concentration, some particles with 5000 nm diameter were fabricated. DLS consider large size aggregates as one big particle which lead to the reporting of larger particle size. Therefore, some small nanoparticles can aggregate and form an aggregate with large size. When concentration decreases, particle size was expected to decrease because of increased intermolecular distance and decreased collision frequency. From this point of view, the increase in size of β -elemene NPs with respect to concentration started at 0.005 mM β -elemene concentration, indicating the smallest possible size for β -elemene was around 30 nm. To demonstrate this smallest possible size, POPC was introduced to fabricate POPC coated nanoparticles.

Table 3-1 % Intensity distribution of 0.001 mM β -elemene NPs

Number of measurements	Pk 1 mean diameter (nm)	Pk 1 area percentage (%)	Pk 2 mean diameter (nm)	Pk 2 area percentage (%)
1	31.93	96.3	5452	3.7
2	29.74	90	4889	10
3	30.98	91.7	5070	8.3

3.1.2.2 POPC coated β -elemene nanoparticles

3.1.2.2.1 Effect of injection volume ratio of ethanol to water on the particle size and polydispersity of POPC coated β -elemene nanoparticles

Before fabricating POPC coated nanoparticles, the effect of different concentrations (%volume) of ethanol-water mixtures was investigated. In this experiment, nanoparticles were fabricated using direct injection (1:9) by following the set method for each approach as described in **section 2.2.1.2**. Experiments were carried out by using ethanolic β -elemene solutions at the concentration of 0.5 mM, 1 mM, 2 mM, 5 mM and 10 mM.

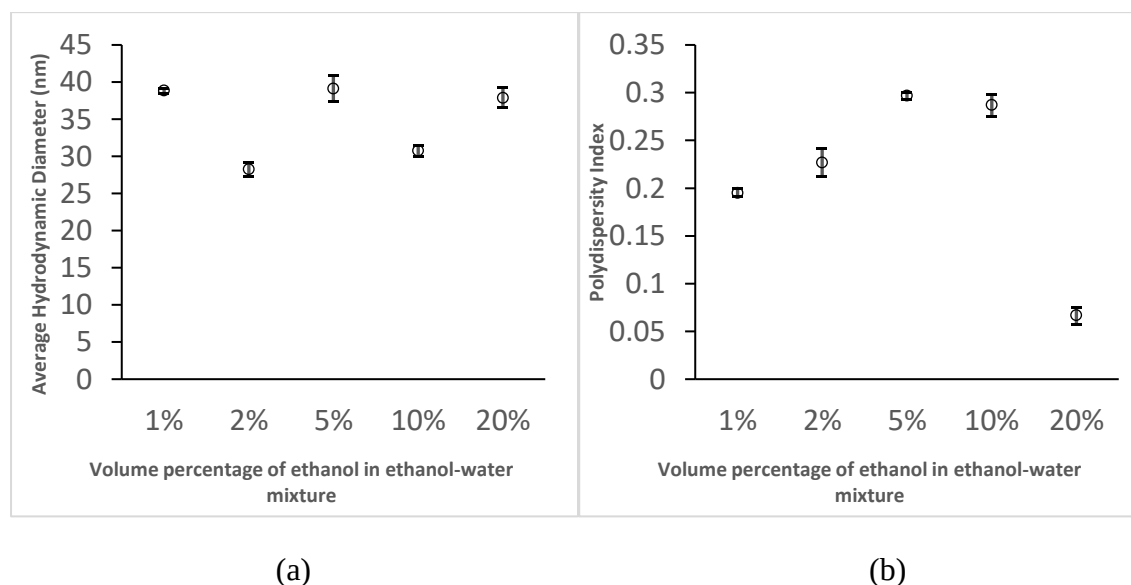


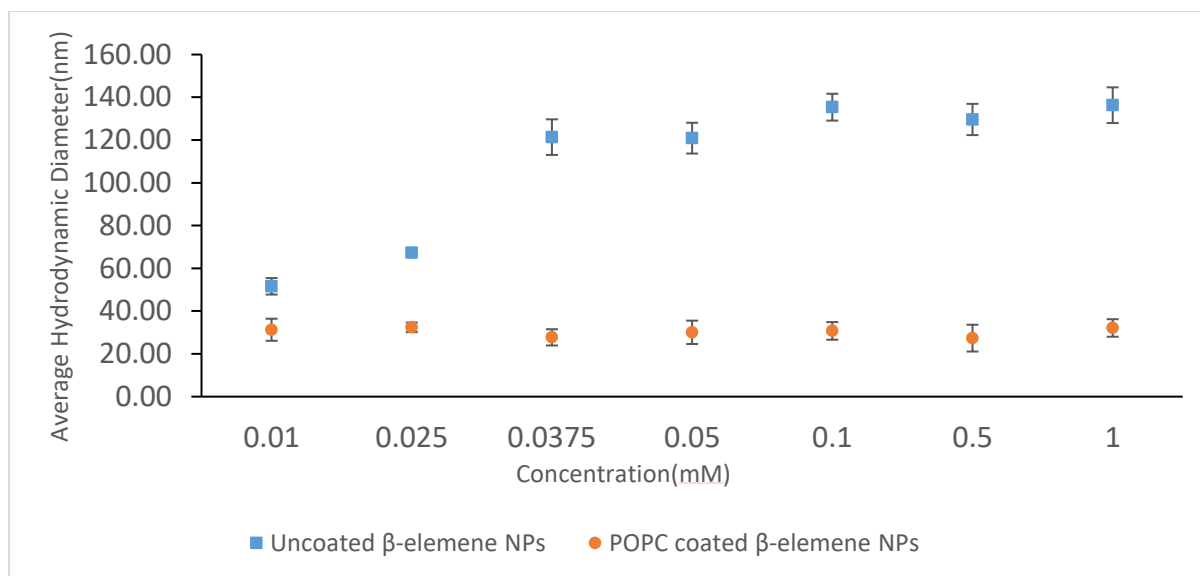
Figure 3-2 β -elemene and POPC in ethanol-water mixture using different volume % of ethanol (a) Particle size data of POPC coated β -elemene nanoparticles obtained from the DLS in the form of average hydrodynamic diameter were plotted against different injection ratio (x-axis). (b) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against different injection ratio (x-axis). Measurements for samples with 1:4 mixing ratio were carried out in 20% ethanol-water while for mixing ratio of 1:9,

1:19, 1:49 and 1:99, measurements were carried out in 10%, 5%, 2% and 1% ethanol-water separately.

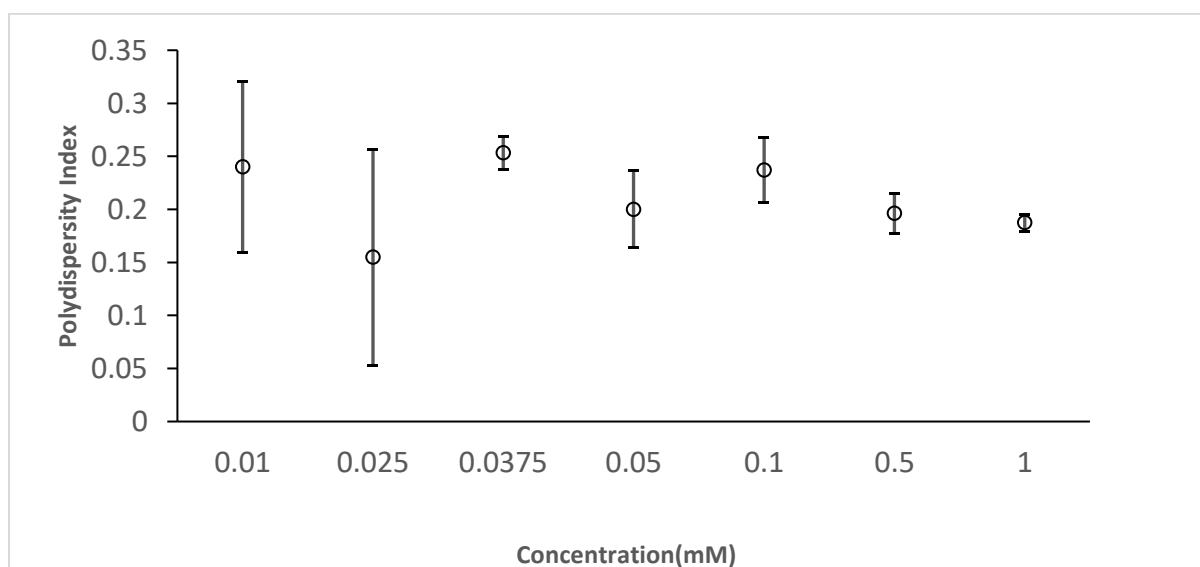
Figure 3-2 illustrates particle size data obtained for the β -elemene through solvent exchange method. It is apparent that at ethanol volume percentage of 2%, the smallest particle size (28.22 nm) can be obtained. Meanwhile, 30.73 nm nanoparticles, which is also acceptable, can be obtained at ethanol volume percentage of 10%. PDI value was found to be highest at ethanol volume percentage of 5%, which could be resulted from low particle concentration. It should be noted that for ethanol-water mixing ratio of 1:49, the initial concentration gets 50 times diluted. In other words, if 1 mM final concentration of β -elemene nanoparticles were aimed to be fabricated, 50 mM β -elemene ethanolic solution will be injected directly into water. However, the saturated mol concentration of POPC in ethanol is around 33 mM at room temperature. Hence, taking solubility, particle size and PDI into consideration, the results favoured 1:9 mixing ratio of ethanol and water.

3.1.2.2.2 Effect of concentration of β -elemene in ethanol on the particle size and PDI of POPC coated β -elemene nanoparticles

Investigation of injection volume was followed by POPC coated β -elemene NPs fabrication. To investigate the effect of concentration of β -elemene in ethanol, NPs were fabricated using solvent exchange method. As described in **section 2.2.1.2**, β -elemene solutions of initial concentrations 0.1 mM, 0.25 mM, 0.375 mM, 0.5 mM, 1 mM, 5 mM and 10 mM were mixed with POPC, whose concentrations were the same as β -elemene. Then, these premixed solutions were injected into distilled water directly. Particle size data for POPC coated NPs obtained from DLS is presented in the following figure along with the particle size data for uncoated β -elemene NPs.



(a)



(b)

Figure 3-3 Pure β -elemene nanoparticles and POPC coated β -elemene nanoparticles in 10% ethanol-water (a) Particle size data obtained for pure β -elemene NPs (shown as blue squares) and POPC coated β -elemene nanoparticles (shown as orange circles) prepared by using solvent exchange method technique and measured using DLS in 10% ethanol-water are plotted against final β -elemene nanoparticle concentrations (as shown on X-axis). (b) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters of POPC coated β -elemene nanoparticles (shown as black circles) against varying final β -elemene nanoparticles concentrations (as shown on X-axis). Data were obtained in triplicate. Error bars indicates std. deviation of the three individual values.

The Figure above clearly shows the difference of uncoated and coated β -elemene nanoparticles. The average hydrodynamic diameter of coated β -elemene nanoparticles in the range of 0.01 mM to 1 mM was found to be almost constant (27 to 33 nm), thus indicating the smallest size for β -elemene and showing the negligible effect of concentration on the size of coated nanoparticles.

3.2 Characterisation and optimization

3.2.1 Aims and Objectives

1. To characterize particle size and polydispersity index of POPC coated β -elemene nanoparticles with TEM.
2. To determine the influence of injection ratio on the size and PDI of β -elemene nanoparticles.
3. To optimize solvent exchange method and evaluate the physical stability of NPs.

3.2.2 TEM data

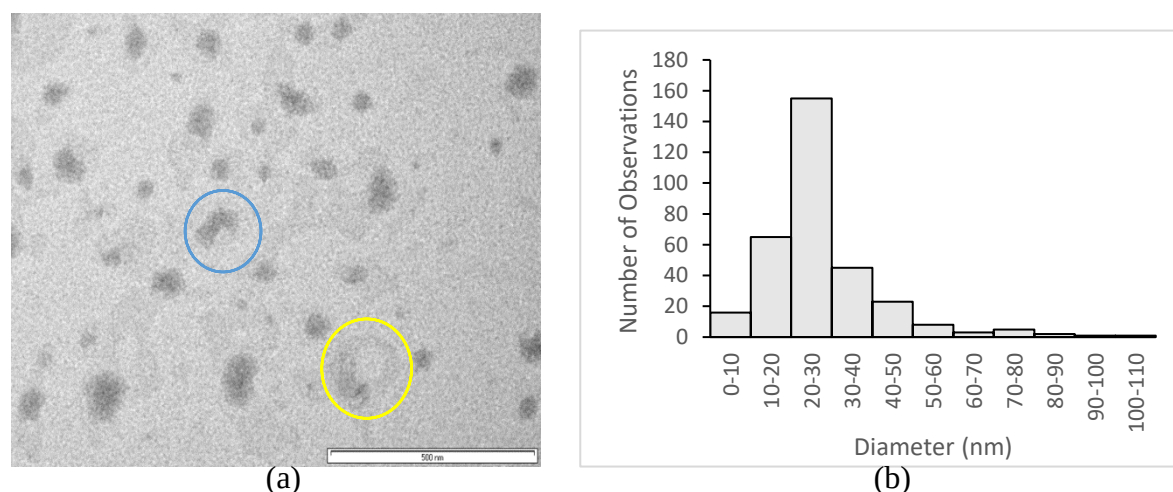


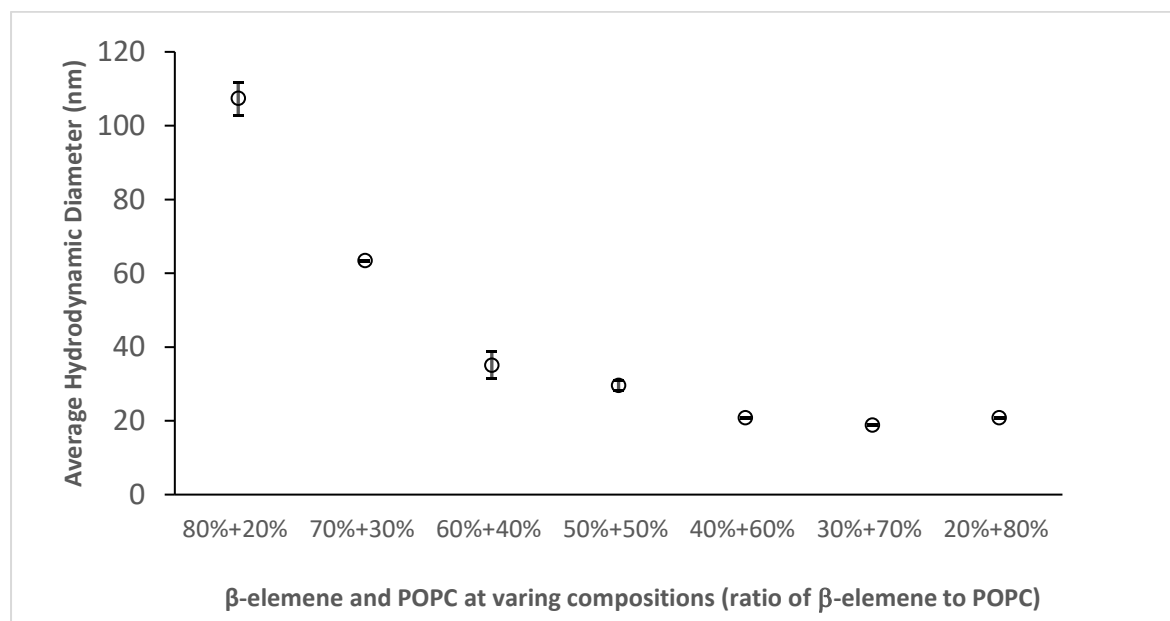
Figure 3-4 TEM image and image analysis results from POPC coated β -elemene nanoparticles (0.15 mM) in 10% ethanol-water (a) The TEM image obtained with Tecnai T12 Biotwin TEM. 13 μ L of a sample containing POPC coated β -elemene nanoparticles (0.15 mM) in water, was deposited on a carbon film. (b) Frequency distribution of the measured 324 particles in the form of histogram.

It clearly shows in the figure that most of the particles are singlet while some particles are aggregate along with some liposomes (yellow circle). These images were analysed by image J Fiji and the distribution of particles were presented in the form of histogram. Aggregates (blue circle) was considered as one large particle when measured with DLS, leading to the reporting of larger particle size.

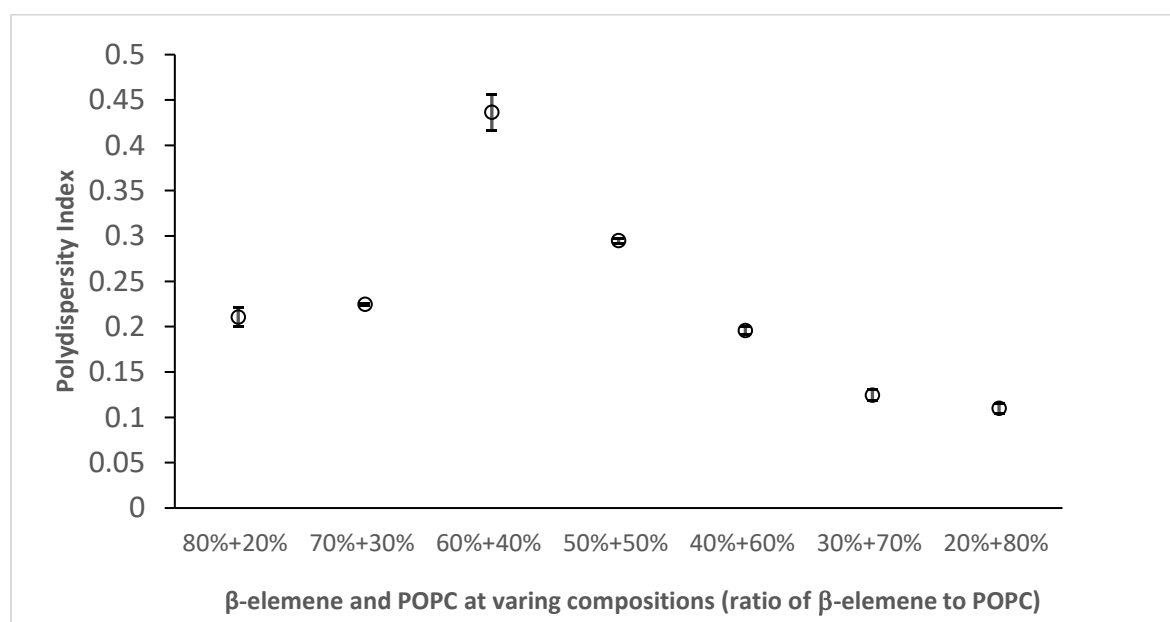
3.2.3 Optimization of β -elemene nanoparticle

3.2.3.1 Effect of solution ratio on the size and stability of β -elemene nanoparticles

Following the fabrication of POPC coated β -elemene nanoparticles, the next step was to optimize the solvent exchange method. To improve the quality of the nanoparticles (in the form of more stable and smaller size), solution ratio of β -elemene to POPC was optimized. Nanoparticles were fabricated by following the method in **section 2.2.2.2**. Nanoparticle size data were plotted in the following **Figure 3-5**.



(a)



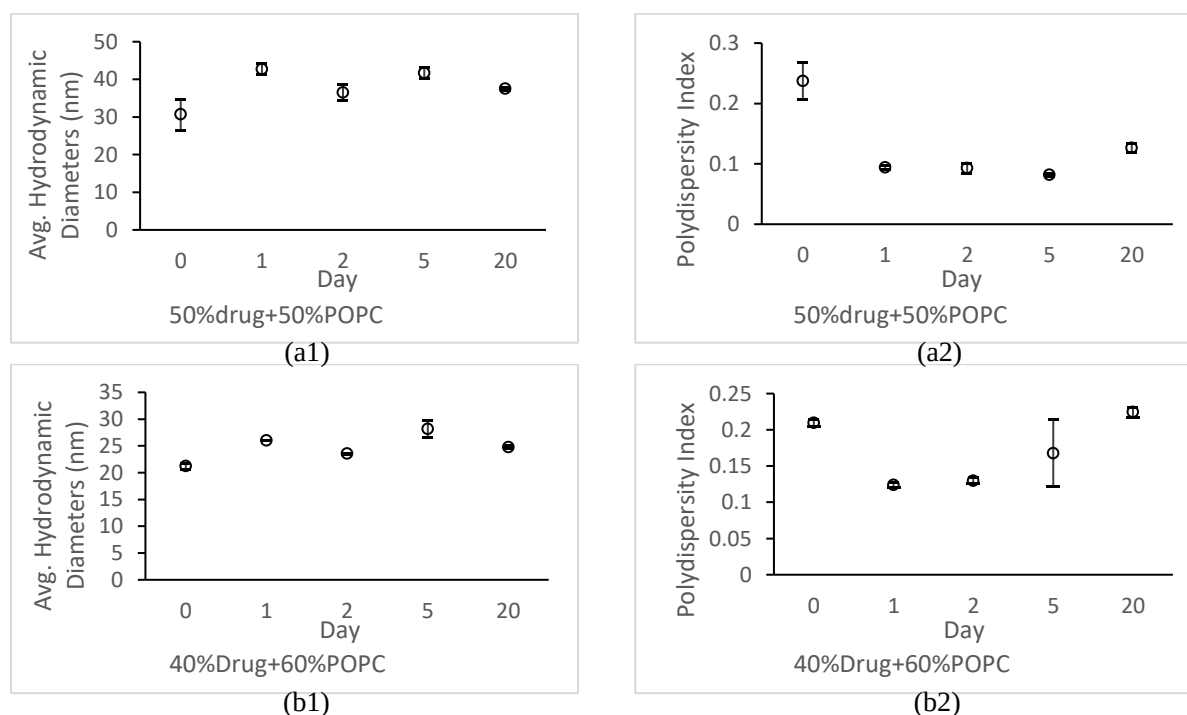
(b)

Figure 3-5 POPC coated nanoparticles of β -elemene and POPC at varying compositions
 (a) Avg. hydrodynamic diameters of POPC coated nanoparticles (shown on Y-axis) obtained from the cumulant mode of DLS were plotted against varying ratio of β -elemene to POPC (shown on X-axis). (b) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against varying ratio of β -elemene to POPC (as shown on X-axis). Data were obtained in triplicate. Error bars indicates std. deviation of the three individual values.

As shown in the **Figure 3-6**, the average hydrodynamic diameter of POPC coated β -elemene nanoparticles decrease with increasing of POPC composition. It clearly shows that at β -elemene ratio of 30%, the smallest size (18.9 nm) can be obtained. At the β -elemene ratio of 40% and 20%, the average hydrodynamic diameter are 20.87 nm and 20.85 nm respectively. Compared with the β -elemene ratio of 30%, particle sizes at the β -elemene ratio of 40% and 20% are also acceptable. Considering the polydispersity index, the β -elemene ratio of 20% is preferable.

To investigate the stability of β -elemene nanoparticles fabricated using different ratio of β -elemene to POPC, β -elemene nanoparticles at β -elemene ratio of 50%, 40%, 30% and 20% were selected and stored at room temperature (25 °C).

Particle size of β -elemene NPs was measured in the form of average hydrodynamic diameter using DLS at different time point. Sample measurements were carried out at 25 °C. The data obtained from DLS is shown in the following **Figure 3-7**.



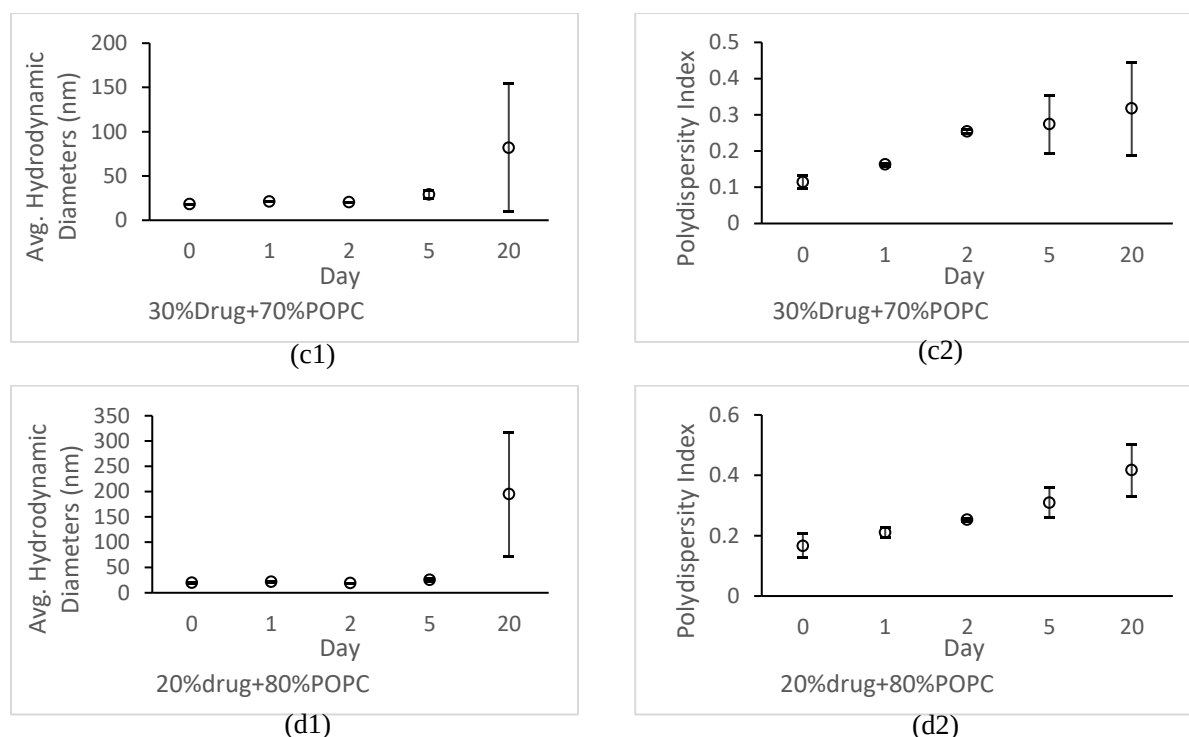


Figure 3-6 Stability of β -elemene nanoparticles in water. (a1) Particle size data of POPC coated NPs of 50% drug and 50% POPC were plotted against days. (a2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. (b1) Particle size data of POPC coated NPs of 40% drug and 60% POPC were plotted against days. (b2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. (c1) Particle size data of POPC coated NPs of 30% drug and 70% POPC were plotted against days. (c2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. (d1) Particle size data of POPC coated NPs of 20% drug and 80% POPC were plotted against days. (d2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. Data were obtained in triplicate. Error bars indicates std. deviation of the three individual values.

Figure 3-7 illustrates that particle size can change over time. For NPs fabricated with 50% β -elemene and 50% POPC or 40% β -elemene and 60% POPC, changes are not sharp. However, for NPs of 30% β -elemene and 20% β -elemene, aggregation is apparent. Ostwald ripening was observed after 5 days storage. Molecules on the surface of small particles detach from small particles and condense on the surface of larger particles, leading to the growth of larger particles. By comparing the stability of POPC coated nanoparticles of β -elemene and POPC

at varying compositions, mole percentage of 50% β -elemene and 40% β -elemene were selected to carry out the following experiments.

3.2.3.2 Effect of Temperature on the Stability of β -elemene Nanoparticles

To investigate effect of temperature on the stability of POPC coated β -elemene nanoparticles, NPs were stored at 4 °C for up to 20 days. Particle size was measured in the form of average hydrodynamic diameter at different time point. The data is shown below.

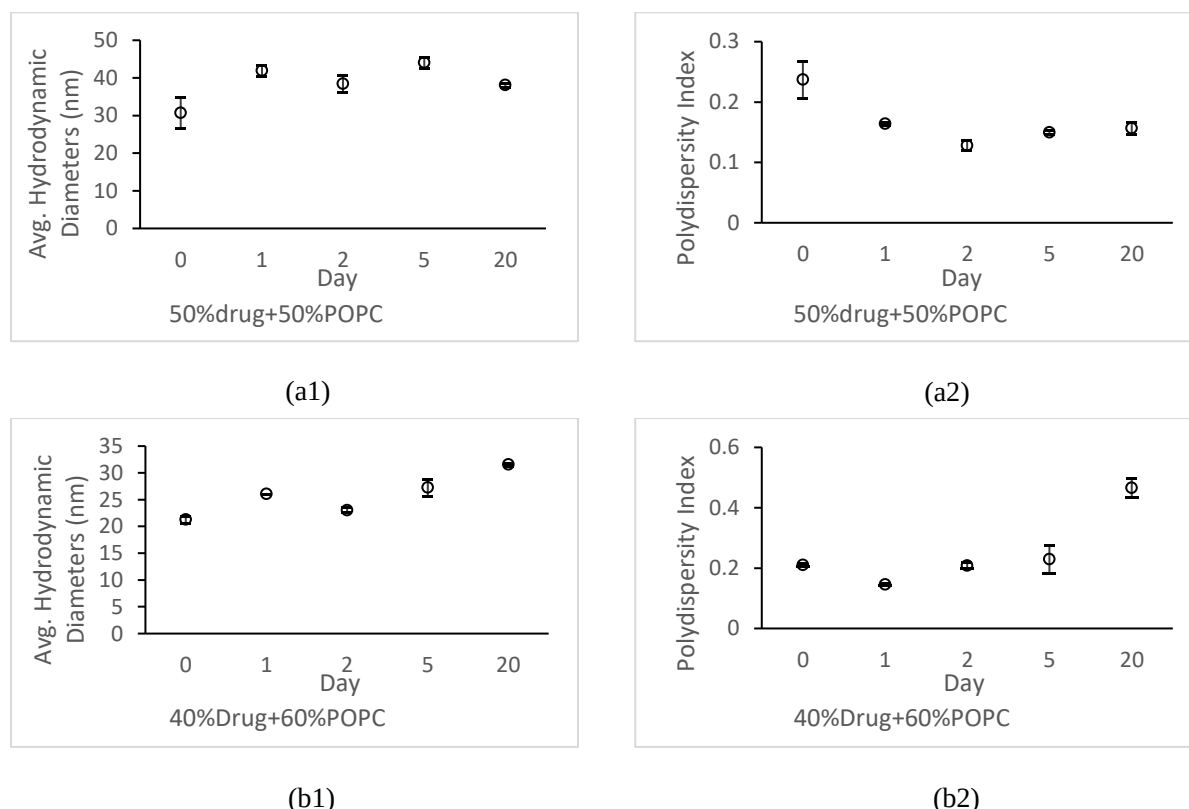


Figure 3-7 Stability of β -elemene nanoparticles in water stored at 4 °C. (a1) Particle size data of POPC coated NPs of 50% drug and 50% POPC were plotted against days. (a2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. (b1) Particle size data of POPC coated NPs of 40% drug and 60% POPC were plotted against days. (b2) The plot of corresponding polydispersity index values of the avg. hydrodynamic diameters against days. Error bars indicate std. deviation of the three individual values.

It can be seen in **Figure 3-8** that β -elemene NPs at the composition ratio of 40:60 aggregated apparently after 20 days storage at 4 °C while NPs at the composition ratio of 50:50 remain stable. Comparing the size data of β -elemene NPs stored at 4 °C and RT up to 20 days, β -elemene NPs at the composition ratio of 50:50 show the best size stability.

3.3 Evaluation

3.3.1 Aims and objectives

1. To compare the cytotoxicity of POPC coated β -elemene NPs with pure β -elemene and uncoated β -elemene NPs.
2. To observe cell uptake of POPC coated β -elemene NPs using confocal microscope.

3.3.2 MTT assay

In order to compare the effect of POPC coated β -elemene nanoparticles, pure β -elemene nanoparticles with free drugs in breast cancer cells, MTT assay was carried as described in **section 2.2.4.1.1**. Human breast cancer cells were exposed to serial dilutions of NPs and to β -elemene alone for 72 hours.

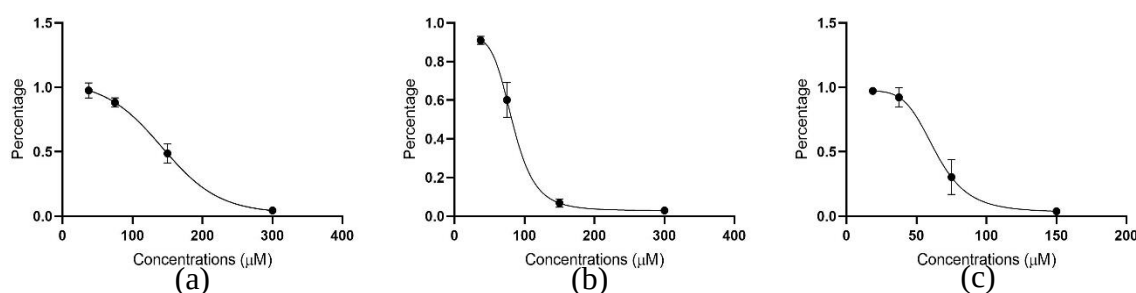


Figure 3-8 Cell growth curves generated from 72 h MTT assay data for MCF-7 cells (a) Growth inhibition data (shown on Y-axis) for POPC coated nanoparticles is plotted against β -elemene concentration (shown on X-axis). (b) Growth inhibition data (shown on Y-axis) for pure β -elemene nanoparticles is plotted against β -elemene concentration (shown on X-axis). (c) Growth inhibition data (shown on Y-axis) for pure β -elemene is plotted against β -elemene concentration (shown on X-axis). Error bars indicate std. deviation of values. Absorbance at 490 nm (optical density – OD490) was read using an Envision 2104 Multilabel Reader.

It can be seen in **Figure 3-8** that the percentage of viable cells approach to 0 at the highest concentration of 300 μM for POPC coated nanoparticles and pure β -elemene nanoparticles. This means at this concentration of β -elemene, almost all cells were inhibited. POPC coated β -elemene NPs shows extremely low cytotoxicity when the concentration is lower than 37.5 μM . For uncoated β -elemene NPs, a sharp increase of the cytotoxicity of appeared along with the concentration of β -elemene increases from 37.5 μM to 100 μM . Compared with uncoated β -elemene NPs, the cytotoxicity of POPC coated NPs increases gently in the process of β -

elemene concentration increasing. IC₅₀ values was obtained from the MTT assay and presented in the table below.

Table 3-2 IC₅₀ data obtained from MTT assay

Type	IC ₅₀ (μM)
Pure β-elemene nanoparticles	83.69
POPC coated β-elemene nanoparticles	157.6
β-elemene in DMSO	63.58
Pure POPC nanoparticles	>300

As shown in the table, pure drug in DMSO has the lowest IC₅₀ values compared with nanoparticles. For POPC NPs without drug, IC₅₀ value is larger than the other three.

3.3.3 Flow cytometry

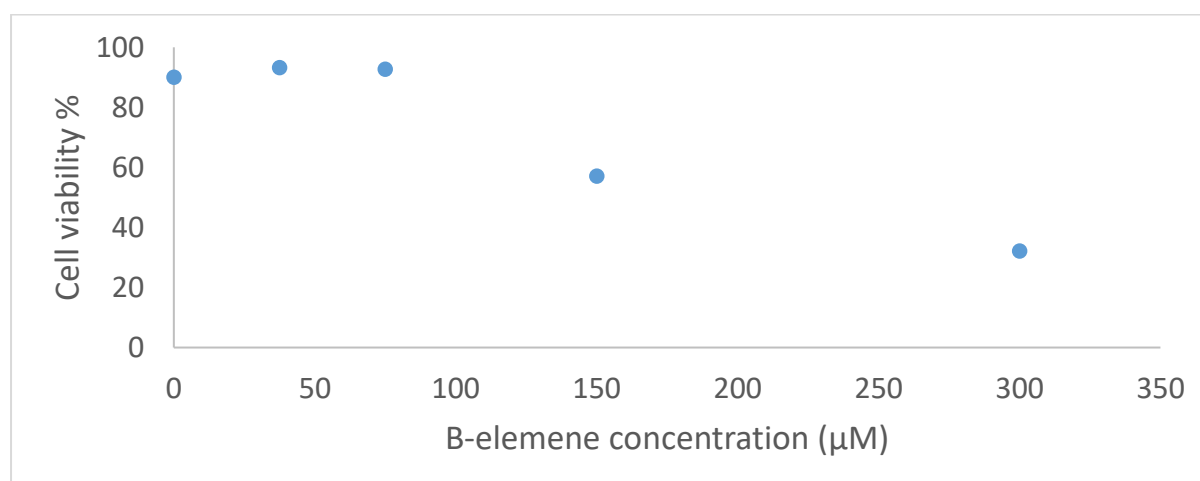
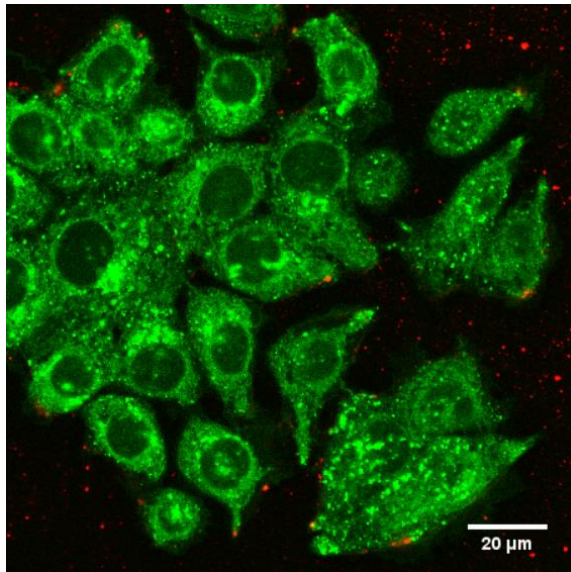


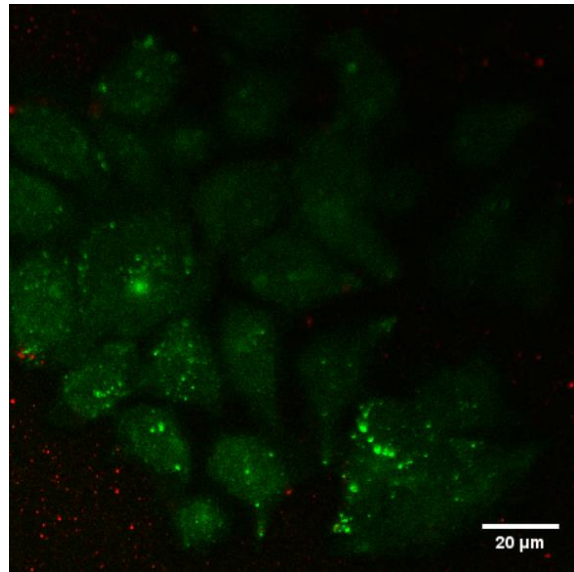
Figure 3-9 Cell viability data obtained from Flow cytometer. Cells were incubated with different concentration of POPC coated β-elemene nanoparticles for 72 hours and stained with propidium iodide.

It can be seen in **Figure 3-9** that POPC coated β-elemene NPs can inhibit cell growth apparently at highest concentration of 300 μM. The cell viability curve decreases gently with increasing of β-elemene concentration.

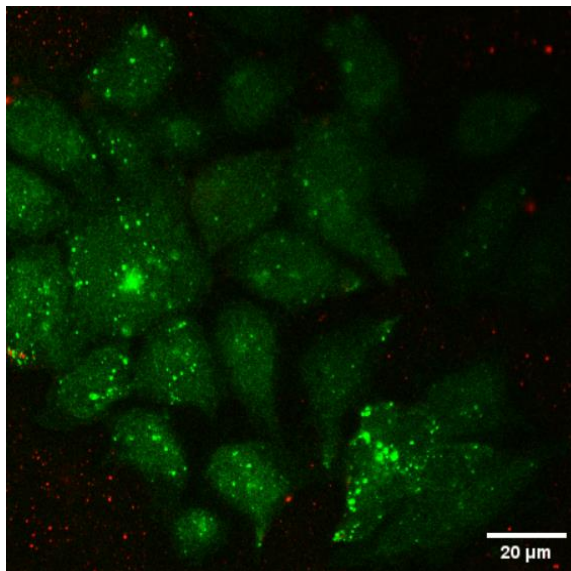
3.3.4 Confocal microscope



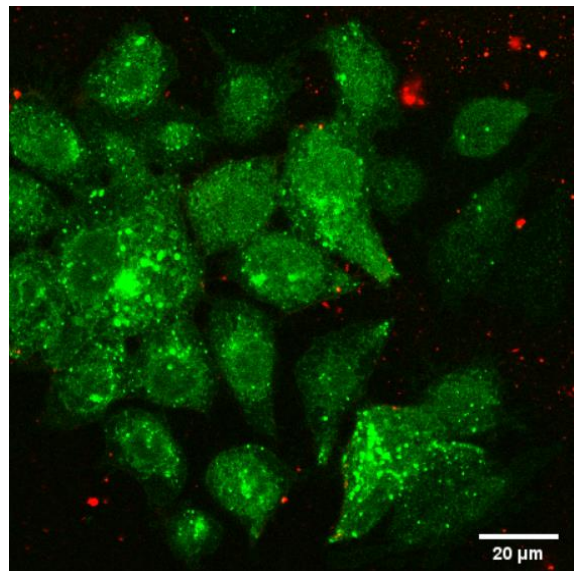
(a)



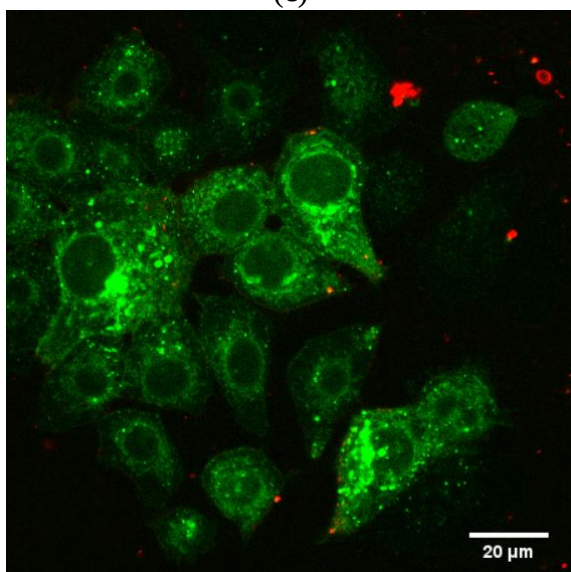
(b)



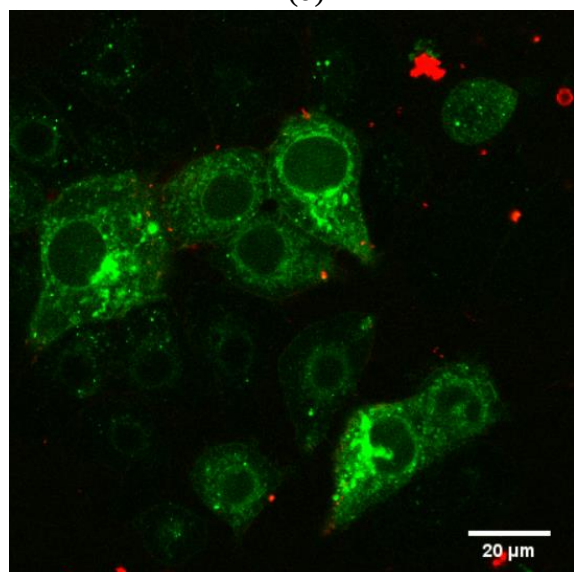
(c)



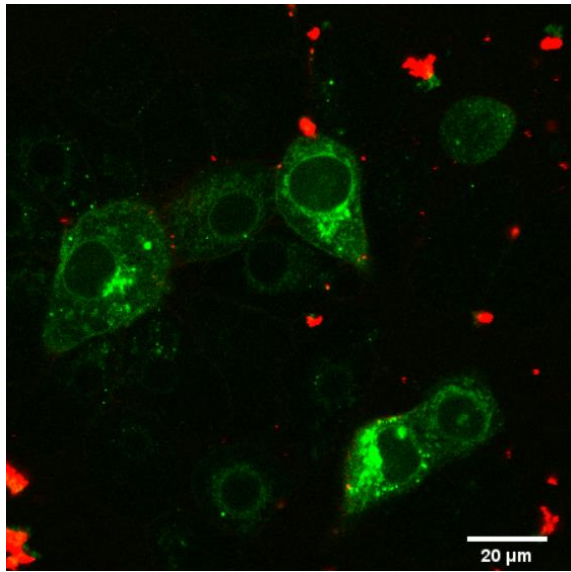
(d)



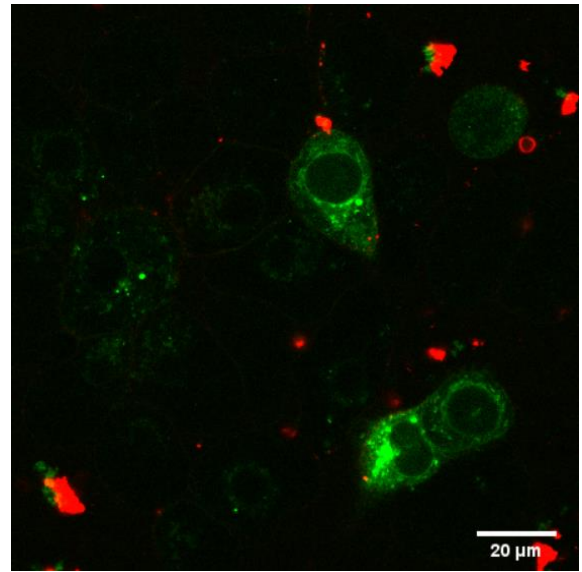
(e)



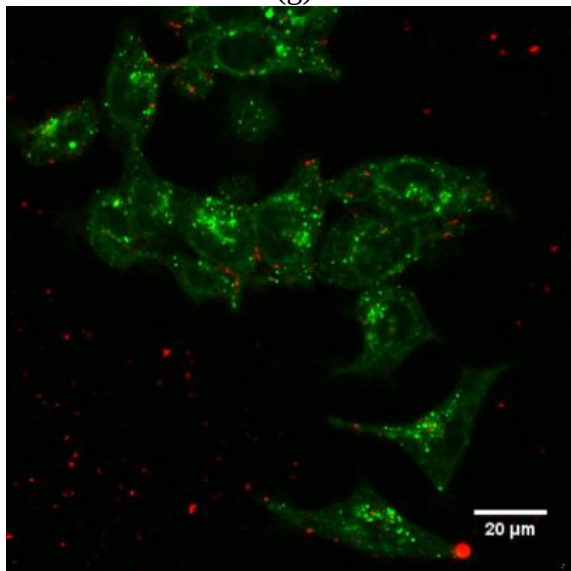
(f)



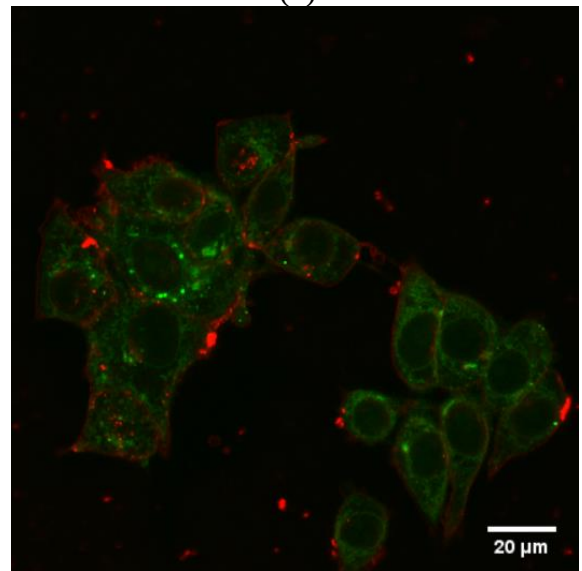
(g)



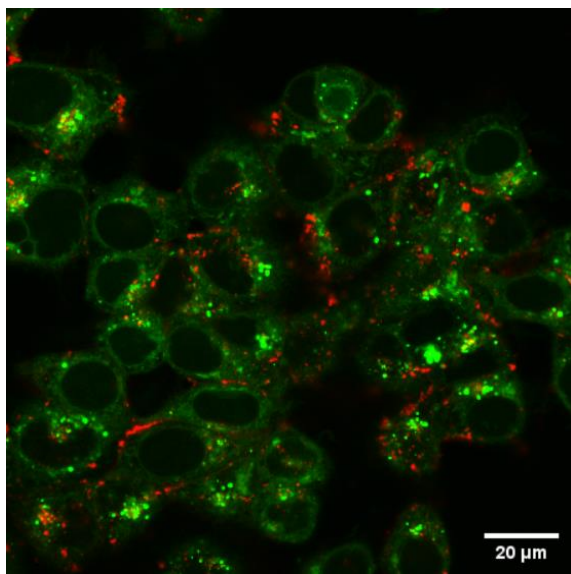
(h)



(i)



(j)



(K)

Figure 3-10 Cell uptake images consisting of cells (green) and nanoparticles (red) were obtained from Confocal microscope. (a) Image obtained at the time point of 5 minutes. (b) Image obtained at the time point of 10 minutes. (c) Image obtained at the time point of 15 minutes. (d) Image obtained at the time point of 20 minutes. (e) Image obtained at the time point of 25 minutes. (f) Image obtained at the time point of 30 minutes. (g) Image obtained at the time point of 35 minutes. (h) Image obtained at the time point of 40 minutes. (I) Image obtained at the time point of 60 minutes. (J) Image obtained at the time point of 120 minutes. (a) Image obtained at the time point of 240 minutes.

As shown in the figure above, cells were surrounded by NPs. At the time point of 5 minutes, some NPs attached to cell membrane while others just stay in the medium. At the time point of 20 minutes, some NPs were taken up by cells while some NPs aggregate and form a large particle. It can be clearly observed that a great number of NPs were inside cells at time point of 60 minutes. By comparing figures at different time points, it can be concluded that NPs started to reach lysosome at 20 minutes.

4 Discussion

In this section, fabrication, characterization and evaluation of β -elemene nanoparticles for anticancer treatment were discussed. This kind of NP fabricated using solvent exchange method is a novel carrier for hydrophobic anticancer drugs especially for β -elemene. The understanding of these NPs is significant for validating the appropriate conditions of fabricating NPs. A brief discussion of the obtained results was presented below.

4.1 β -elemene nanoparticles

4.1.1 Fabrication of β -elemene nanoparticles

In this project, uncoated β -elemene nanoparticles were fabricated first to explore the limit size of NPs. At low enough concentration for β -elemene, the particle size reaches a limit size of about 30 nm. Because of the poor water solubility of β -elemene (0.004 g/L at 25°C)[111], the system was supersaturated after injecting β -elemene solution into the water. In this situation, the dissolved molecules of β -elemene in the solution lead to the formation of the clusters of a new phase which are called as ‘Nuclei’. When the cluster or embryo reaches critical size, it can be regarded as the critical nucleus.

The surface tension of liquid β -elemene is 2.98×10^{-2} N/m while the molecule volume of β -elemene is 3.93×10^{-28} m³. Assuming the degree of supersaturation values for β -elemene is in the range of 1 to 1.3. NPs were fabricated at RT (298.15 K) under stirring. The critical diameters of β -elemene can be predicted using **Equation 1-5**. The predicted diameter (25.50 nm) support the data obtained from DLS, indicating that NPs fabricated in this project is supported by the classical nucleation theory. The difference between data obtained in this project and predicted diameter is negligible, owing to the possibility of difference in the assumed values of supersaturation degree and surface tension with actual values. DLS considers aggregates as one big particle, thus TEM was used to support the data obtained from DLS. Considering the possibility of aggregation, particle size data obtained from DLS match with the limit size.

As shown in **section 3.2.2**, POPC coated NPs were fabricated and a series of diameters data was obtained from DLS. The effect of concentration of β -elemene on particle size is negligible. In other words, POPC coated β -elemene NPs are more stable than uncoated β -elemene NPs. Prasad’s[119] fabricated and characterized lipid-coated stealth nanoparticles of Niclosamide stearate. They measured average hydrodynamic diameters at 0.1 mM and 1 mM, the diameters were found to be 20.4 ± 2.4 nm and 27.4 ± 3.3 nm respectively. Compared with

the work they carried out, β -elemene is a good selection for the core of NPs fabricated using rapid solvent exchange method. Meanwhile, lipid coated NPs fabricated in this project are stable against concentration.

4.1.2 Optimization and stability of β -elemene NPs

In this project, rapid solvent exchange method was used to fabricate NPs. In **section 3.2.2**, the effect of different concentrations (%volume) of ethanol-water mixtures was investigated to optimize the rapid solvent exchange method. As mentioned in **section 1.4.5.1**, the critical nucleation radius is inversely proportional to the natural log of degree of supersaturation and proportional to the surface tension. Lower concentration of ethanol in ethanol-water mixtures during injection period can ensure the system reach supersaturation, leading to smaller particle size. The saturated mol concentration of POPC in ethanol is fixed and hence 1:9 mixing ratio of ethanol and water is favoured.

As presented in **section 3.2.4**, influence of different ratios of β -elemene to POPC in β -elemene-POPC solutions on particle size were investigated. Composition ratio 50:50 of β -elemene NPs was selected as the most stable concentration ratio of β -elemene to POPC, which can remain stable for 20 days at RT or 4 °C while NPs fabricated with other composition ratio aggregated. Compared with some marketed nanomedicines like Myocet®, Doxil® and Abraxane®, which are supplied as power and in-use stability after reconstitution is reported to be around a week. For example, Chemical and physical in-use stability of Myocet® after reconstitution has been demonstrated for up to 8 hours at 25°C, and for up to 5 days at 2°C – 8°C[120]. The stability of POPC coated β -elemene NPs were considered to be good.

4.2 Evaluation of POPC coated β -elemene nanoparticles

In this project, the activity of NPs against cancer cell line was evaluated. In the section of cytotoxicity assay, Activity of NPs against MCF-7 cells were assessed. Although the IC₅₀ value of POPC coated β -elemene is larger than the value of pure β -elemene, the cell growth inhibition curve is also acceptable. Data obtained from *in vitro* experiments just show the potency of drugs in MCF-7 cells. The aim of fabricating this LDL like NPs is targeting to the tumour site. So, it can be said that the efficiency of POPC coated β -elemene and efficiency of pure β -elemene are comparable. To determine the actual potency of POPC coated β -elemene NPs, an *in vivo* experiment become the next important task for the future work.

Cell uptake of materials from the cell surface to lysosomes starts with the process of endocytosis, in which materials are enclosed by a small portion of the cell membrane and bud

off inside the cell. Endocytosis can be classified as two main types: phagocytosis and pinocytosis. Pinocytosis, which involves the invagination of the cell membrane and ingestion of fluid and solutes, internalize small particles within the range of nanometre. As a type of pinocytosis, Clathrin-mediated endocytosis is the cellular entry mechanism to internalize nutrients including cholesterol by low-density lipoprotein receptor. As shown in **section 3.5**, NPs bound to the surface of the cell membrane at time point of 5 minutes. At 25 minutes, NPs eventually reach lysosomes via the endo-lysosomal pathway. After this time point, more and more NPs were invested by MCF-7 cells. By conducting confocal microscope, cancer cell uptake of POPC coated β -elemene NPs was confirmed.

5 Conclusions and future perspectives

5.1 Conclusions

This project focused on fabricating a kind of NPs which can target tumour site with hydrophobic drugs. Motivated from the properties and structures of LDL, this thesis provides a detailed insight into the fabrication, characterization and evaluation of β -elemene NPs.

This project can be summarized in three sections: “design and fabrication”, “characterization and optimization” and “evaluation”. In the “design and fabrication” section, β -elemene was selected as the hydrophobic core and POPC was selected as the out shell. By injecting β -elemene ethanolic solution into water under stirring, nanoparticles of hydrophobic drug β -elemene were fabricated. For solvent exchange method, there is a principle underlying it. When a hydrophobic solute, dissolving in a suitable solvent, is exposed to an excess of an anti-solvent rapidly, this solute must precipitate out of solution due to change in the solubility. Fabrication of β -elemene NPs reveals that initial concentration of β -elemene in ethanol can affect the diameter of uncoated β -elemene NPs while diameters of POPC coated NPs almost remain unchanged.

In the “characterization and optimization” section, particle size and polydispersity index were measured using DLS. Besides, TEM was used properly to obtain parameters of nanoparticles. By optimizing the injection volume ratio of ethanol to water, 1:9 injection ratio was preferred for later injection. Meanwhile, some parameters were investigated to optimize the solvent exchange method. In order to investigate the most preferable composition ethanolic used properly to obtain parameters of nanoparticles solution, different ratio of β -elemene to POPC was investigated. Considering the stability of NPs, 1:1 ratio was selected as the most preferable ratio of β -elemene to POPC.

In the “evaluation” section, nanoparticles were evaluated through carrying out *in vitro* experiment. Cytotoxicity assay was carried out to test the efficiency of β -elemene NPs. For MTT assay, MCF-7 cells were incubated with β -elemene NPs for 72 hours at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ followed by adding MTT and reading absorbance at 490 nm. An Envision 2104 Multilabel Reader was used to read the absorbance. Meanwhile, Flow cytometry was used to evaluate cell viability of β -elemene nanoparticles. Cell uptake of β -elemene nanoparticles was also of interest in this project. By using a confocal microscope, cell uptake of β -elemene NPs can be observed clearly against time.

5.2 Future work

Encapsulation efficiency is an important parameter to ensure drug amounts at a therapeutic level. Hong Yao, who is a visiting PhD student and worked on β -elemene NPs in our laboratory before, had researched the POPC coated β -elemene nanoparticles including encapsulation efficiency. However, measuring the encapsulation efficiency using another method would still be beneficial.

Cytotoxicity evaluation of β -elemene NPs on cells was carried out in this project. However, the activity of NPs in wild type cancer cell lines and selectivity of NPs towards non-cancerous cells remains unexplored. These works will be the next task for the future study. It is important to note that these simulated *in vitro* conditions are far from *in vivo* conditions, so the stability of POPC coated β -elemene NPs in plasma and drug release in tumour site experiments should be done. Further work should also involve evaluating the effect of POPC coated β -elemene NPs in metastasis model.

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Appendices

A1 TEM Images

1.1 Uncoated β -elemene Nanoparticles

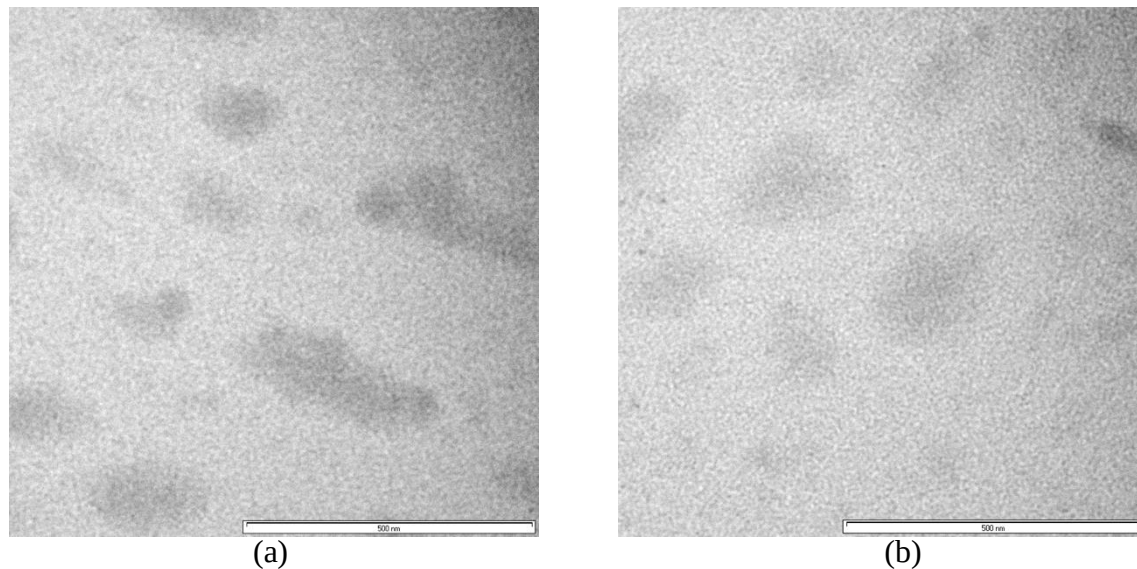


Figure A3 TEM images of pure β -elemene nanoparticles (0.15 mM) in water

The TEM image obtained with Tecnai T12 Biotwin TEM. 13 μ L of a sample containing pure β -elemene nanoparticles (0.15 mM) in water, was deposited on a carbon film.

1.2 POPC Coated β -elemene Nanoparticles

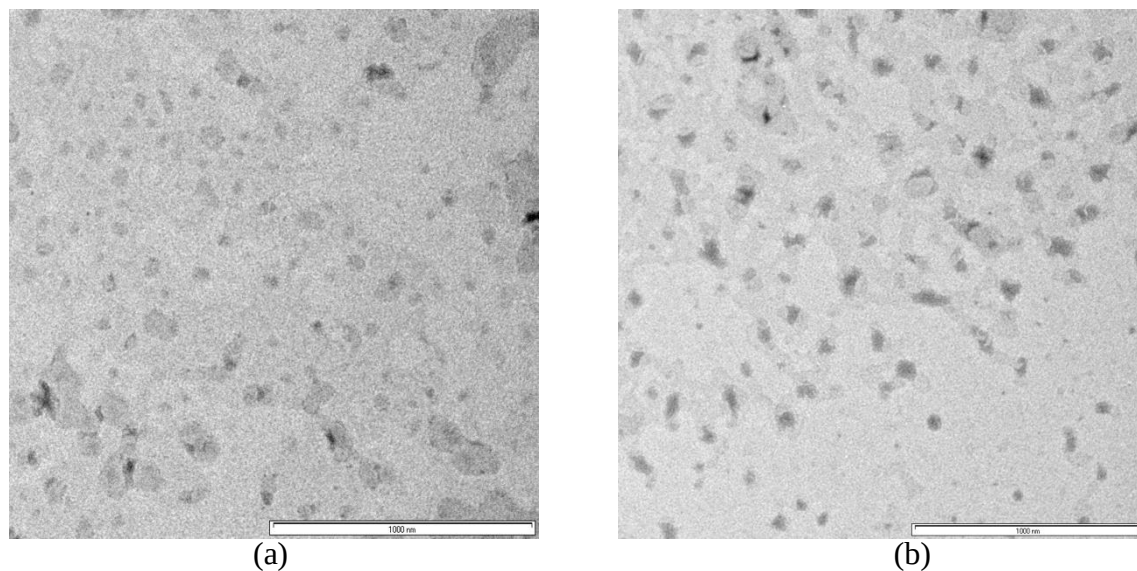


Figure A4 TEM images of POPC coated β -elemene nanoparticles (0.15 mM) in water

The TEM image obtained with Tecnai T12 Biotwin TEM. 13 μ L of a sample containing POPC coated β -elemene nanoparticles (0.15 mM) in water, was deposited on a carbon film.

1.3 Pure POPC Nanoparticles

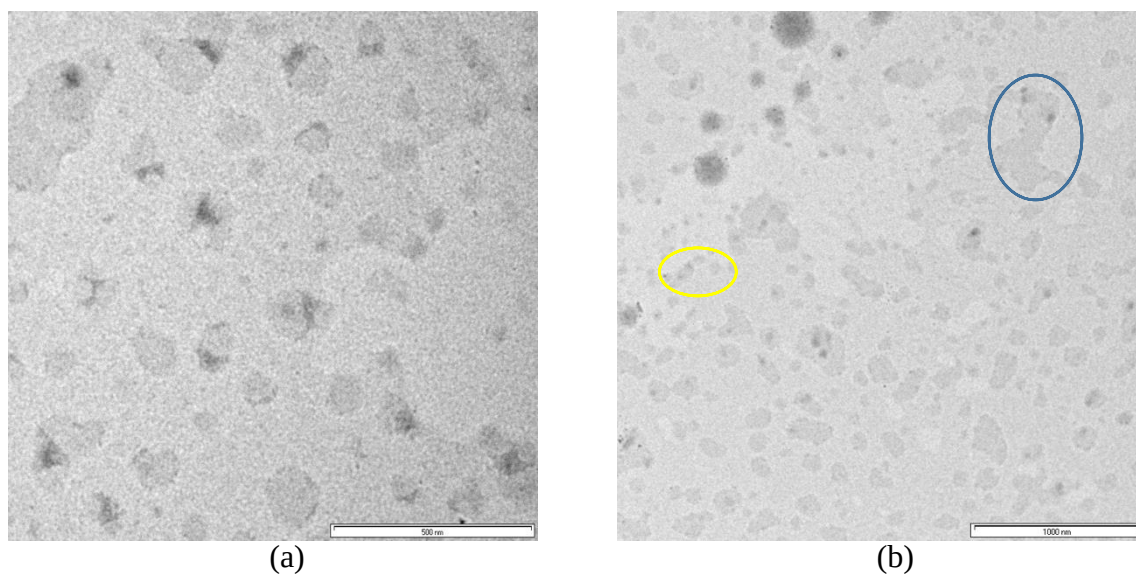


Figure A5 TEM images of pure POPC nanoparticles (0.15 mM) in water

The TEM image obtained with Tecnai T12 Biotwin TEM. 13 μL of a sample containing pure POPC nanoparticles (0.15 mM) in water, was deposited on a carbon film.

The image above shows pure POPC nanoparticles, along with some odd shapes (shown in blue circles). If the aggregate, shown in yellow circle, measured in DLS, it will be considered as one particle.