ATRACTYLODIN-LOADED PLGA NANOPARTICLES AND CYTOTOXIC EFFECTS IN CHOLANGIOCARCINOMA CELLS

BY

MISS NADDA MUHAMAD

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (BIOCLINICAL SCIENCES)
CHULABHORN INTERNATIONAL COLLEGE OF MEDICINE
THAMMASAT UNIVERSITY
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was approved as partial fulfillment of the requirements for
the degree of Master of Science in Bioclinical Sciences

on May 1, 2018

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ABSTRACT

The anti-cholangiocarcinoma activity of atractylodin, a major natural active constituent of the rhizomes of *Atractylodes lancea* (Thunb.) DC, has been demonstrated in a series of studies. However, this compound has poor water solubility and needs to be dissolved in organic solvent before used. Poly (lactic-co-glycolic acid) (PLGA) is currently extensively developed as polymeric nanoparticles for delivering hydrophobic drugs and enhancing its solubility in water. This present study aimed to develop atractylodin-loaded PLGA nanoparticles (AL-loaded PLGA NPs) and investigate *in vitro* cytotoxic activity against cholangiocarcinoma cell lines. The nanoparticle formulations were prepared using solvent displacement method. The encapsulation and loading efficiency were characterized and particle size, and zeta potential were determined by dynamic light scattering technique. Drug release was assessed *in vitro*. All NPs were found to be freely dispersible in water without aggregation. The size (mean±SD of diameter) of the prepared AL-loaded PLGA NPs using Resomer® RG502 and Resomer® RG504 were 158.33±0.21 nm and 161.27±1.87 nm, respectively with

narrow size distribution. The zeta potential values of both nanoparticle formulations were observed to be lower than -20 mV which would facilitate stability of NPs. The % encapsulation efficiency (%EE) and % loading efficiency (%LE) of AL-loaded PLGA NPs using Resomer® RG502 were 52.02±1.64% and 2.30±0.07%, respectively and found to be significantly higher than that of using Resomer® RG 504. Drug release from AL-loaded PLGA NPs both formulations were observed up to 88% in 72 hours with biphasic manner. AL-loaded PLGA NPs using Resomer® RG502 were selected to study the stability and to investigate *in vitro* cytotoxic activity against cholangiocarcinoma cell lines, CL-6 and HuCCT-1. The best condition for storage of NPs was found to be stored in ultrapure water at 4 °C. For the *in vitro* cytotoxic activity, AL-loaded PLGA NPs were concentration- and time-dependent. The IC_{50} (mean±SD) of AL-loaded PLGA NPs at 72 hours were 38.28±2.62 µg/ml on CL-6 and 48.08±1.07 µg/ml on HuCCT-1 which were lower than that of atractylodin dissolved with water (IC_{50}; mean±SD: 42.83±2.97 µg/ml and 63.71±3.90 µg/ml, respectively). Moreover, the toxicity on normal cell line, OUMS-36T-1F, of AL-loaded PLGA NPs were not found to be different from atractylodin dissolved with water. Besides, the blank PLGA NPs were observed to be non-toxic to both cholangiocarcinoma and normal cell lines. From these findings, the AL-loaded PLGA NPs were successfully developed and had potential to be used as drug delivery systems for the treatment of cholangiocarcinoma.

Keywords: Atractylodin, Poly (lactic-co-glycolic acid) (PLGA), Nanoparticles, Solvent displacement method, Cholangiocarcinoma

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Miss Nadda Muhamad

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
5-FU	5-fluorouracil
μg	Microgram
μM	Micromolar
CCA	Cholangiocarcinoma
CL-6	Human cholangiocarcinoma cell line
DDS	Drug Delivery System
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation efficiency
FBS	Fetal Bovine Serum
Gd-DTPA	Diethylenetriaminepentaacetic acid gadolinium (III)
g	Gram
h	Hour
HUCCT-1	Human cholangiocarcinoma cell line
IC_{50}	Half maximal inhibitory concentration
IL	Interleukin
LE	Loading efficiency
mg	Milligram
MIC	Minimal Inhibitory Concentration
ml	Milliliter
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MW	Molecular weight
nm	Nanometer
NPs	Nanoparticles
OUMS-36T-1F	Normal fibroblast cell line

Symbols/Abbreviations	Terms
PBS	Phosphate Buffer Solution
PCL	Poly epsilon-caprolactone
PDI	Polydispersity index
PEG	Polyethylene glycol
PEO	Polyethyleneoxide
PLA	Poly (lactic acid)
PLGA	Poly (lactic-co-glycolic acid)
PPO	Polypropyleneoxide
rpm	Round per minute
RPMI	Roswell Park Memorial Institute
SPIO	Super paramagnetic iron oxide
Tg	Glass transition temperature
TPGS	D-alpha-Tocopheryl polyethylene glycol 1000 succinate
US FDA	United States Food and Drug Administration
UV	Ultraviolet

CHAPTER 1
INTRODUCTION

Cholangiocarcinoma (CCA) or bile duct cancer arises from the epithelial cells of the intrahepatic or extrahepatic bile ducts and it is classified based on anatomical location including intrahepatic, extrahepatic i.e. perihilar and distal CCA.[1,2] This cancer is one of the most mortality and morbidity cancer in Thailand especially in northeastern area with the incidence rate 85 cases per 100,000 people per year. Whereas incidence rate in American, European, and another Asian countries less than 10 cases per 100,000 people per year.[3] CCA can caused by cirrhosis, viral hepatitis B and C and liver fluke *(Opisthorchis viverrini, Clonorchis sinensis)* infection.[3,4] The most common cause of CCA in Thailand is *O. viverrini* infection that caused by consuming raw food or uncooked food from freshwater cyprinoid fish, *koi-pla* or *pla-ra*, that contains *O. viverrini*.[5]

The treatment of CCA including surgery, radiotherapy, and chemotherapy. Most of CCA patients presented unresectable or metastatic stage of the disease that have to be treated with chemotherapy or chemoradiotherapy.[6,7] In the recent, chemotherapeutic regimens for the treatment of CCA are 5-fluorouracil (5-FU) base and gemcitabine base in combination with another chemotherapeutic drugs or targeted therapy.[3,6,7] The combination of chemotherapeutic drugs can prolonged survival time of CCA patients. However, the overall survival of patients who received combination regimen still less than 12 months.[6]

Atractylodin is an active compound contained in rhizome of traditional Chinese medicine, *Atractylodes lancea* (*A. lancea*) (Thunb.) DC. It has been found anti-cholangiocarcinoma activity greater than the standard drug, 5-FU.[8,9] However, atractylodin insoluble in water and has to be dissolved in organic solvent[10,11] which is harmful to administer via oral route and might have poor oral bioavailability.

Nanoparticles (NPs) are the particulate dispersions or solid particles with size in range of 10-1000 nm. They extensively used as drug delivery system (DDS) to increase efficacy and safety of various chemotherapeutic drugs.[12-15] Furthermore, NPs are helpful to enhance water solubility of hydrophobic drugs[12-13] and improve

pharmacokinetic[14-16] and biodistribution profiles.[13] NPs are not only used to be DDS of chemotherapeutic drugs but also traditional medicines or natural active compounds.[17-20]

Poly (lactic-co-glycolic acid) (PLGA) is a biocompatible and biodegradable copolymer which is approved to be used in clinical by US FDA.[21,22] This copolymer is widely used to prepare polymeric NPs to deliver chemotherapeutic drugs.[13,15,16] Traditional medicines or natural active compounds that have anticancer activities, for instance, curcumin and quercetin were also delivered by PLGA NPs.[20,23] Moreover, PLGA copolymer has potential to deliver both hydrophobic and hydrophilic drugs[13-16] and can be administered via both oral and parenteral route.

There are many studies of development of NPs to deliver chemotherapeutic drugs and targeted therapy for the treatment of CCA, for instance, 5-FU-loaded gold NPs[24], sorafenib-incoperated dextran/PLGA NPs[25], and vorinostat-incoperated PLGA-PEG NPs.[26] However, there is not a study of development of NPs to deliver atracylodin. Thus, from the benefits of NPs and great properties of PLGA, development of atracylodin-loaded PLGA NPs could help to enhance water solubility and efficacy of this compound on cholangiocarcinoma cells.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Cholangiocarcinoma

Cholangiocarcinoma (CCA) or bile duct cancer arises from the epithelial cells of the intrahepatic or extrahepatic bile ducts, and it is classified based on anatomical location including intrahepatic, extrahepatic, i.e., perihilar and distal CCA. Intrahepatic CCA is located at proximally to second-degree bile ducts in the liver, perihilar CCA is located between second-degree bile ducts and insertion of cystic duct into the common bile duct, and distal CCA located between the origin of cystic duct and ampulla of Vater **(Figure 2.1)**.[1] Perihilar CCA is the most common cancer, around 60%-70%, whereas intrahepatic CCA is the least common cancer, about 5%-10%.[2] Most of CCA patients presented unresectable or metastatic disease and resulted in poor survival rate.[27]

2.1.1 Epidemiology and risk factor

CCA is public health problem of Thailand particularly in the northeastern area, and it is the cause of morbidity and mortality with incidence rate 85 cases per 100,000 people per year. Whereas the incidence rate in Europe, America, and another country in Asia less than 10 cases per 100,000 people per year.[3] The risk factor of CCA are cirrhosis, viral hepatitis B, and C that causes intrahepatic CCA, primary sclerosing cholangitis, gallstones and liver fluke *(Opisthorchis viverrini*, *Clonorchis sinensis)* infection.[4-6] In Thailand, the primary cause of CCA of northeastern Thai populations is *O. viverrini* infection. Because of their cultural traditional consuming raw food or uncooked food from freshwater cyprinoid fish, *koi-pla* or *pla-ra*, that contains *O. viverrini*.[5] There is a study reported that from 85,927 cases who screened for *O. viverrini*, 25,445 are infected with this liver fluke and have CCA.[5] The life cycle of *O. viverrini* is shown in **Figure 2.2**.

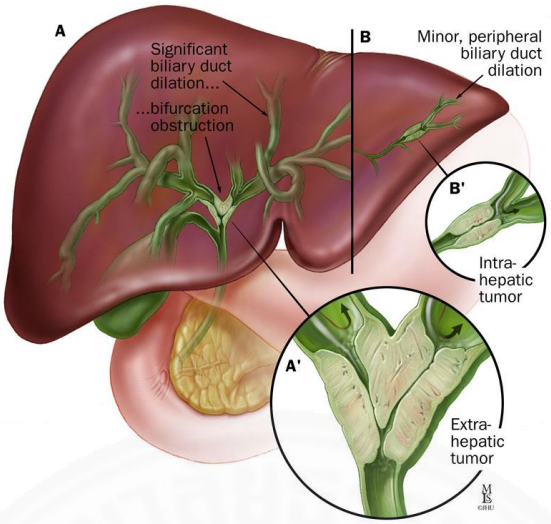

Figure 2.1 Location of cholangiocarcinoma. [28]

A location of perihilar CCA, between second-degree bile ducts and insertion of cystic duct into the common bile duct. **B** location of intrahepatic CCA, proximally to second-degree bile ducts in the liver.

2.1.2 Treatments

The treatments of CCA are individuals based on the stages of the disease. For localized CCA patient, first of all, need to be assessed for potential resectability. Because surgical tumor resection is a significant treatment of CCA. In patients who might benefit from surgery are selected for surgical resection. After resection, treated with adjuvant therapy, chemotherapy, and chemoradiotherapy, are needed for patients who present with microscopically positive resection margins or complete resection patients but node-positive disease. Whereas locally advanced CCA patients, who present with macroscopic residual disease after surgical resection, locally recurrent disease after potentially curative treatment, and locally unresectable disease are needed to treat with chemotherapy or chemoradiotherapy as well as in metastatic stage.[7] Most of CCA patients present with locally unresectable or metastatic disease that have to be treated with chemotherapy or chemoradiotherapy which can prolong overall survival of patients compared to best supportive care alone.[6] The chemotherapeutic regimens for CCA patients are 5-fluorouracil-based chemotherapy in combination with, for example, cisplatin, oxaliplatin, and etoposide or gemcitabine base in combination with, for example, cisplatin, oxaliplatin, and capecitabine.[4,6] However, the first-line drug for the treatment of advanced CCA is gemcitabine plus cisplatin or oxaliplatin.[6] A study in England reported that prolonged overall survival was observed in patients who treated with gemcitabine plus cisplatin compared to gemcitabine alone.[29] However, the research in Korea reported that the overall survival was not significantly different between patients who treated with gemcitabine plus cisplatin or gemcitabine alone.[30] Moreover, there are studies conducted for finding the best regimens for both locally advanced and metastatic CCA to increase survival time.[31,32]

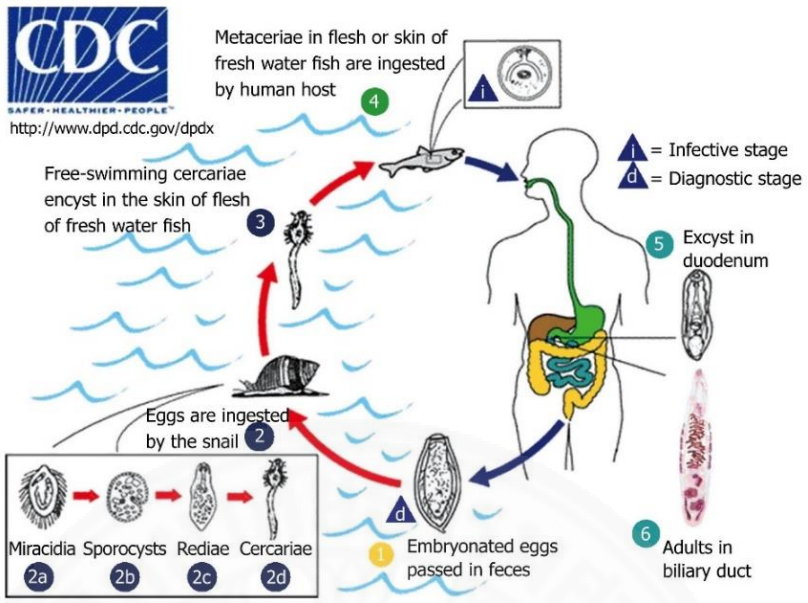

Figure 2.2 Life cycle of *O. viverrini*.[33]

Embryonated eggs, diagnostic stage, are passed in biliary duct and feces (1) and ingested by intermediate host snail (2). After ingested by snail, miracidia stage (2a) is released from the eggs and develop to sporocysts (2b), rediae (2c), and cercariae (2d) stages respectively and cercariae stage is released from snail to water. Free-swimming cercariae (3) encyst in the skin of flesh of freshwater cyprinoid fish and develop to the metaceriae stage, infectious stage (4). Human ingests metaceriae stage by eating raw or uncooked food from freshwater cyprinoid fish, *koi-pla* or *pla-ra*, and then excyst in the duodenum and ascends biliary tract through the ampulla of vater and develop to be adult in biliary duct.

2.2 Atractylodin

Atractylodin is a polyethylene alkyne bioactive compound contained in rhizome of traditional Chinese medicine, *Atractylodes lancea* (*A. lancea*) (Thunb.) DC. It is a significant component (14%) which is associated with pharmacological activities of this rhizome.[34] There is a review study reported that *A. lancea* has activities on cardiovascular system, nervous system, gastrointestinal system and anticancer activity and all of these activities associated with this compound.[35]

2.2.1 Pharmacological activities of atractylodin

Atractylodin has been found in many pharmacological activities particularly anticancer activity. There is a study investigated activity of this compound on cholangiocarcinoma cell lines, CL-6. They discovered that atractylodin exhibited anti-cholangiocarcinoma activity more potent than standard drug, 5-FU with half maximal inhibitory concentration (IC_{50}) values of 41.66 ± 2.51 µg/ml and 98.41 µg/ml, respectively. Moreover, this study also reported that atractylodin had ability to induce cell apoptosis in cancer cells, CL-6, with percentage of 27.21%.[34] Apart of anticancer activity, there is a study investigated the effect of atractylodin on gastrointestinal system in rat. They found that atractylodin had an effect on delayed gastric emptying time in a dose-dependent manner and the maximum activity was observed at 0.3 mg/kg.[36] Furthermore, another study reported that atractylodin had antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* with minimal Inhibitory Concentration (MIC) value of 20 µg/ml.[37]

2.2.2 Physical and chemical properties of atractylodin

The physical and chemical properties of atractylodin are shown in **Table 2.1**.

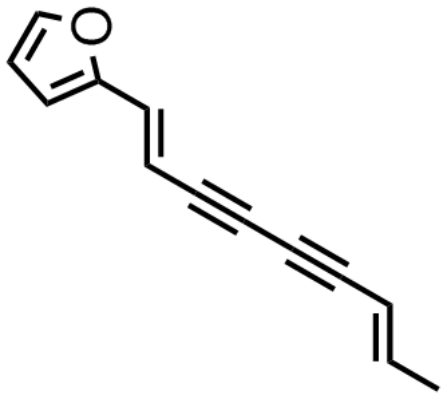

Figure 2.3 Chemical structure of atractylodin.[38]

Table 2.1 Physical and chemical properties of atractylodin.[10,11,39]

Property name	**Property value/Description**
Molecular weight	182.222 g/mol
Formula	$C_{13}H_{10}O$
Physical description	Brown powder
Absorption wavelength	340 nm
Solvent	Chloroform, Dichloromethane, Ethyl Acetate, DMSO, Acetone
Storage	Powder: 24 months at 2-8 ℃, 36 months at -20 ℃ Solvent: 6 months at -80 ℃, 1 months at -20 ℃

2.3 Nanoparticles

Nanoparticles (NPs) are the particulate dispersions or solid particles with size in range of <10-1000 nm. In biomedical area, they are mostly used for diagnosis[40-41] and drug delivery system.[12-17] In the recent, there are many studies were conducted on chemotherapeutic drug delivery system application of NPs to increase drug efficacy and safety. NPs enhance water solubility of hydrophobic drug[12,13] and improve pharmacokinetics profile[14-16] as well as biodistribution profiles to reduce toxicity to normal cells.[13] Furthermore, several studies had developed NPs to be drug delivery system of natural active compounds. Because some of natural active compounds have been found anticancer activity but have poor water solubility and pharmacokinetic profiles.[17-20]

There are various types of NPs are used as drug delivery systems. However, drugs- or natural active compounds-loaded NPs are different based on physicochemical properties of the drugs/compounds and the applications of each types of NPs are also different. The types of NPs used as chemotherapeutic drugs or natural active compounds delivery system are:

2.3.1 Polymeric nanoparticles

Polymeric NPs are spherical NPs produced from polymer which are widely used to develop drug delivery system of chemotherapeutic drugs for both hydrophilic[14-16] and hydrophobic properties.[12,13,43] The types of polymer are used to prepared polymeric NPs can be both natural or synthesized polymer, for instance, chitosan[42], polylactic acid (PLA)[12], poly(lactic-*co*-glycolic acid) (PLGA)[13,15,16], and poly epsilon-caprolactone (PCL).[43] Moreover, on the surface of NPs can be conjugated with targeting ligand to increase tumor cell specificity, for instance, folic acid[44] and aptamer[45] or conjugated with polyethylene glycol (PEG)[12,13] to improve pharmacokinetic profiles by increasing blood circulation half-life of the drugs. There are many studies of development of polymeric NPs to deliver chemotherapeutic drugs to increase their efficacy and safety. For instance, development of docetaxel-loaded PLA-PEG NPs to enhance water solubility, efficacy and reduce side effects of the drug[12] or docetaxel-loaded PLGA-PEG NPs to improve pharmacokinetic and

biodistribution profiles of the drug by increasing blood circulation half-life and lowering drug accumulation in normal tissue.[13] Furthermore, polymeric NPs are also used to develop as drug delivery system of natural compounds. For instance, curcumin-loaded PLGA-PEG NPs for enhancing water solubility, anticancer activity, and blood circulation half-life.[20]

2.3.2 Polymeric micelles

Polymeric micelles are spherical of self-assemble amphiphilic block of copolymer. The hydrophobic block act as hydrophobic core and hydrophilic block act as hydrophilic corona of polymeric micelle. This type of NPs can deliver hydrophobic drugs which are loaded in the core of NPs. There are many studies of hydrophobic drugs-loaded polymeric micelles to enhance water solubility and efficacy of the drugs. For instance, docetaxel-loaded Pluronic P123 (poloxamer) micelles to increase anticancer activity both *in vitro* and *in vivo*[46] and paclitaxel-loaded Pluronic P123 and F127 polymeric micelles to increase anticancer activity and prolong survival time.[47] Moreover, there is a study of curcumin-loaded poloxamer and D-alpha-Tocopheryl polyethylene glycol 1000 succinate (TPGS) polymeric micelles to enhance anticancer activity in multidrug resistant ovarian cancer compared to free-curcumin.[19]

2.3.3 Liposomes

Liposomes are spherical lipid bilayer, produced from phospholipid and cholesterol[48,49], that can deliver both hydrophobic[50,51] and hydrophilic drugs.[49] Hydrophobic drugs are loaded in hydrophobic tail of phospholipid and hydrophilic drugs are loaded inside hydrophilic core of liposomes. Moreover, on the surface of liposome can be conjugated or grafted with targeting ligands, for instance, peptide, carbohydrate, antibody, and small molecule.[48-53] Not only chemotherapeutic drugs are delivered by liposomes but also traditional medicines, for instance, baicalin-, traditional Chinese medicine, loaded folate-PEGylated liposome as tumor-targeting drug delivery system and to improve pharmacokinetic profiles.[17] Some of chemotherapeutic drug-loaded liposomes are approved by US FDA to use for cancer treatment. For instance, Doxil®, doxorubicin-loaded PEGylated liposome, approved for the treatment of Kaposi's sarcoma in 1995, ovarian cancer in 1999 and breast cancer in 2003. DaunoXome®, daunorubicin-loaded liposome, approved for the treatment of Kaposi's

sarcoma in 1996. Marqibo®, vincristine-loaded liposome, approved for the treatment of acute lymphoblastic leukemia in 2012.[54]

2.3.4 Solid lipid nanoparticles

Solid lipid NPs are spherical solid lipid core surrounded by phospholipid monolayer and/or surfactant. This type of NPs can deliver only hydrophobic drugs[55,56] which are loaded into solid lipid core. However, on the surface of NPs can be conjugated with PEG to enhance blood circulation half-life of the drugs, for instance, prolong blood circulation half-life of paclitaxel by loaded into PEGylated solid lipid NPs.[55] Moreover, solid lipid NPs can deliver natural active compounds as well. For instance, curcumin-loaded solid lipid NPs to improve therapeutic efficacy for cancer treatment.[18]

2.3.5 Dendrimers

Dendrimers are extensively branches of macromolecules present with three dimension.[57] Dendrimers are used to deliver both hydrophobic and hydrophilic drugs.[58,59] Moreover, this type of NPs can be grafted with targeting ligand to increase tumor cell specificity. For instance, hyaluronic acid grafted topotecan-loaded dendrimer to enhance cellular uptakes efficiency and improve pharmacokinetic profiles of the drug[58] and IL-6 antibody grafted doxorubicin-loaded dendrimer to enhance cellular uptake efficiency.[59]

2.3.6 Metallic nanoparticles

Metallic NPs are mostly prepared from silver and gold. Metallic act as a core of NPs and it can deliver both hydrophobic and hydrophilic drug.[24,60,61] Moreover, it can be conjugated with targeting ligand to increase tumor cell specificity or conjugated with PEG to enhance blood circulation half-life of the drug.[24,61] For instance, PEG and folic acid conjugated doxorubicin-gold NPs to increase cellular uptake efficiency and prolong blood circulation half-life.[61]

2.3.7 Magnetic nanoparticles

Magnetic NPs consist of magnetic core, iron, that can be conjugated with polymer, targeting ligand, and drugs. It can be used for both diagnosis tool and drug delivery system. For therapeutic purpose, magnetic NPs not only deliver hydrophobic drugs[62] but also hydrophilic drugs.[63] For instance, paclitaxel delivered by

magnetic NPs to target tumor site and enhance drug efficacy[62] or methotrexate-conjugated magnetic NPs to target tumor site and enhance cytotoxicity to cancer cells.[63]

2.3.8 Carbon nanotubes

Carbon nanotubes produced from single or multiple rolled graphene sheet. As drug delivery system, the drugs are loaded in the tube or conjugated on the surface of nanosystems. Moreover, it can be conjugated with targeting ligands to improve efficacy and safety of the drugs.[64,65] For instance, chitosan and folic acid conjugated docetaxel-loaded carbon nanotube for targeting lung cancer and increasing drug efficacy[64] or chitosan conjugated paclitaxel-loaded carbon nanotube to control drug release and target tumor site.[65]

2.4 Poly (lactic-co-glycolic acid) (PLGA)

Poly (lactic-co-glycolic acid) (PLGA) is biocompatible and biodegradable copolymer which is approved to use in clinical by US FDA as drug delivery system of various types of drugs and natural active compounds.

2.4.1 Physicochemical properties

PLGA is linear copolymer of two monomer, lactic acid and glycolic acid, link together with ester linkages **(Figure 2.4)**. This copolymer is identified from weight ratio of lactic acid to glycolic acid. For instance, PLGA 50:50 composed of 50% of lactic acid and 50% of glycolic acid or PLGA 75:25 composed of 75% of lactic acid and 25% of glycolic acid. The glass transition temperature (Tg) of PLGA is 40-60 °C which is decreased with lactic acid weight ratio in copolymer decrease. The inherence viscosity is 0.5-0.8 MPa and PLGA structure is amorphous.[66] PLGA is degraded to lactic acid and glycolic acid by hydrolysis reaction in an aqueous environment **(Figure 2.5)**. Both lactic acid and glycolic acid are metabolized by tricarboxylic acid cycle and eliminated from the body as carbon dioxide and water which are the biocompatible and toxicologically safe by-products.[67,68] The molecular weight of this copolymer affects mechanical strength which is rule the biodegradation rate of copolymer. Moreover, biodegradation rate is also affected by the difference of lactic acid to glycolic acid weight ratio in copolymer.[69] There is a study found that weight ratio of lactic acid to

glycolic acid 50:50 exhibited fastest degradation followed by 65:35 and 75:25 respectively.[70] And another study reported that weight ratio of lactic acid to glycolic acid 50:50 exhibited 2-fold faster degradation compared to 75:25.[70] However, higher weight ratio of lactic acid in copolymer exhibited higher hydrophobicity of copolymer which is benefit for hydrophobic drugs loading in NPs.[71] Molecular weight of PLGA copolymer directly affect size of NPs. Size will be increased when molecular weight increase for hydrophilic drugs.[72,73] Whereas size of NPs loading hydrophobic drug decreased when molecular weight increased. Because of higher molecular weight leads to higher hydrophobicity and longer aliphatic chain.[74] Moreover, molecular weight also affects drug release from NPs. In many studies found that higher molecular weight resulted in lower drug release from NPs.[72-74] Because of high molecular weight PLGA composes of high amount of lactic acid which is hydrophobic part of NPs.

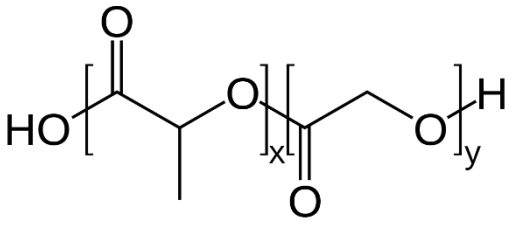

Figure 2.4 Chemical structure of Poly (lactic-co-glycolic acid) (PLGA). (x is the number of lactic acid units and y is number of glycolic acid units).[75]

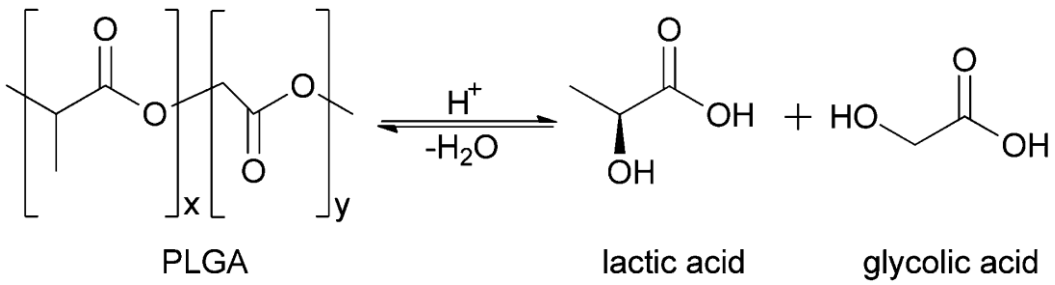

Figure 2.5 Hydrolysis reaction of PLGA.[76]

PLGA is degraded to lactic acid and glycolic acid by hydrolysis reaction in an aqueous environment and metabolized by tricarboxylic acid cycle and eliminated from the body as carbon dioxide and water.

2.4.2 Applications

2.4.2.1 Drug delivery systems

PLGA is widely used to prepare polymeric NPs to deliver various types of chemotherapeutic drugs for the treatment of cancers. There are many studies of development of PLGA NPs to enhance water solubility of hydrophobic drugs, increase efficacy and safety and improve pharmacokinetic profiles of chemotherapeutic drugs. For instance, development of docetaxel-loaded PLGA NPs to enhance water solubility and increase anticancer activity and pharmacokinetic profile of the drugs[13] or gemcitabine-loaded PLGA NPs to enhance oral bioavailability and drug absorption in the gastrointestinal tract.[14] Moreover, on the surface of PLGA NPs can be conjugated with targeting ligand to increase tumor cell specificity that help to increase efficacy of chemotherapeutic drugs and reduce side effect to normal cells. For instance, PLGA NPs conjugated with folic acid[44], aptamer[45,], hyaluronic acid[77], and antibody[78] or improve pharmacokinetic profile of the drug by conjugated with PEG to enhance blood circulation half-life.[13,20,44] PLGA NPs were also developed for delivering traditional medicines or natural active compounds. For instance, curcumin-loaded PLGA NPs to enhance water solubility and increase anticancer activity[20], quercetin-loaded PLGA NPs to increase anticancer activity[23], ethanolic extract of *Polygala senega*-loaded PLGA NPs to increase anticancer activity against lung cancer.[79]

2.4.2.2 Diagnosis and Imaging

PLGA NPs are also used for imaging by conjugated with imaging probe or radiolabeled on the surface of NPs. For instance, development of biotinylated F-18 prosthetic groups labeled on the surface of PLGA NPs for brain imaging in rat.[80] Moreover, contrast media can be loaded in the PLGA NPs, for instance, development of diethylenetriaminepentaacetic acid gadolinium (III) (Gd-DTPA)-loaded PLGA NPs to enhance magnetic resonance imaging (MRI) for atherosclerosis detection[81] and superparamagnetic iron oxide (SPIO)-loaded PLGA NPs for imaging brain-penetrating of NPs by using MRI.[82]

2.4.2.3 Theranostic

PLGA NPs are developed as theranostic in cancer therapy which is the combination between therapy and diagnosis. Chemotherapeutic drugs and imaging agents are loaded in NPs and targeting ligands are conjugated on the surface

of NPs to increase cancer cell specificity. For instance, development of doxorubicin and magnetic loaded PLGA NPs and conjugated with HER antibody for the treatment of breast cancer[83] or development of gemcitabine and SPIO-loaded PLGA NPs for cancer therapeutic and diagnosis.[84]

2.5 Poloxamer 407

Poloxamer 407 is one of popular stabilizer that used to prepare NPs in many studies.[85,86] It composed of triblock of polyethyleneoxide (PEO) and polypropylene oxide (PPO; PEO-PPO-PEO) (**Figure 2.6**). The concentration of poloxamer directly affects the size of NPs. There are many studies found that size of NPs decreased when the concentration of poloxamer increase but when the concentration of poloxamer is very high, size of NPs will increase.[85] That caused from the higher viscosity of stabilizer solution and leads to particle size shift. Poloxamer 407 is also used as co-stabilizer or co-surfactant with another stabilizer, for instance, polyvinyl alcohol, poloxamer 188, and tween 80.[87-89] Moreover, poloxamer is also used to prepare polymeric micelles, for instance, paclitaxel-loaded Pluronic F127 (poloxamer 407) polymeric micelles to increase anticancer activity and prolong survival time[47] and paclitaxel and lapatinib-loaded Pluronic F127 polymeric micelles for co-delivery against metastatic breast cancer.[90]

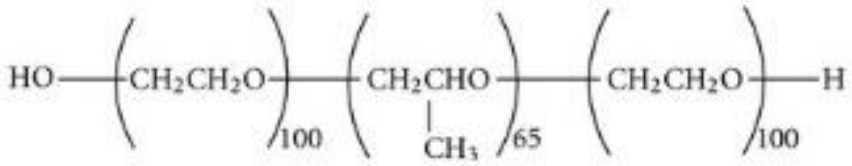

Figure 2.6 Chemical structure of poloxamer 407.[91] Poloxamer 407 composes of triblock of polyethyleneoxide-polypropyleneoxide (PEO-PPO-PEO).

2.6 Solvent displacement method

Solvent displacement method also known as solvent diffusion and nanoprecipitation method. This method is widely used to prepared hydrophobic drug-loaded polymeric NPs. Polymers are dissolved in the polar or water-miscible solvent, mostly use acetone, acetonitrile, methanol, and ethanol. The solutions are added drop-wise in an aqueous phase containing surfactant or stabilizer and continuous stirring. Solvent will rapidly diffuse and NPs are formed. Then organic solvents are removed by evaporation.[92] Using this method to prepare NPs, there are several parameters that have to be concerned including;

Polymer concentration: size of NPs increase correlated with polymer concentration.[92]

Molecular weight and copolymer ratio: size of NPs increase when molecular weight of copolymer increase for hydrophilic drugs. But for hydrophobic drugs, size of NPs decrease when molecular weight of copolymer decrease. And polymer ratio are not significantly affects size of NPs in similar molecular weight.[74,92]

Solvent nature: there is not the best solvent for preparing NPs by solvent displacement method. But criteria to select suitable solvent depends on dissolving capacity of polymers, water-miscible, and low boiling point to be evaporated quickly. Mostly used solvent are acetone, acetonitrile, methanol, and ethanol.[92]

Surfactant: concentration of surfactant increase resulting in decreased in size of NPs. The surfactant or stabilizer that mostly used are poloxamer and polyvinyl alcohol.

CHAPTER 3
OBJECTIVES

3.1 General objectives

This present study aimed to develop atractylodin-loaded PLGA nanoparticles (AL-loaded PLGA NPs) and investigate *in vitro* cytotoxic activity against cholangiocarcinoma cell lines.

3.2 Specific objectives

1. To prepare the AL-loaded PLGA NPs and blank PLGA NPs.
2. To characterize the physiological properties of AL-loaded PLGA NPs and blank PLGA NPs.
3. To determine drug encapsulation and drug loading efficiency of AL-loaded PLGA NPs.
4. To investigate drug releasing profile of AL-loaded PLGA NPs.
5. To investigate the stability of AL-loaded PLGA NPs and blank PLGA NPs in ultrapure water, phosphate buffer solution, and culture medium.
6. To investigate *in vitro* cytotoxic activity of AL-loaded PLGA NPs on cholangiocarcinoma cell lines, CL-6 and HuCCT-1 and normal cell line, OUMS-36T-1F.

CHAPTER 4
RESEARCH METHODOLOGY

4.1 Materials and chemicals

Atractylodin and 5- fluorouracil (5-FU) were purchased from WAKO, Osaka, Japan. PLGA (50:50) MW 12,000 (Resomer® RG502) and MW 48,000 (Resomer® RG504), D-mannitol, and (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) reagent were purchased from Sigma-Aldrich, MO, USA. Acetone and DMSO for solvent were purchased from Fisher scientific, Co. LLC, USA. Poloxamer 407 was obtained from BASF, USA. Dialysis membrane MWCO 50,000 Da was purchased from Spectrum Laboratory Products Inc., Rancho Dominguez, CA, USA. The CL-6 cell lines were obtained by Associate Professor Dr. Adisak Wongkajornsilp, Department of Pharmacology, Faculty of Medicine (Siriraj Hospital). The normal fibroblast cell line, OUMS-36T-1F, was purchased from JCRB Cell Bank (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan). RPMI, DMEM, FBS, and antibiotic-antimycotic solution were purchased from Invitrogen, CA, USA. The PBS and DMSO for cryoprotectant were purchased from Ameresco, OH, USA.

4.2 PLGA NPs preparation and evaluations

4.2.1 AL-loaded PLGA NPs and blank PLGA NPs preparation

Atractylodin-loaded PLGA nanoparticles (AL-loaded PLGA NPs) and blank PLGA nanoparticles (blank PLGA NPs) were prepared by solvent displacement method with modification.[74] One milligram of atractylodin and 22.5 mg of PLGA (Resomer® RG502; MW 12,000 and Resomer® RG504; MW 48,000) were dissolved in 0.5 ml and 1 ml of acetone, respectively and thoroughly mixed. The mixture was then added drop-wise into 15 ml of 1% poloxamer 407 by using syringe pump (KD Scientific, USA) under magnetic stirrer of 405 rpm. The excess surfactant was removed by dialysis (MWCO 50,000 Da) against a 0.2% D-mannitol solution for

2 hours. The NPs were kept in the form of NP suspension at 4 ºC for further use. Blank PLGA NPs were prepared by the same method without atractylodin. The formulations of AL-loaded PLGA NPs and blank PLGA NPs are shown in **Table 4.1**.

4.2.2 PLGA NPs characterization

NPs were investigated by size, polydispersity index (PDI), and zeta potential. One hundred microliters of 1.4 mg/ml of AL-loaded PLGA NP suspensions or blank PLGA NP suspensions were added into 900 µl of ultrapure water. Then NPs were investigated size, PDI, and zeta potential using Zetasizer (Malvern, USA).

4.2.3 Drug encapsulation and drug loading efficiency determination

Five hundred microliters of 1.4 mg/ml of AL-loaded PLGA NP suspensions were added into the microcentrifuge tube and centrifuged at 13,000 rpm (4 ºC) for 15 minutes. The supernatants were discarded, and 500 microliters of DMSO were added to dissolve drug and polymer. The mixtures were sonicated until becoming the solution. Then the solutions were determined the amount of atractylodin spectroscopically using UV absorbance reader (Spectramax microplate reader, Molecular Devices, USA) at the wavelength of 340 nm. The amount of atractylodin was calculated by compare concentration with the standard curve. Then %encapsulation efficiency (%EE) and %loading efficiency (%LE) were calculated by following equations.

$$\text{Encapsulation efficiency (\%EE)} = \frac{\text{Amount of drug loaded in NPs}}{\text{Amount of drug added}}\ x\ 100\%$$

$$\text{Loading efficiency (\%LE)} = \frac{\text{Amount of drug in NPs}}{\text{Amount of NPs}}\ x\ 100\%$$

4.3 Drug releasing study

4.3.1 Drug releasing profile of AL-loaded PLGA NPs

Four hundred microliters of 1.4 mg/ml of AL-loaded PLGA NP suspensions were added into 400 µl of phosphate buffer solution, pH 7.4. The samples were incubated at 37 ºC and collected at each time interval. Samples were then centrifuged at 13,000 rpm (4 ºC) for 15 minutes. The supernatants were discarded, and

400 µl of DMSO was added to dissolve drug and polymer. The mixtures were sonicated until becoming the solution. Then the solutions were determined the amount of atractylodin spectroscopically using UV absorbance reader (Spectramax microplate reader, Molecular Devices, USA) at the wavelength of 340 nm. The amount of atractylodin was calculated to compare concentration with the standard curve. Then % cumulative release was calculated by the following equation.

$$\text{Cumulative release (\%)} = \frac{\text{DL} - \text{DR}}{\text{DL}} \text{ x } 100\%$$

Where DL: Amount of drug loaded in NPs

DR: Amount of drug remained in NPs

4.4 Stability study

The NPs prepared by using biodegradable polymer can be degraded all the time in an aqueous environment. Thus, the NP formulations have to be investigated stability in aqueous solutions.

4.4.1 Stability of NPs in ultrapure water

One hundred microliters of 1.4 mg/ml of AL-loaded PLGA NP suspensions and blank PLGA NP suspensions were added into 900 µl of ultrapure water. The samples were then stored at 4℃, 30 ℃, and 37 ℃ for four weeks and collected at each time interval and investigated size, PDI, and zeta potential using Zetasizer (Malvern, USA).

4.4.2 Stability of NPs in phosphate buffer solution

One hundred microliters of 1.4 mg/ml of AL-loaded PLGA NP suspensions and blank PLGA NP suspensions were added into 900 ul of phosphate buffer solution, pH 7.4. The samples were then stored at 4 ℃, 30 ℃, and 37 ℃ for four weeks and collected at each time interval and investigated size and PDI using Zetasizer (Malvern, USA).

4.4.3 Stability of NPs in the medium

One hundred microliters of 1.4 mg/ml of AL-loaded PLGA NP suspensions and blank PLGA NP suspensions were added into 900 ul of RPMI, and DMEM complete media and serum-free media. The samples were then stored at 37 ℃ for 24 hours and collected at each time interval and investigated size and PDI using Zetasizer (Malvern, USA).

4.5 *In vitro* cytotoxic activity

4.5.1 Cell culture

The human cholangiocarcinoma (CCA) cell lines, CL-6 and HuCCT-1, as well as normal fibroblast cell line, OUMS-36T-1F, were used for *in vitro* cytotoxic activity. The CCA cell lines were cultured in RPMI 1640 medium and OUMS-36T-1F cell line was cultured in DMEM. Both cultures were prepared as complete media with supplement of 10% (v/v) heated fetal bovine serum (FBS) and 1% of 100 IU/ml antibiotic-antimycotic solution. All cell cultures were maintained at 37 ºC under 5% CO_2 atmosphere and 95% humidity (HERRACELL 150i, Thermo Scientific, MA, USA). All cells were preserved in 10% DMSO supplemented with 90% FBS and stored in a liquid nitrogen tank.

4.5.2 AL-loaded PLGA NPs, blank PLGA NPs, atractylodin and 5-fluorouracil working solution preparation

4.5.2.1 AL-loaded PLGA NPs and blank PLGA NPs working solution preparation

The AL-loaded PLGA NPs were suspended in deionized water. Then stock solution serially diluted (1:2) with serum-free media to obtain working solution at eight final concentrations of 42.19-5,400 µg/ml which is equivalent to atractylodin 1-125 µg/ml.

The blank PLGA NPs were suspended in deionized water. Then stock solution serially diluted (1:2) with serum-free media to obtain working solution at eight final concentrations of 42.19-5,400 µg/ml.

4.5.2.2 Atractylodin working solution preparation

Atractylodin was dissolved with absolute ethanol or with water. Then stock solution serially diluted (1:2) with serum-free media to obtain working solution at eight final concentrations of 1-125 µg/ml.

4.5.2.3 5-Fluorouracil working solution preparation

5-FU was dissolved with absolute ethanol. Then stock solution serially diluted (1:2) with serum-free media to obtain working solution at eight final concentrations of 1-125 µg/ml.

4.5.3 MTT assay

The CCA and normal cell lines were treated with 100 µl of AL-loaded PLGA NPs, blank PLGA NPs, atractylodin dissolved with water or ethanol, and 5-FU (positive control) at various eight concentrations and 100 µl of culture medium (negative control) in a 96-well microtiter plate (Corning, VA, USA). Cell viability was measured by MTT assay.[93] Briefly, 8,000 cells were seeded into each well and incubated at 37 °C in 5% CO_2 for 24 hours. After that culture medium was replaced with AL-loaded PLGA NPs, blank PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU in serum-free media at different concentrations and incubated at 37 °C in 5% CO_2 for 24 hours. After incubation time, cell viability was measured or serum-free media containing drugs and NPs were replaced with 100 µl of complete media and further incubated at 37 °C in 5% CO_2 up to 48, and 72 h. For cell viability measurement, serum-free media containing drugs and NPs or complete media were replaced with 100 µl of 0.5 mg/ml MTT reagent in complete media and further incubated for an additional 2 hours. MTT reagent was discarded and 100 µl of DMSO were added. The absorbance of cell suspension was measured at 550 nm by using UV absorbance reader (Vario skan flash, Thermo Fisher Scientific, MA, USA). The % cell viability was calculated by the following equation, and the concentration-effect curve will be analyzed, and the IC_{50} will be determined by using GraphPad Prism version 7.04.[93]

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of sample}}{\text{absorbance of negative control}}\ x\ 100\%$$

4.6 Statistical analysis

Statistical evaluation of data was performed using the analysis of variance (one-way ANOVA). Bonferroni was used as a post-hoc test to assess the significant difference. To compare the significance of the difference between the means of two groups, a t-test was performed. The statistical significance level was set at $p = 0.05$.

Table 4.1 Formulations of AL-loaded PLGA NPs and blank PLGA NPs.

Formulation code	Ingredients				
	Atractylodin (mg)	**Acetone (ml)**	**PLGA MW 12,000 (Resomer® RG502) (mg)**	**PLGA MW 48,000 (Resomer® RG504) (mg)**	**1% Poloxamer 407 (w/v) (ml)**
F1	-	1.5	22.5	-	15
F2	1	1.5	22.5	-	15
F3	-	1.5	-	22.5	15
F4	1	1.5	-	22.5	15

CHAPTER 5
RESULTS

5.1 PLGA NPs preparation and evaluations

5.1.1 AL-loaded PLGA NPs and blank PLGA NPs preparation

NPs were prepared from PLGA copolymer MW of 12,000 (Resomer® RG502), and 48,000 (Resomer® RG504) by solvent displacement method and all NPs were kept in the form of NP suspensions at 4 ºC for further use.

5.1.2 PLGA NPs characterization

The prepared NPs were examined for particle size, size distribution or polydispersity index (PDI) and zeta potential by dynamic light scattering technique and shown in **Table 5.1**. All formulations provided particle size diameter in the range of 155-160 nm with narrow size distribution (0.068-0.095). The size of AL-loaded PLGA NPs prepared from Resomer® RG504 (F4) were found to be significantly higher than blank PLGA NPs (F3) with the size of 161.27±1.87 nm and 156.10±1.54 nm, respectively ($p < 0.05$). However, the size of AL-loaded PLGA NPs prepared from Resomer® RG502 (F2) were not found to be different from blank PLGA NPs (F1). Moreover, the size of NPs made from Resomer® RG502 and Resomer® RG504 were not found to be different. The zeta potential values of all formulations were lower than -20 mV and there were not found to be significantly different between AL-loaded PLGA NPs and blank PLGA NPs.

5.1.3 Drug encapsulation and drug loading efficiency determination

The percent of drug encapsulation efficiency (%EE) and percent of drug loading efficiency (%LE) of F2 and F4 were determined. Both formulations could encapsulate atractylodin in NPs, and the %EE and %LE of both formulations were found to be 50% and 2%, respectively. However, the %EE and %LE of F2 were significantly higher than F4 ($p < 0.05$) and results are summarized in **Table 5.2**.

Table 5.1 Particle size, PDI, and zeta potential values of AL-loaded PLGA NPs and blank PLGA NPs. Data are presented as mean±SD values from three experiments.

Formulation code	Size (nm)	PDI	Charge (mV)
F1	159.43±0.68	0.089±0.023	-24.60±1.21
F2	158.33±0.21	0.076±0.003	-23.80±0.75
F3	156.10±1.54	0.095±0.023	-29.13±0.32
F4	161.27±1.87*	0.068±0.015	-28.83±0.35

* Indicates $p < 0.05$ compared with F3.

Table 5.2 Drug encapsulation efficiency (%) and drug loading efficiency (%) of AL-loaded PLGA NPs. Data are presented as mean±SD values from three experiments.

Formulation code	%Encapsulation efficiency (%)	%Loading efficiency (%)
F2	52.02±1.64	2.30±0.07
F4	47.68±0.83*	2.12±0.04*

* Indicates $p < 0.05$ compared with F2.

5.2 Drug releasing study

5.2.1 Drug releasing profile of AL-loaded PLGA NPs

The AL-loaded PLGA NPs were investigated for drug release *in vitro*. The NPs were incubated in phosphate buffer solution, pH 7.4 at 37 °C and collected at each time interval until 72 hours. Drug released from NPs of both NP formulations occurred in a biphasic manner with initial burst release followed by sustained release. During the first 4 hours, drug burst released from NPs were 40% and 35% for F2 and F4, respectively. Drug released from both NP formulations reaching 82% in 24 hours, with the maximum value of 88% in 72 hours. The different amount of drug release of both NP formulations was not observed, and drug release profiles are shown in **Figure 5.1**.

From the results of a drug-releasing study of AL-loaded PLGA NPs prepared from Resomer® RG502 (F2) and Resomer® RG504 (F4) exhibited that drug released from both formulations were not found to be different. However, the %EE and %LE of F2 were significantly higher than F4. Thus, the AL-loaded PLGA NPs prepared from Resomer® RG502 (F2) were selected to study the stability and to investigate *in vitro* cytotoxic activity.

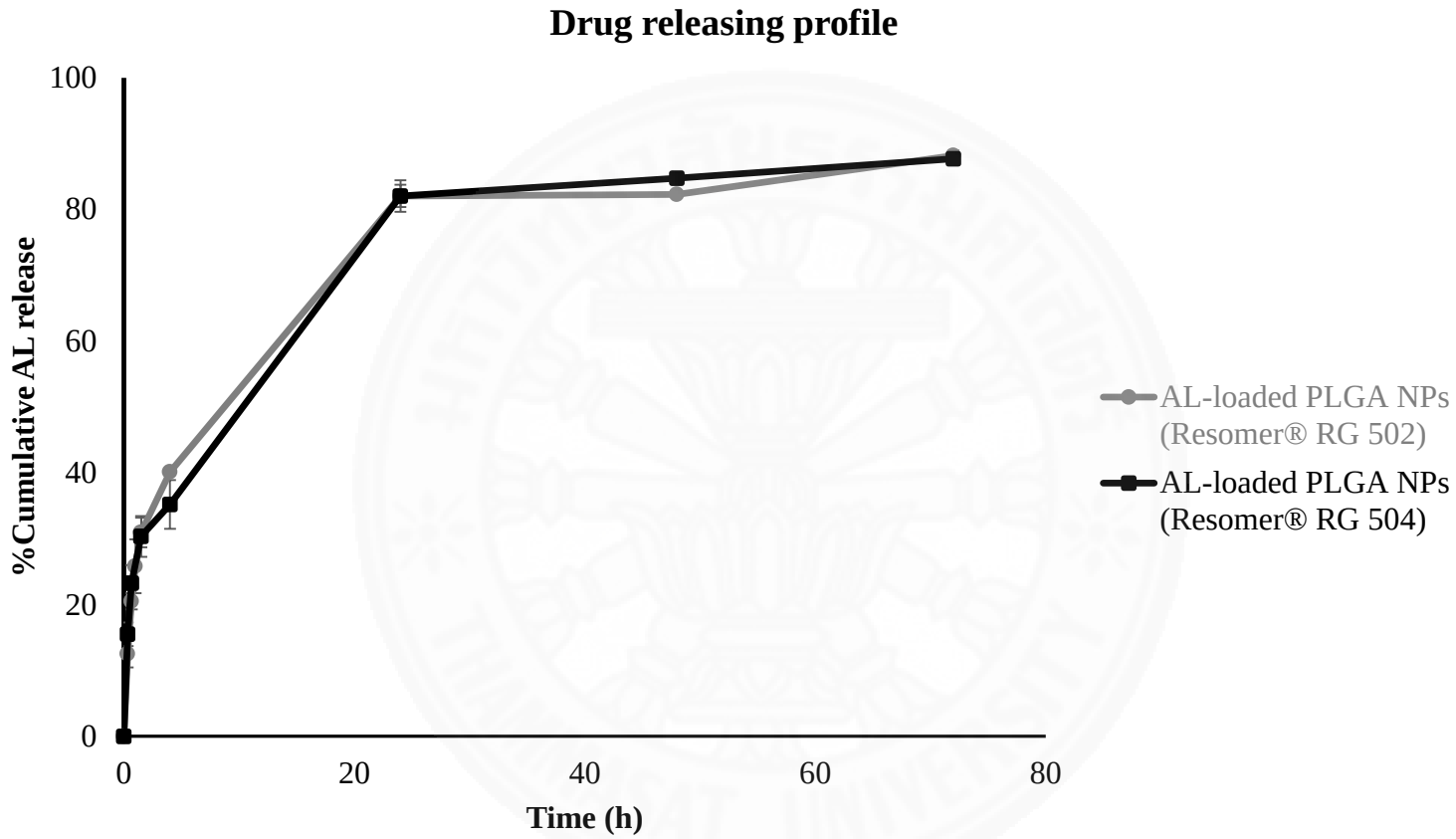

Figure 5.1 Drug releasing profile of AL-loaded PLGA NPs.

5.3 Stability study

The NP formulations F1 and F2 were selected to investigate stability in ultrapure water, PBS (pH 7.4) and RPMI, and DMEM complete media and serum-free media. After the specific time, the samples were then collected and investigated size, polydispersity index (PDI), and zeta potential.

5.3.1 Stability of NPs in ultrapure water

The AL-loaded PLGA NPs which were stored in ultrapure water at 4 °C for four weeks was not found the significant change in size, PDI, and zeta potential compared with freshly prepared NPs. Whereas, the sizes of NPs were found to be significantly decreased after storage at 30 °C and 37 °C for three and four weeks ($p<0.05$). Moreover, the zeta potentials of AL-loaded PLGA NPs were found the significantly changed after storage at 37 °C for one and three weeks compared with freshly prepared NPs ($p<0.05$).

The blank PLGA NPs which were stored in ultrapure water were found to be significantly decreased in size after stored at 4 °C for one week, 30 °C for two weeks, and 37°C for one week compared with freshly prepared NPs ($p<0.05$). Moreover, zeta potentials were also found the significantly changed after stored at 4 °C for three weeks and 37 °C for one week compared with freshly prepared NPs ($p<0.05$).

The Size, PDI and zeta potential values of AL-loaded PLGA NPs and blank PLGA NPs after storage in ultrapure water at 4 °C, 30 °C, and 37 °C for four weeks are summarized in **Table 5.3 -5.8**.

5.3.2 Stability of NPs in phosphate buffer solution

The AL-loaded PLGA NPs which were stored in PBS were found the significant decrease in size after stored at 4 °C and 30 °C for one week and 37 °C for three weeks compared with freshly prepared NPs. However, the sizes of NPs were decreased after one week of storage at 37 °C. Moreover, the size distribution of AL-loaded PLGA NPs was found the significantly increase after stored at 37 °C for four weeks.

The blank PLGA NPs which were stored in PBS were found the significant decrease in size after stored at 4 °C for one week and 30 °C and 37 °C for three weeks compared with freshly prepared NPs ($p<0.05$). Moreover, PDI of blank PLGA NPs was found the significantly increase after stored at 37 °C for four weeks ($p<0.05$).

The Size and PDI of AL-loaded PLGA NPs and blank PLGA NPs after storage in PBS, pH 7.4 at 4 °C, 30 °C, and 37 °C for four weeks are summarized in **Table 5.9 -5.14**.

5.3.3 Stability of NPs in the medium

The size of AL-loaded PLGA NPs and blank PLGA NPs after storage in RPMI and DMEM complete media were found the significantly decrease compared with freshly prepared NPs after 1 hour of storage ($p<0.05$). Moreover, PDI of both NP formulations was also found the increase substantially compared with newly prepared NPs ($p<0.05$). The AL-loaded PLGA NPs which were stored in RPMI serum-free media for three hours and in DMEM serum-free media for two hours were found the significant increase in size compared with freshly prepared NPs but changing in PDI was not observed at all time point. However, blank PLGA NPs which were stored in RPMI and DMEM serum-free media were not found to be changed in size and size distribution.

The Size and PDI of AL-loaded PLGA NPs and blank PLGA NPs after storage in RPMI and DMEM complete media at 37 °C for 24 hours are summarized in **Table 5.15 -5.18**.

The Size and PDI of AL-loaded PLGA NPs and blank PLGA NPs after storage in RPMI and DMEM serum-free media at 37 °C for 24 hours are summarized in **Table 5.19 -5.22**.

Table 5.3 Size, PDI and zeta potential values of AL-loaded PLGA NPs after storage in ultrapure water at 4 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**	**Zeta potential (mV)**
Freshly prepared	158.33±0.21	0.076±0.003	-23.80±0.75
1 week	157.80±0.60	0.065±0.017	-23.57±0.74
2 weeks	161.33±0.67	0.050±0.034	-23.07±0.75
3 weeks	154.90±0.56	0.073±0.025	-24.40±0.92
4 weeks	154.70±2.46	0.059±0.013	-26.10±0.82

Table 5.4 Size, PDI and zeta potential values of AL-loaded PLGA NPs after storage in ultrapure water at 30 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**	**Zeta potential (mV)**
Freshly prepared	158.33±0.21	0.076±0.003	-23.80±0.75
1 week	156.13±2.44	0.047±0.008	-20.63±0.65
2 weeks	154.43±1.62	0.066±0.029	-23.70±2.65
3 weeks	150.80±1.08*	0.081±0.012	-24.27±2.45
4 weeks	153.57±0.25*	0.079±0.028	-25.40±2.31

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.5 Size, PDI and zeta potential values of AL-loaded PLGA NPs after storage in ultrapure water at 37 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**	**Zeta potential (mV)**
Freshly prepared	158.33±0.21	0.076±0.003	-23.80±0.75
1 week	156.10±2.13	0.097±0.031	-18.90±1.78*
2 weeks	158.70±0.46	0.057±0.020	-19.47±1.37
3 weeks	152.13±0.78*	0.056±0.009	-18.20±1.32*
4 weeks	152.40±1.31*	0.089±0.026	-20.10±2.43

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.6 Size, PDI and zeta potential values of blank PLGA NPs after storage in ultrapure water at 4 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**	**Zeta potential (mV)**
Freshly prepared	159.43±0.68	0.089±0.023	-24.60±1.21
1 week	154.53±0.75*	0.072±0.023	-25.50±0.82
2 weeks	155.40±0.61*	0.068±0.009	-26.60±0.26
3 weeks	155.90±0.95*	0.071±0.014	-27.50±1.13*
4 weeks	158.20 ±1.15	0.083±0.006	-27.83±0.47*

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.7 Size, PDI and zeta potential values of blank PLGA NPs after storage in ultrapure water at 30 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**	**Zeta potential (mV)**
Freshly prepared	159.43±0.68	0.089±0.023	-24.60±1.21
1 week	160.73±2.32	0.069±0.024	-23.80±3.55
2 weeks	152.67±2.89*	0.063±0.010	-22.70±0.95
3 weeks	152.10±0.62*	0.077±0.009	-20.27±9.55
4 weeks	154.30±1.91	0.091±0.027	-15.13±5.16

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.8 Size, PDI and zeta potential values of blank PLGA NPs after storage in ultrapure water at 37 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**	**Zeta potential (mV)**
Freshly prepared	159.43±0.68	0.089±0.023	-24.60±1.21
1 week	153.10±2.15*	0.068±0.021	-19.03±0.61*
2 weeks	155.20±1.30	0.050±0.019	-18.63±0.55*
3 weeks	152.97±2.46*	0.065±0.023	-21.27±1.42
4 weeks	155.90±0.72	0.074±0.008	-23.83±2.05

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.9 Size and PDI values of AL-loaded PLGA NPs after storage in PBS pH 7.4 at 4 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	158.33±0.21	0.076±0.003
1 week	152.93±1.12*	0.075±0.028
2 weeks	157.53±0.25	0.073±0.011
3 weeks	154.07±0.35*	0.060±0.023
4 weeks	151.37±0.85*	0.085±0.020

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.10 Size and PDI values of AL-loaded PLGA NPs after storage in PBS pH 7.4 at 30 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	158.33±0.21	0.076±0.003
1 week	148.47±2.11*	0.048±0.027
2 weeks	151.50±2.27*	0.052±0.021
3 weeks	145.73±1.31*	0.101±0.022
4 weeks	142.70±2.66*	0.084±0.024

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.11 Size and PDI values of AL-loaded PLGA NPs after storage in PBS pH 7.4 at 37 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	158.33±0.21	0.076±0.003
1 week	149.03±4.60	0.082±0.003
2 weeks	150.53±1.82	0.050±0.025
3 weeks	147.63±1.00*	0.067±0.007
4 weeks	129.60±5.35*	0.189±0.008*

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.12 Size and PDI values of blank PLGA NPs after storage in PBS pH 7.4 at 4 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	159.43±0.68	0.089±0.023
1 week	153.10±1.08*	0.064±0.015
2 weeks	157.43±1.90	0.070±0.026
3 weeks	154.07±1.53*	0.089±0.012
4 weeks	151.07±1.19*	0.106±0.009

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.13 Size and PDI values of blank PLGA NPs after storage in PBS pH 7.4 at 30 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	159.43±0.68	0.089±0.023
1 week	154.17±2.02	0.065±0.017
2 weeks	153.80±1.39	0.049±0.007
3 weeks	152.17±0.80*	0.074±0.027
4 weeks	143.90±4.01*	0.134±0.014

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.14 Size and PDI values of blank PLGA NPs after storage in PBS pH 7.4 at 37 °C for 4 weeks. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	159.43±0.68	0.089±0.023
1 week	151.87±2.20	0.077±0.022
2 weeks	152.17±2.37	0.068±0.005
3 weeks	149.50±1.14*	0.071±0.014
4 weeks	132.97±4.62*	0.168±0.023*

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.15 Size and PDI values of AL-loaded PLGA NPs after storage in RPMI complete media at 37 °C for 24 hours. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	158.33±0.21	0.076±0.003
1 hour	140.80±1.80*	0.220±0.007*
2 hours	142.17±0.75*	0.223±0.006*
3 hours	141.93±1.37*	0.224±0.013*
24 hours	138.03±1.10*	0.209±0.008*

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.16 Size and PDI values of blank PLGA NPs after storage in RPMI complete media at 37 °C for 24 hours. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	159.43±0.68	0.089±0.023
1 hour	139.00±0.66*	0.237±0.001*
2 hours	138.37±1.61*	0.233±0.010*
3 hours	138.87±2.73*	0.241±0.002*
24 hours	136.50±1.73*	0.217±0.011*

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.17 Size and PDI values of AL-loaded PLGA NPs after storage in DMEM complete media at 37 °C for 24 hours. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	158.33±0.21	0.076±0.003
1 hour	144.80±2.17*	0.213±0.014*
2 hours	140.70±0.82*	0.218±0.015*
3 hours	144.10±1.22*	0.206±0.004*
24 hours	142.40±1.06*	0.181±0.005*

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.18 Size and PDI values of blank PLGA NPs after storage in DMEM complete media at 37 °C for 24 hours. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	159.43±0.68	0.089±0.023
1 hour	139.00±3.53*	0.241±0.007*
2 hours	140.40±1.76*	0.241±0.014*
3 hours	139.70±3.82*	0.237±0.005*
24 hours	140.83±1.03*	0.187±0.003*

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.19 Size and PDI values of AL-loaded PLGA NPs after storage in RPMI serum-free media at 37 °C for 24 hours. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	158.33±0.21	0.076±0.003
1 hour	164.67±0.32*	0.065±0.013
2 hours	163.17±2.90*	0.076±0.016
3 hours	165.43±1.07*	0.079±0.022
24 hours	158.20±1.97	0.050±0.025

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.20 Size and PDI values of blank PLGA NPs after storage in RPMI serum-free media at 37 °C for 24 hours. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	159.43±0.68	0.089±0.023
1 hour	160.93±1.16	0.075±0.027
2 hours	161.43±1.50	0.068±0.004
3 hours	161.40±1.65	0.067±0.017
24 hours	160.17±0.81	0.058±0.009

Table 5.21 Size and PDI values of AL-loaded PLGA NPs after storage in DMEM serum-free media at 37 °C for 24 hours. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	158.33±0.21	0.076±0.003
1 hour	162.17±1.94	0.060±0.021
2 hours	167.13±0.35*	0.076±0.022
3 hours	161.97±2.91	0.058±0.029
24 hours	157.90±1.25	0.071±0.017

* Indicates $p < 0.05$ compared with freshly prepared NPs

Table 5.22 Size and PDI values of blank PLGA NPs after storage in DMEM serum-free media at 37 °C for 24 hours. Data are presented as mean±SD values from three experiments.

	Size (nm)	**PDI**
Freshly prepared	159.43±0.68	0.089±0.023
1 hour	161.40±0.53	0.071±0.023
2 hours	162.40±3.69	0.061±0.006
3 hours	162.60±3.99	0.053±0.009
24 hours	160.43±2.81	0.089±0.018

5.4 *In vitro* cytotoxic activity

The cholangiocarcinoma (CCA) cell lines, CL-6 and HuCCT-1 and normal cell lines, OUMS-36T-1F were treated with AL-loaded PLGA NPs, blank PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol and 5-FU. The cytotoxic activities were then observed at 24, 48, and 72 hours after treated.

After 24 hours of treatment, the cytotoxic activity of AL-loaded PLGA NPs against CL-6 and HuCCT-1 were significantly lower than that of blank PLGA NPs, atractylodin dissolved with water and atractylodin dissolved with ethanol and 5-FU ($p<0.05$). Whereas, cytotoxic activities of atractylodin dissolved with water or atractylodin dissolved with ethanol and 5-FU were not found to be different. However, the cytotoxic activity against OUMS-36T-1F of AL-loaded PLGA NPs was found to be significantly higher than that of blank PLGA NPs and 5-FU ($p<0.05$). When compare to atractylodin dissolved with water and atractylodin dissolved with ethanol, however, the cytotoxic activity against OUMS-36T-1F was not found to be different.

In vitro cell viability (%) of AL-loaded PLGA NPs, blank PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) on CL-6, HuCCT-1 and OUMS-36T-1F cell lines after 24 hours of incubation are presented in **Figure 5.2-5.7**.

The cytotoxic activities (presented as IC_{50}) of the AL-loaded PLGA NPs, blank PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) against CL-6, HuCCT-1, and OUMS-36T-1F cell lines after 24 hours are summarized in **Table 5.23 and 5.24**.

After 48 hours of treatment, the cytotoxic activities against CL-6 and HuCCT-1 were found to be higher than that of 24 hours for almost all of the treatments except atractylodin dissolved with ethanol. The cytotoxic activity of AL-loaded PLGA NPs against CL-6 was not different from atractylodin dissolved with water or atractylodin dissolved with ethanol. However, it was found to be significantly higher than blank-PLGA NPs and lower than 5-FU ($p<0.05$). The cytotoxic activity against HuCCT-1 of AL-loaded

PLGA NPs was found to be substantially higher than blank PLGA NPs and lower than atractylodin dissolved with ethanol and 5-FU. Whereas, it was not different from atractylodin dissolved with water. Moreover, atractylodin dissolved with ethanol exhibited significantly greater than atractylodin dissolved with water on both CL-6 and HuCCT-1 ($p<0.05$). The cytotoxic activities of AL-loaded PLGA NPs, atractylodin dissolved with water and atractylodin dissolved with ethanol against OUMS-36T-1F were not found to be different. However, it was significantly higher than that of 5-FU ($p<0.05$).

In vitro cell viability (%) of AL-loaded PLGA NPs, blank PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) on CL-6, HuCCT-1 and OUMS-36T-1F cell lines after 48 hours of incubation are presented in **Figure 5.8-5.13**.

The cytotoxic activities (presented as IC_{50}) of the AL-loaded PLGA NPs, blank PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) against CL-6, HuCCT-1, and OUMS-36T-1F cell lines after 48 hours are summarized in **Table 5.25 and 5.26**.

After 72 hours of treatment, cytotoxic activities of almost all of the treatments were found to be decreased from 48 hours on CL-6, HuCCT-1, and OUMS-36T-1F except 5-FU. The cytotoxic activity of AL-loaded PLGA NPs was significantly lower than atractylodin dissolved with ethanol and 5-FU on all cell lines ($p<0.05$). However, it was significantly higher than blank PLGA NPs ($p<0.05$). Moreover, the cytotoxic activities of atractylodin dissolved with ethanol were significantly higher than atractylodin dissolved with water ($p<0.05$).

In vitro cell viability (%) of AL-loaded PLGA NPs, blank PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) on CL-6, HuCCT-1 and OUMS-36T-1F cell lines after 72 hours of incubation are presented in **Figure 5.14-5.19**.

The cytotoxic activities (presented as IC_{50}) of the AL-loaded PLGA NPs, blank PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-

FU (positive control) against CL-6, HuCCT-1, and OUMS-36T-1F cell lines after 72 hours are summarized in **Table 5.27 and 5.28.**

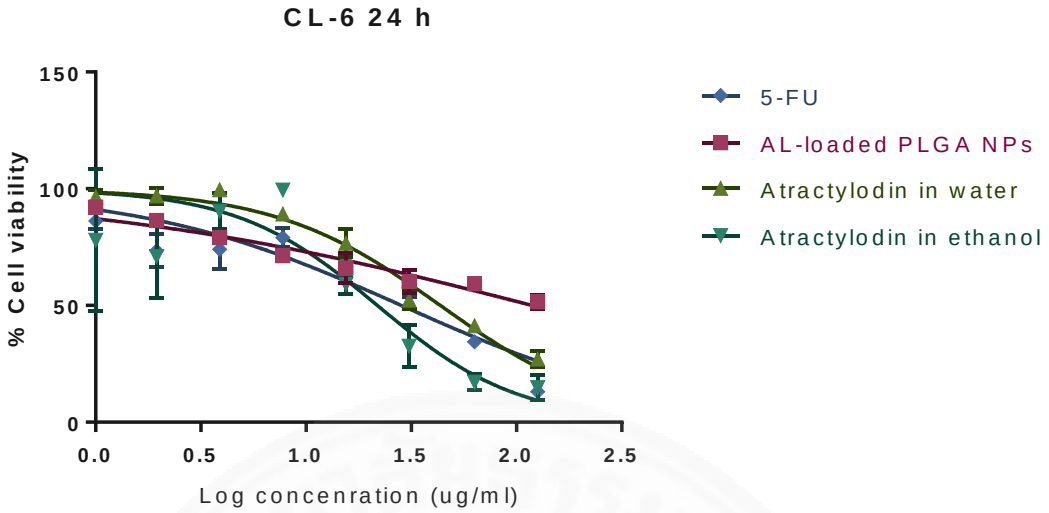

Figure 5.2 *In vitro* cell viability (%) of AL-loaded PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) on CL-6 after 24 hours of incubation.

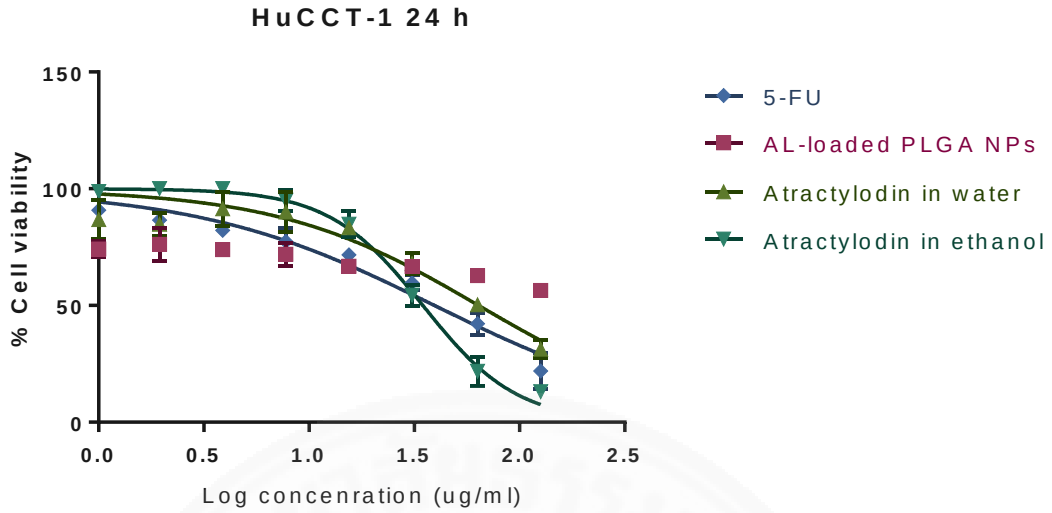

Figure 5.3 *In vitro* cell viability (%) of AL-loaded PLGA NPs, atractylodin dissolved with water, and atractylodin dissolved with ethanol, and 5-FU (positive control) on HuCCT-1 after 24 hours of incubation.

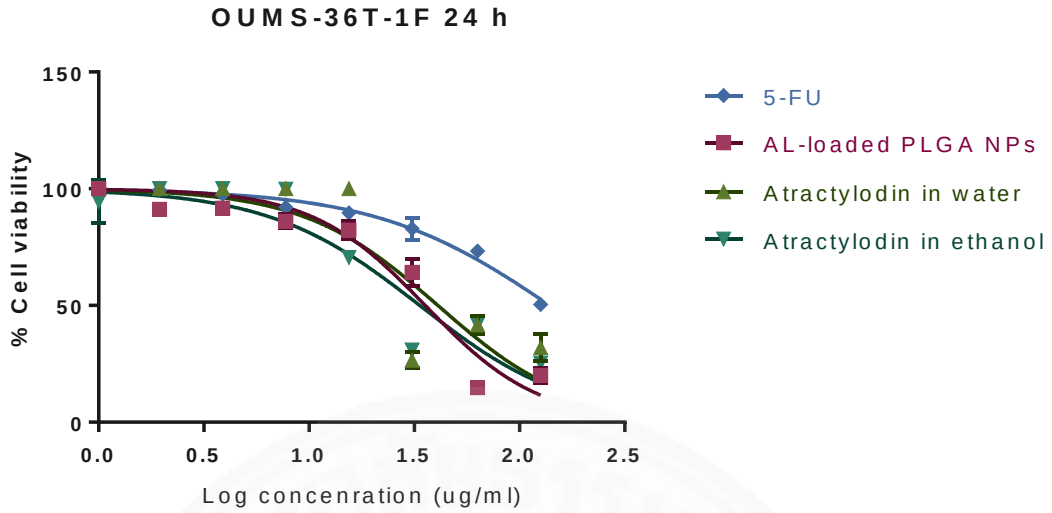

Figure 5.4 *In vitro* cell viability (%) of AL-loaded PLGA NPs, atractylodin dissolved with water, and atractylodin dissolved with ethanol, and 5-FU (positive control) on OUMS-36T-1F after 24 hours of incubation.

Table 5.23 Cytotoxic activities (presented as IC_{50}) of the AL-loaded PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control), against CL-6, HuCCT-1 and OUMS-36T-1F cell lines after 24 hours of incubation using MTT assays. Data are presented as mean±SD values of IC_{50} from three replications.

Drugs	**CL-6**	**HuCCT-1**	**OUMS-36T-1F**
		IC_{50} (µg/ml)	
AL-loaded PLGA NPs	> 125	> 125	35.65±3.21
Atractylodin dissolved with water	43.55±1.20*	64.27±4.04*	41.17±2.93
Atractylodin dissolved with ethanol	20.98±2.95*	34.95±4.95*	33.61±0.38
5-FU	28.41±4.56*	40.22±8.73*	> 125*,**,***

* Indicates $p < 0.05$ compared with AL-loaded PLGA NPs

** Indicates $p < 0.05$ compared with atractylodin dissolved with water

*** Indicates $p < 0.05$ compared with atractylodin dissolved with ethanol

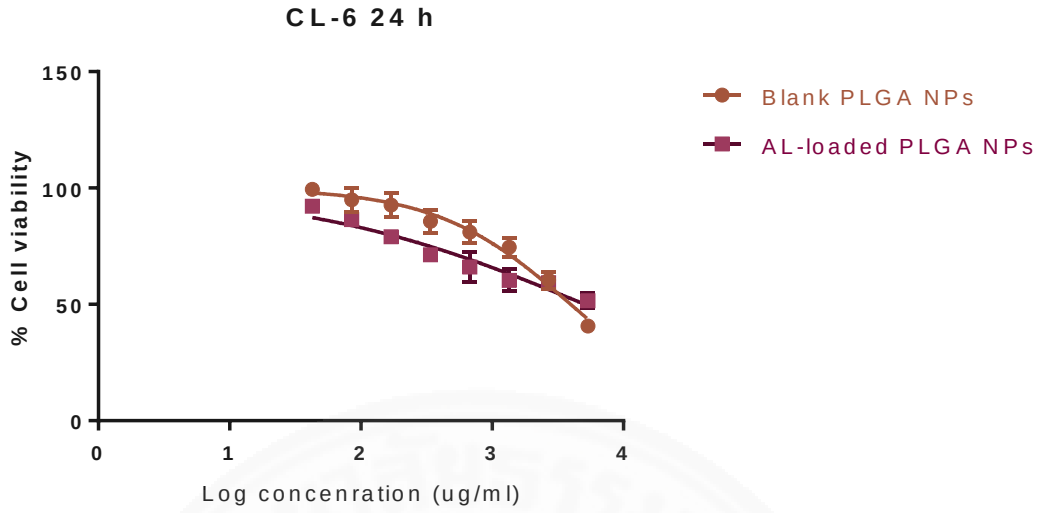

Figure 5.5 *In vitro* cell viability (%) of AL-loaded PLGA NPs and blank PLGA NPs on CL-6 after 24 hours of incubation.

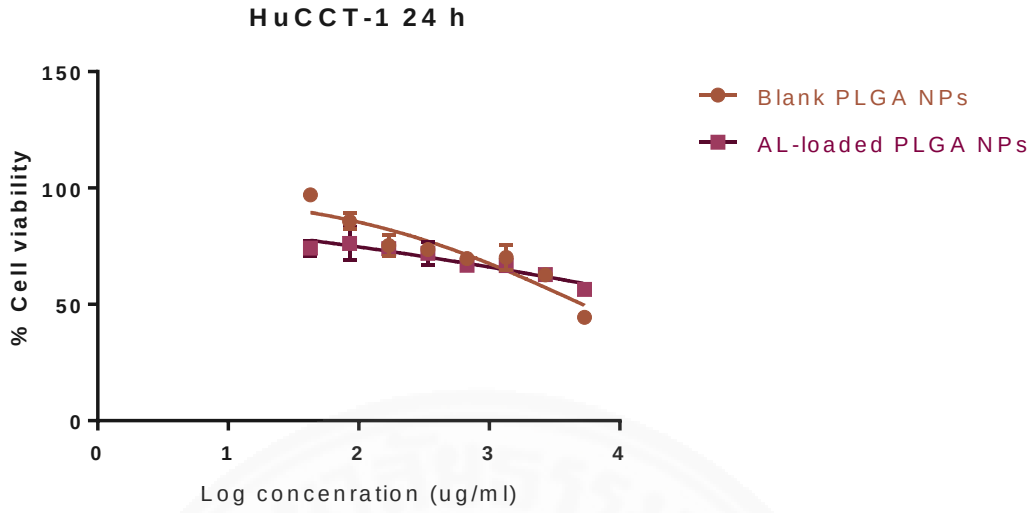

Figure 5.6 *In vitro* cell viability (%) of AL-loaded PLGA NPs and blank PLGA NPs on HuCCT-1 after 24 hours of incubation.

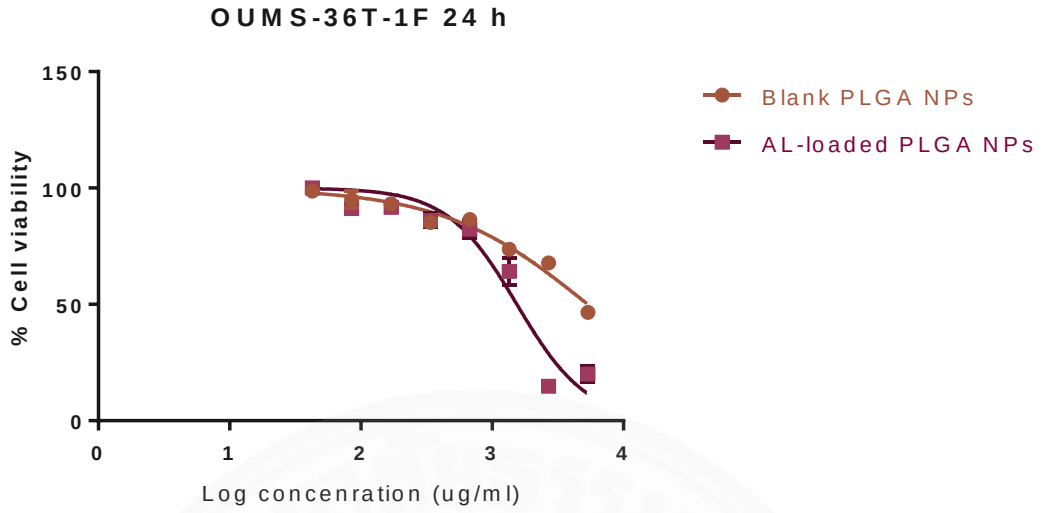

Figure 5.7 *In vitro* cell viability (%) of AL-loaded PLGA NPs and blank PLGA NPs on OUMS-36T-1F after 24 hours of incubation.

Table 5.24 Cytotoxic activities (presented as IC_{50}) of the AL-loaded PLGA NPs and blank PLGA NPs against CL-6, HuCCT-1 and OUMS-36T-1F cell lines after 24 hour of incubation using MTT assays. Data are presented as mean±SD values of IC_{50} from three replications.

Drugs	**CL-6**	**HuCCT-1**	**OUMS-36T-1F**
		IC_{50} (µg/ml)	
blank PLGA NPs	3,929.67±411.30	> 5,400	5,350.33±263.64
AL-loaded PLGA NPs	> 5,400	> 5,400	1,543.00±137.66*

* Indicates $p < 0.05$ compared with blank PLGA NPs

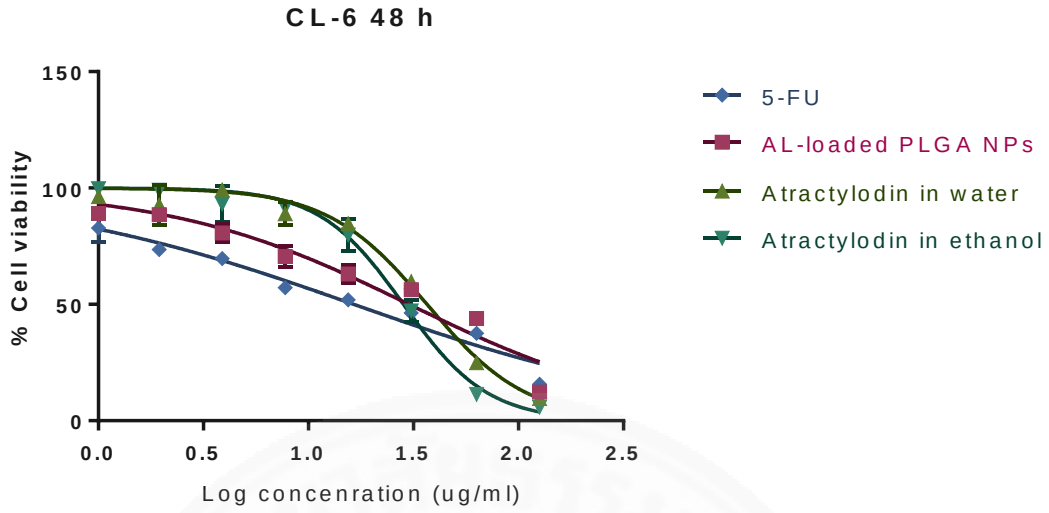

Figure 5.8 *In vitro* cell viability (%) of AL-loaded PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) on CL-6 after 48 hours of incubation.

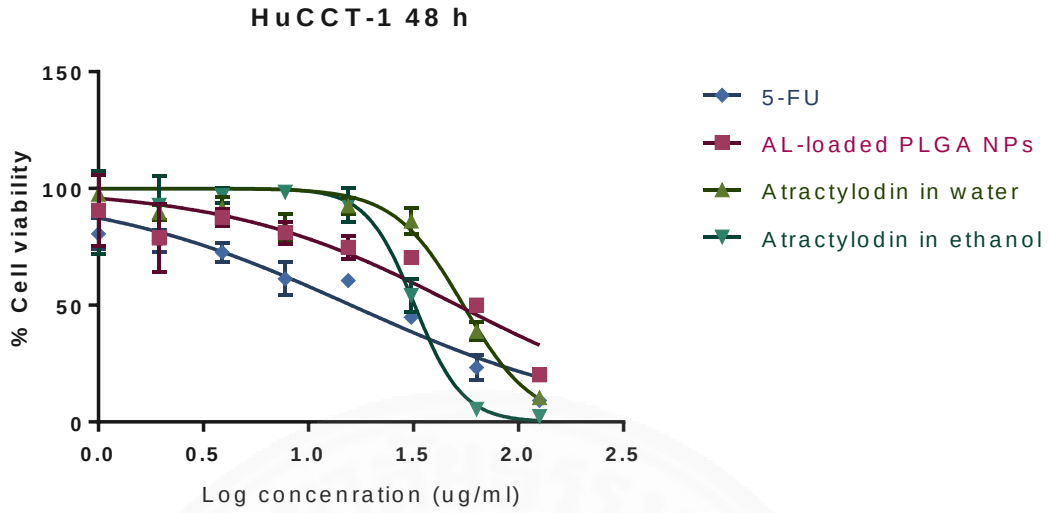

Figure 5.9 *In vitro* cell viability (%) of AL-loaded PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) on HuCCT-1 after 48 hours of incubation.

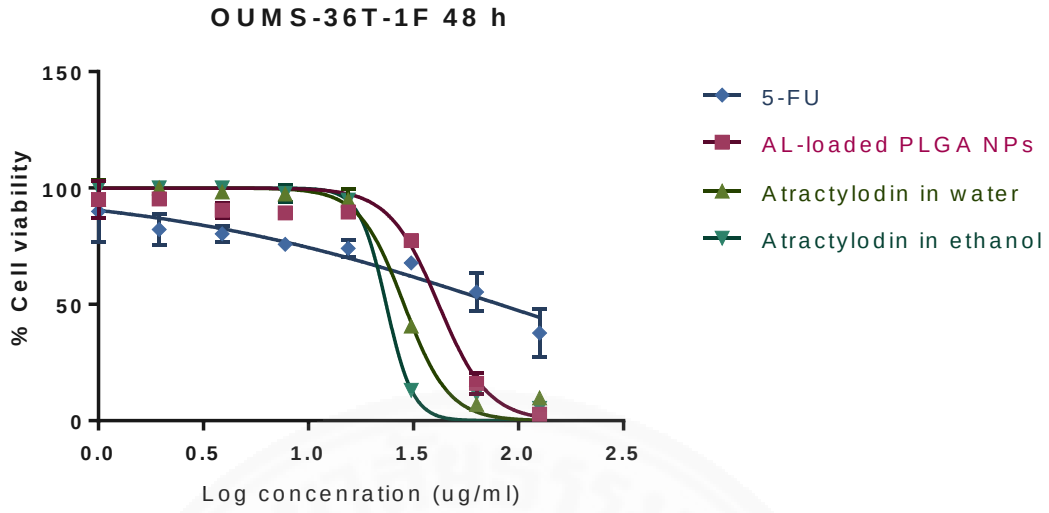

Figure 5.10 *In vitro* cell viability (%) of AL-loaded PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) on OUMS-36T-1F after 48 hours of incubation.

Table 5.25 Cytotoxic activities (presented as IC_{50}) of the AL-loaded PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control), against CL-6, HuCCT-1 and OUMS-36T-1F cell lines after 48 hours of incubation using MTT assays. Data are presented as mean±SD values of IC_{50} from three replications.

Drugs	**CL-6**	**HuCCT-1**	**OUMS-36T-1F**
		IC_{50} (µg/ml)	
AL-loaded PLGA NPs	30.20±2.93	51.19±4.45	41.72±1.39
Atractylodin dissolved with water	36.73±0.66	54.55±4.23	28.58±0.62
Atractylodin dissolved with ethanol	28.31±3.19**	31.76±2.80*,**	23.53±0.90
5-FU	16.51±1.91*,**,***	16.13±3.04*,**,***	85.61±30.58*,**,***

* Indicates $p < 0.05$ compared with AL-loaded PLGA NPs

** Indicates $p < 0.05$ compared with atractylodin dissolved with water

*** Indicates $p < 0.05$ compared with atractylodin dissolved with ethanol

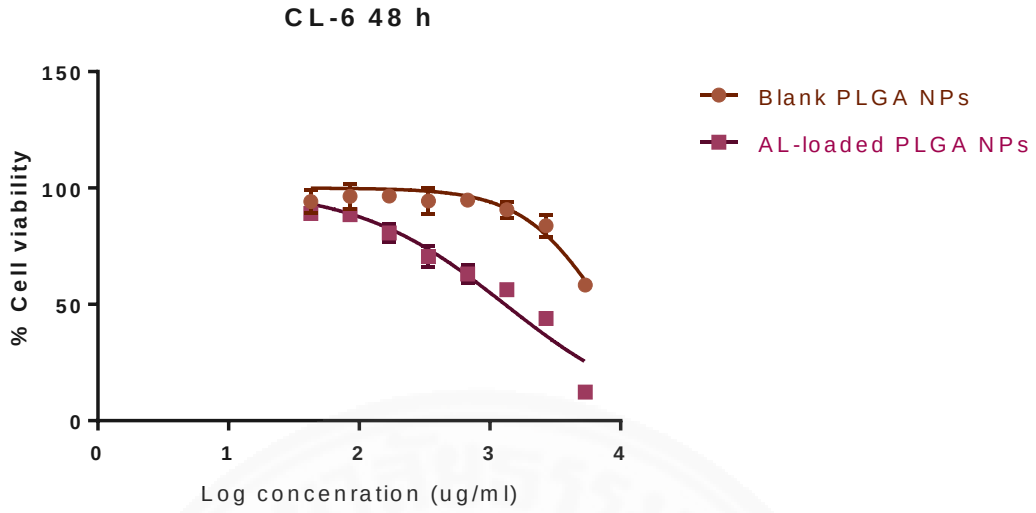

Figure 5.11 *In vitro* cell viability (%) of AL-loaded PLGA NPs and blank PLGA NPs on CL-6 after 48 hours of incubation.

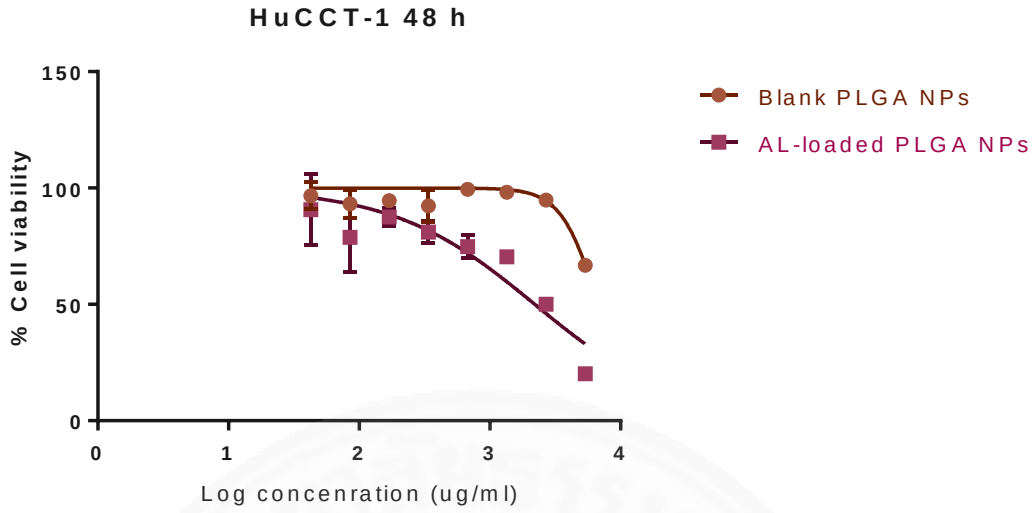

Figure 5.12 *In vitro* cell viability (%) of AL-loaded PLGA NPs and blank PLGA NPs on HuCCT-1 after 48 hours of incubation.

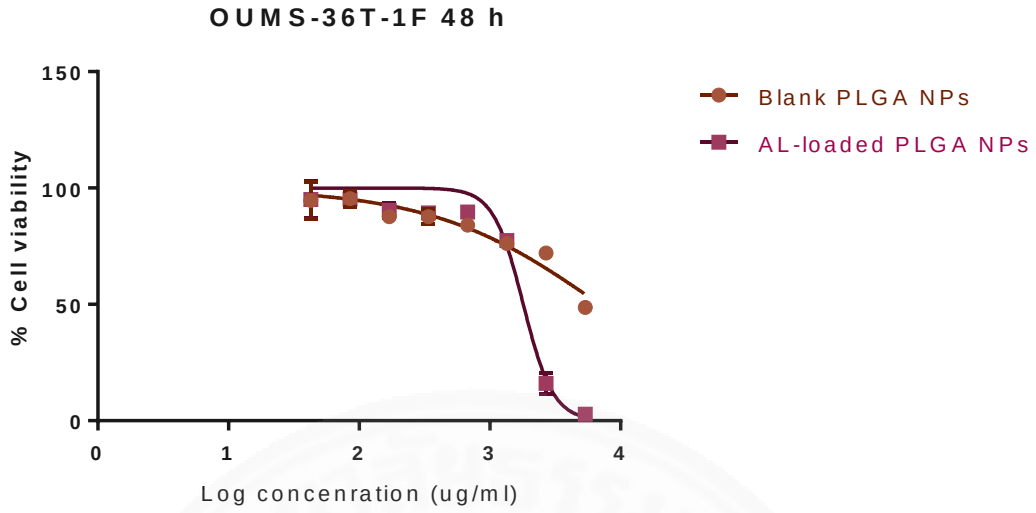

Figure 5.13 *In vitro* cell viability (%) of AL-loaded PLGA NPs and blank PLGA NPs on OUMS-36T-1F after 48 hours of incubation.

Table 5.26 Cytotoxic activities (presented as IC_{50}) of the AL-loaded PLGA NPs and blank PLGA NPs against CL-6, HuCCT-1 and OUMS-36T-1F cell lines after 48 hours of incubation using MTT assays. Data are presented as mean±SD values of IC_{50} from three replications.

Drugs	**CL-6**	**HuCCT-1**	**OUMS-36T-1F**
		IC_{50} (µg/ml)	
blank PLGA NPs	> 5,400	> 5,400	> 5,400
AL-loaded PLGA NPs	1,306.00±126.35*	2,205.00±189.52*	1,804.67±58.23*

* Indicates $p < 0.05$ compared with blank PLGA NPs

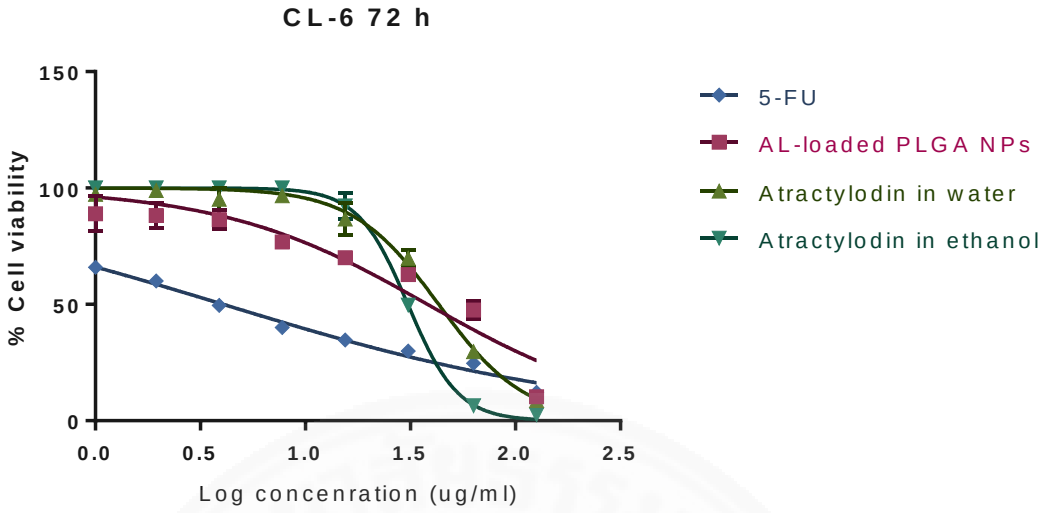

Figure 5.14 *In vitro* cell viability (%) of AL-loaded PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) on CL-6 after 72 hours of incubation.

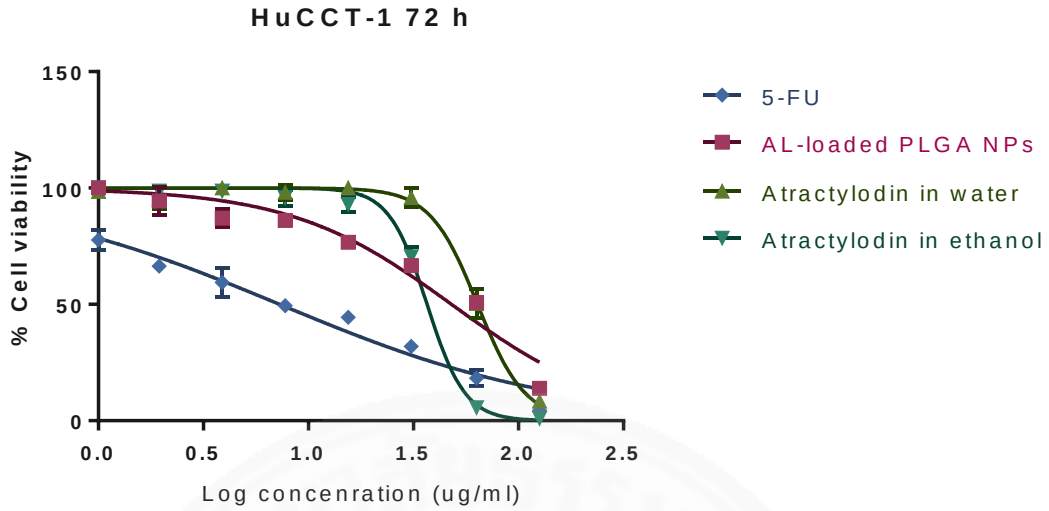

Figure 5.15 *In vitro* cell viability (%) of AL-loaded PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) on HuCCT-1 after 72 hours of incubation.

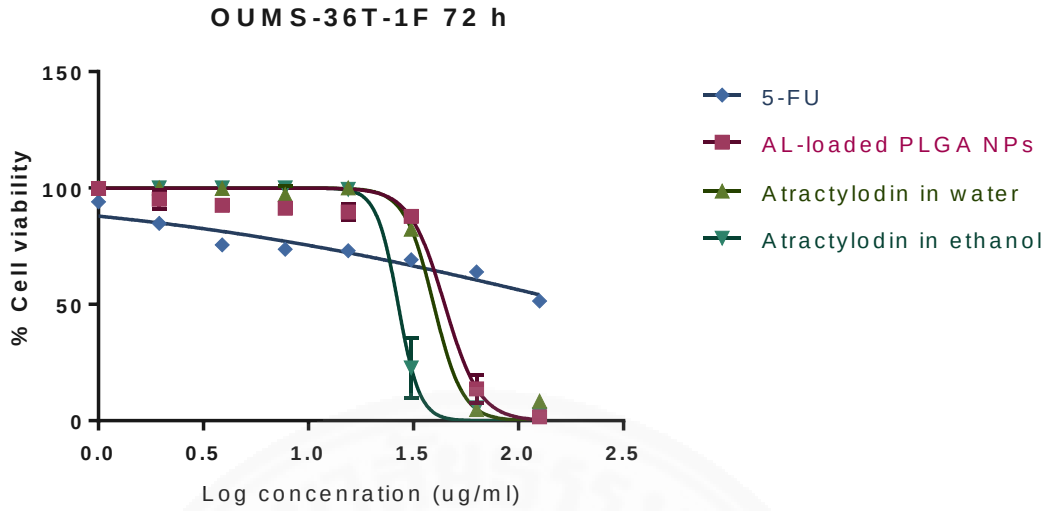

Figure 5.16 *In vitro* cell viability (%) of AL-loaded PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control) on OUMS-36T-1F after 72 hours of incubation.

Table 5.27 Cytotoxic activities (presented as IC_{50}) of the AL-loaded PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU (positive control), against CL-6, HuCCT-1 and OUMS-36T-1F cell lines after 72 hours of incubation using MTT assays. Data are presented as mean±SD values of IC_{50} from three replications.

Drugs	**CL-6**	**HuCCT-1**	**OUMS-36T-1F**
		IC_{50} (µg/ml)	
AL-loaded PLGA NPs	38.28±2.62	48.08±1.07	44.47±1.82
Atractylodin dissolved with water	42.83±2.97	63.71±3.90*	39.48±0.24
Atractylodin dissolved with ethanol	30.64±1.01*,**	36.78±1.52*,**	26.84±1.76*,**
5-FU	4.07±0.60*,**,***	7.30±1.12*,**,***	> 125*,**,***

* Indicates $p < 0.05$ compared with AL-loaded PLGA NPs

** Indicates $p < 0.05$ compared with atractylodin dissolved with water

*** Indicates $p < 0.05$ compared with atractylodin dissolved with ethanol

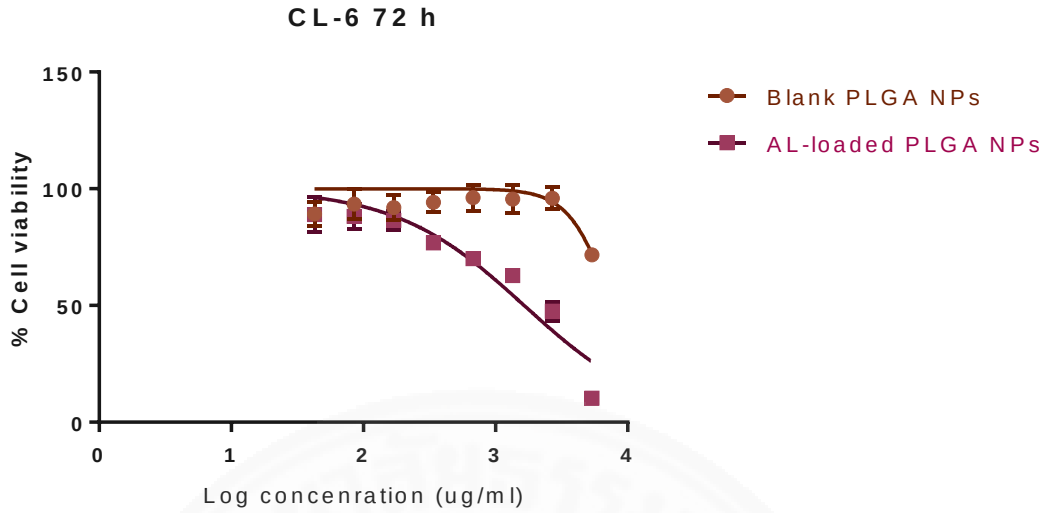

Figure 5.17 *In vitro* cell viability (%) of AL-loaded PLGA NPs and blank PLGA NPs on CL-6 after 72 hours of incubation.

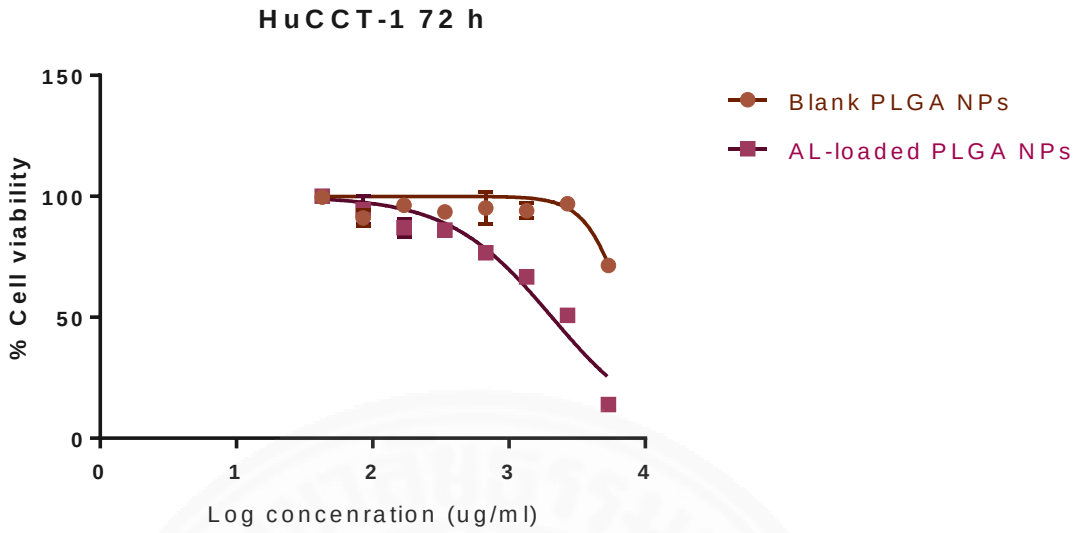

Figure 5.18 *In vitro* cell viability (%) of AL-loaded PLGA NPs and blank PLGA NPs on HuCCT-1 after 72 hours of incubation.

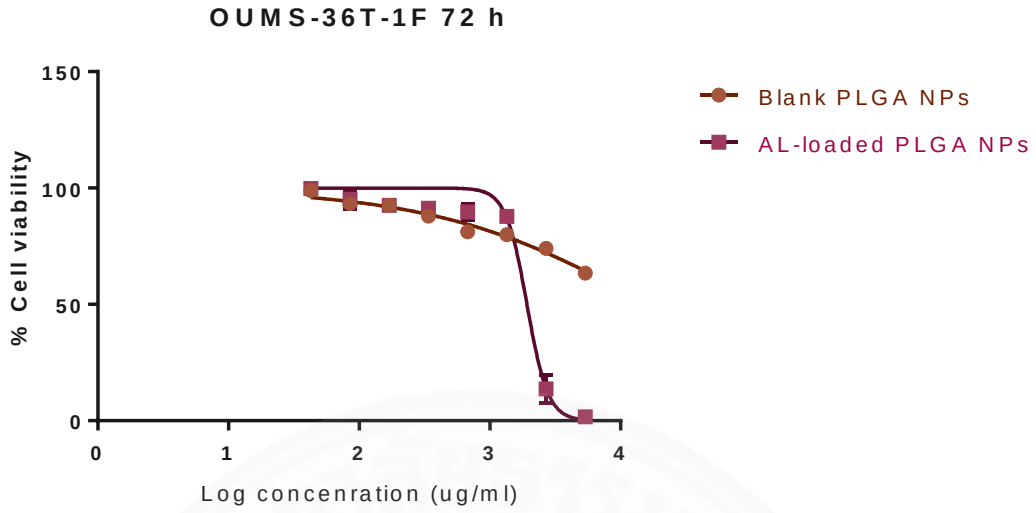

Figure 5.19 *In vitro* cell viability (%) of AL-loaded PLGA NPs and blank PLGA NPs on OUMS-36T-1F after 72 hours of incubation.

Table 5.28 Cytotoxic activities (presented as IC_{50}) of the AL-loaded PLGA NPs and blank PLGA NPs against CL-6, HuCCT-1 and OUMS-36T-1F cell lines after 72 hours of incubation using MTT assays. Data are presented as mean±SD values of IC_{50} from three replications.

Drugs	**CL-6**	**HuCCT-1**	**OUMS-36T-1F**
		IC_{50} (µg/ml)	
Blank PLGA NPs	> 5,400	> 5,400	> 5,400
AL-loaded PLGA NPs	1,654.00±112.07*	2,072.00±45.53*	1,919.33±76.79*

* Indicates $p < 0.05$ compared with blank PLGA NPs

CHAPTER 6
DISCUSSIONS

Atractylodin is an active compound contained in the rhizome of traditional Chinese medicine, *Atractylodes lancea* (*A. lancea*) (Thunb.) DC. It has been found an excellent anti-cholangiocarcinoma activity.[8,9] However, atractylodin insoluble in water and has to be dissolved in organic solvent.[11,12] Nowadays, PLGA copolymer is widely used to prepared as polymeric NPs for delivering hydrophobic drugs to increase the solubility of the drugs in water. For instance, docetaxel-loaded PLGA-PEG NPs to improve pharmacokinetic and biodistribution profiles of the drug by increasing blood circulation half-life and lowering drug accumulation in normal tissue.[13] Furthermore, PLGA was also used to prepare NPs as drug delivery system of natural active compounds. For instance, curcumin-loaded PLGA-PEG NPs for enhancing water solubility, anticancer activity, and blood circulation half-life.[20] However, PLGA NPs delivering atractylodin has never been developed. Thus, this present study aimed to develop atractylodin-loaded PLGA NPs (AL-loaded PLGA NPs) as a drug delivery system to enhance solubility in water.

In this study, atractylodin was developed in the form of NP suspensions to enhance water solubility. The AL-loaded PLGA NPs and blank PLGA NPs were prepared from PLGA copolymer MW 12,000 (Resomer® RG502) and 48,000 (Resomer® RG504) by using solvent displacement method. All NPs were found to be freely dispersible in water without aggregation. The small sizes and narrow size distributions of NPs were observed in all NP formulations. The sizes of AL-loaded PLGA NPs prepared from Resomer® RG502, and Resomer® RG504 were not found to be different. In the previous study, however, the smaller size was observed in NPs loaded hydrophobic drugs prepared from higher MW of PLGA due to the longer aliphatic chain.[74] For the surface charge of NPs, all NP formulations provided zeta potential lower than -20 mV which was sufficient stabilization of NPs. The zeta potential is an important parameter which influences the stability of NP suspensions. There is a study reported that high positive or negative value of zeta potential could prevent NPs aggregation and easy redispersion. Moreover, NPs prepared by using high

molecular weight surfactant, for instance, poloxamer407, the zeta potential -20 mV or much lower can provide sufficient stability for NP suspensions.[94] For % encapsulation efficiency (%EE) and % loading efficiency (%LE), both AL-loaded PLGA NP formulations (F2 and F4) had the ability to encapsulate atractylodin in NPs. The %EE and %LE of both formulations were about 50% and 2%, respectively. However, the %EE and %LE of AL-loaded PLGA NPs prepared from Resomer® RG502 were observed to be higher than that of NPs prepared from Resomer® RG504.

The drug releasing study was then investigated. Drug releasing profile of AL-loaded PLGA NPs both formulations (F2 and F4) occurred in a biphasic manner with initial burst release followed by sustained release. The burst release might occur from drug adsorbed on the surface of NPs diffuse through the polymer matrix and from a large surface to volume ratio of the NPs. The sustained release might be from drug diffusion and polymer degradation.[95,96] This observation supports the previous study of hydrophobic drug-loaded PLGA NPs. For instance, cannabinoid-loaded PLGA NPs[74] and docetaxel-loaded PLGA NPs.[13] From the results, the percent of drug released from AL-loaded PLGA NPs both formulations were not found to be different (88% in 72 hours). In a review, the study reported that, however, MW of PLGA affects drug release from NPs. Moreover, in several studies also found that higher MW resulted in lower drug release from NPs.[74,97] Because of high MW, PLGA composes of the high amount of lactic acid which is hydrophobic part of NPs.

In spite of the drug released from NPs were not found to be different in both formulations the %EE and %LE of AL-loaded PLGA NPs prepared from Resomer® RG502 were found to be higher than AL-loaded PLGA NPs prepared from Resomer® RG504. Thus, AL-loaded PLGA NPs prepared from Resomer® RG502 were selected for further studies. Because higher drug loading indicates the smaller amount of NPs used in the treatment.

The prepared AL-loaded PLGA NPs and blank PLGA NPs were kept in the form of NP suspensions, and PLGA is a biodegradable copolymer which can be degraded all the time in an aqueous environment. Thus, the stability of NPs in an aqueous solution was investigated. The significantly changed in sizes of NPs was observed after storage in almost all of the conditions except AL-loaded PLGA NPs which were stored in ultrapure water at 4 °C. However, sizes of all NPs still were in the

nanometer range and acceptable. For PDI, most of the NPs exhibited PDI less than 0.1 which indicated the narrow distribution of NPs.[97] In some conditions of storage, PDI was found to be significantly increased especially the NPs which were stored in RPMI and DMEM complete media. This might be affected by the components in cell culture medium, for instance, protein supplemented and electrolytes that can interact with NPs.[98] According to the zeta potential is an important parameter which influences the stability of NP suspensions, the NPs stored in ultrapure water were investigated zeta potential. Only AL-loaded PLGA NPs and blank PLGA NPs which were stored in ultrapure water at 37 °C exhibited zeta potentials significantly increased to above -20 mV which NPs can easily aggregate. From all stability results, both AL-loaded PLGA NPs and blank PLGA NPs should be stored in ultrapure water at 4 °C and have to be used as soon as possible after preparation to avoid NPs aggregation.

AL-loaded PLGA NPs and blank PLGA NPs were then investigated anti-cholangiocarcinoma activity using MTT assays. The AL-loaded PLGA NPs, blank PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-fluorouracil (5-FU; positive control) were incubated with cholangiocarcinoma (CCA) cell lines, CL-6 and HuCCT-1. The cytotoxic activities against these two cell lines of all treatments were concentration- and time-dependent. The cytotoxic activities of AL-loaded PLGA NPs against CL-6 and HuCCT-1 after 24 hours of incubation found to be lower than atractylodin dissolved with water, atractylodin dissolved with ethanol and 5-FU. After 48 and 72 hours of treatment, however, it was found to be higher than 24 hours. This might be due to the higher amount of drug released from NPs which bound to the cells. Moreover, the cytotoxic activities of AL-loaded PLGA NPs were not significantly different from atractylodin dissolved in water after 48 and 72 hours against CL-6 and 48 hours against HuCCT-1. However, it was found to be lower than atractylodin dissolved in ethanol after 48 hours against CL-6 and 48 and 72 hours against HuCCT-1. Besides, cytotoxic activities of atractylodin dissolved in ethanol were greater than atractylodin dissolved with water at all time points. This because the atractylodin was readily soluble in ethanol. Whereas, it is insoluble in water and need to be sonicated to enhance drug disintegration. Moreover, for AL-loaded PLGA NPs formulation, even though the AL-loaded PLGA NPs could be freely dispersible in water, however, the drug needs to be released from NPs which could not expose to the

cells immediately. Thus, the atractylodin dissolved with ethanol exhibited cytotoxic activities against CL-6 and HuCCT-1 greater than atractylodin dissolved with water and AL-loaded PLGA NPs. However, the half maximal inhibitory concentration (IC_{50}) values of all forms of atractylodin against CL-6 at 48 hours were observed to be closed to the IC_{50} of atractylodin reported in the previous study. The mean IC_{50} ± SD of AL-loaded PLGA NP, atractylodin dissolved with water, and atractylodin dissolved with ethanol were 30.20±2.93 μg/ml, 36.73±0.66, and 28.31±3.19, respectively. The mean IC_{50} ± SD of atractylodin in the previous study was 41.66 ± 2.51 μg/ml.[34] These IC_{50} values indicate that all forms of atractylodin provide moderately cytotoxic to CCA cell lines.[99] To investigate the toxicity of the treatments to the normal cells, the AL-loaded PLGA NPs, blank PLGA NPs, atractylodin dissolved with water, atractylodin dissolved with ethanol, and 5-FU were incubated with OUMS-36T-1F. The cytotoxic activities of AL-loaded PLGA NPs against OUMS-36T-1F were found to be lower than atractylodin dissolved with water and atractylodin dissolved with ethanol at 48 and 72 hours even though the significantly different were not observed. This indicates that AL-loaded PLGA NPs had lower toxicity to the normal cells compared with other forms of atractylodin. For the cytotoxic activities of blank PLGA NPs against CL-6, HuCCT-1, and OUMS-36T-1F were found to be significantly lower than AL-loaded PLGA NPs at 48 and 72 hours and % cell viability almost reached 100%. This confirms that the cytotoxic activities against CL-6 and HuCCT1 were from the atractylodin but not from PLGA and blank PLGA NPs did not become toxic to normal cells. The cytotoxic activities of 5-FU against CL-6 and HuCCT-1 were found to be higher than all forms of atractylodin. Moreover, 5-FU exhibited lower toxic to the normal cell. In the previous study, however, reported that 5-FU exhibited lower cytotoxic activities against CL-6 and higher toxic to normal cell compared with atractylodin.[34] This might be due the different protocol of MTT assays was used to investigate cytotoxic activities of these treatments.

CHAPTER 7
CONCLUSIONS AND RECOMMENDATIONS

The results obtained from this present study provide the information of atractylodin-loaded PLGA nanoparticles (AL-loaded PLGA NPs) preparation, NPs characterization and drug release *in vitro* as well as the stability and *in vitro* cytotoxic activity of NPs against cholangiocarcinoma (CCA) cell lines. Main findings obtained could be summarized as follows:

(1) All NPs provide small size with narrow size distribution and sufficient zeta potential that facilitate stability of NPs.

(2) Both AL-loaded PLGA NPs prepared from Resomer® RG502, and Resomer® RG504 could encapsulate atractylodin in NPs, and the drug can be released from NPs.

(3) The AL-loaded PLGA NPs prepared from Resomer® RG502 were selected to study stability in aqueous solutions and to investigate *in vitro* cytotoxic activities against CCA cell lines, CL-6 and HuCCT-1, and normal cell line, OUMS-36T-1F.

(4) The optimal condition for storage of NPs was storage in ultrapure water at 4°C.

(5) The *in vitro* cytotoxic activities of AL-loaded PLGA NPs were concentration- and time-dependent with moderately cytotoxic to CL-6 and HuCCT-1.

(6) The cytotoxic activities of AL-loaded PLGA NPs against OUMS-36T-1F was not found to be significantly different from atractylodin dissolved with water and atractylodin dissolved with ethanol at 48 and 72 hours.

(7) Blank PLGA NPs were not toxic to both cholangiocarcinoma and normal cell lines.

From all findings, the AL-loaded PLGA NPs were successfully developed and have potential to be used as drug delivery systems for the treatment of cholangiocarcinoma. However, this NP formulation should be further developed to

improve drug encapsulation and loading efficiency, the stability of NPs and toxicity to normal cells.

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