Preparation of Novel Soybean Oil Based Polymers and Investigation of Their Application in the Delivery of Anticancer Drugs

by

Yi Ji Wu

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmaceutical Sciences
University of Toronto

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Master of Science
Graduate Department of Pharmaceutical Sciences
2013

Abstract

Previously our group has developed a polymer-lipid hybrid nanoparticle (PLN) system that can effectively deliver the anticancer drugs. However, the polymer, hydrolyzed polymer of epoxide soybean oil (HPESO), used in this system, has low water solubility at neutral pH, which complicates the process of making the PLNs. Therefore, this thesis focuses on the synthesis of new soybean oil based polymers that exhibit better water-solubility and investigation of their application in the delivery of the anticancer drug cisplatin. Three new polymers were synthesized: a) alcohol-based polymer; b) aspartic acid-based and c) taurine-based polymers. These polymers had similar molecular weights, but had higher aqueous solubility and better pH-dependent solubility profile as compared to HPESO. The loading cisplatin into the polymer was likely via covalent conjugation between the polymers and the platinum atom. Platinum drug-loaded PLNs were prepared using lipid and taurine-based polymer and showed a sustained drug release profile similar to Miriplatin.
I would like to thank my supervisor, Professor Shirley Wu, for accepting me as a part time graduate student in her lab and for being such a wonderful mentor. Your patience and continuous support was much appreciated. I can’t thank you enough for the opportunity which can broaden my experience with novel nanoparticle formulation. This definitely increases my knowledge and confidence as a scientist.

Special thanks to Drs. Ping Lee, Peter O’Brien, and A. Michael Rauth for taking their time to attend my annual committee meetings and providing me guidance and detailed recommendations. Special thanks to Dr. Lakshmi Kotra who kindly accepted to be a member of my MSc. thesis examination committee.

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Lastly, I would like to give my warmest thanks to my wife, Grace Yang, my parents and my lovely child, Vincent Wu, for believing in me. As a part time graduate student, without their support, I would not have been able to finish this thesis work.
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<th>Abbreviation</th>
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<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
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<tr>
<td>ATP7A</td>
<td>ATPase A</td>
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<tr>
<td>AAS</td>
<td>atomic absorption spectroscopy</td>
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<tr>
<td>BER</td>
<td>base excision repair</td>
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<td>CaCl₂</td>
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<td>Acronym</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DPPG</td>
<td>dipalmitoyl phosphatidyl glycerol</td>
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<td>DDI</td>
<td>distilled, deionized</td>
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<td>DA</td>
<td>dodecanoic acid</td>
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<td>DOX</td>
<td>doxorubicin</td>
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<td>DMsPLN</td>
<td>doxorubicin and mitomycin C co-encapsulated stealth polymer lipid hybrid nanoparticle</td>
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<td>DLS</td>
<td>dynamic light scattering</td>
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<td>EPR</td>
<td>enhanced permeability and retention effect</td>
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<td>ERCC1</td>
<td>excision repair cross-complementation group 1</td>
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<td>ethanol</td>
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<td>fatty acid ethyl ester</td>
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<td>FDA</td>
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<td>FTIR</td>
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<td>GG918</td>
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<td>$t_{1/2}$</td>
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<td>HCC</td>
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<td>HPESO</td>
<td>hydrolyzed polymer of epoxide soybean oil</td>
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<td>HPMA</td>
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<td>HSQC</td>
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<td>ICP-OES</td>
<td>inductively coupled plasma - optical emission spectrometer</td>
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<tr>
<td>IC$_{50}$</td>
<td>inhibitory concentration for 50% effect</td>
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<tr>
<td>i.v.</td>
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<td>magnesium chloride</td>
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<td>mPEG2000-DSPE</td>
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<td>MDR</td>
<td>multidrug-resistant</td>
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<td>mismatch repair</td>
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<td>MMC</td>
<td>mitomycin C</td>
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<td>number average molecular weight</td>
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<tr>
<td>Mw</td>
<td>molecular weight</td>
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<td>MWCO</td>
<td>molecular weight cut off</td>
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<tr>
<td>NSCLC</td>
<td>non-small-cell-lung cancer</td>
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<td>NER</td>
<td>nucleotide excision repair</td>
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<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
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<td>Myrj</td>
<td>poly(ethylene glycol)-co-stearate block copolymer</td>
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<td>(\text{cis-}[\text{PtII}(\text{NH}_3)_2(\text{N-AcLys})])</td>
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<td>RFI</td>
<td>radio-frequency interference</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>r.t.</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SLN</td>
<td>solid lipid nanoparticle</td>
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<td>Abbreviation</td>
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<tr>
<td>SPC-3</td>
<td>soy phosphatidyl choline</td>
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<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>TNBC</td>
<td>triple negative breast cancer</td>
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<td>VRP</td>
<td>verapamil hydrochloride</td>
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<td>v/v</td>
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<td>water/oil</td>
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</tr>
<tr>
<td>w/v</td>
<td>weight by volume</td>
</tr>
<tr>
<td>ζ</td>
<td>zeta potential</td>
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Chapter 1
Introduction

1.1 An introduction to the anticancer drug cisplatin

Cisplatin \([\text{cis- diamminedichloroplatinum (II); CDDP}]\) has been one of the most effective and widely used antitumor drugs since its first clinical application in the early 70’s. It remains as the first line chemotherapy for various types of cancers, such as lung cancer, ovarian cancer, colon cancer, bladder cancer, testicular cancer and head and neck cancer. Cisplatin is particularly effective against testicular cancer with a cure rate of more than 90%. In addition, cisplatin is used as a second line drug against most other advanced cancers, such as breast, prostate and liver tumors. Cisplatin combination therapy with radiation therapy as well as other anticancer drugs such as taxane or gemcitabine is also commonly used in the clinic to enhance efficacy.\(^1\) One study also found that the combination of cisplatin and docetaxel was particularly effective against triple negative breast cancer (TNBC).\(^2\)

1.1.1 Mechanism of drug action

Cisplatin has two chloride ligands, which act as leaving groups of a platinum (II) atom. Under physiological conditions, aquation of cisplatin, which is the ligand exchange between chloride and water, can generate different platinum species (Figure 1). It is widely believed that mono-aquated compound, \(\text{cis-Pt}[(\text{H}_2\text{O})\text{Cl}((\text{NH}_3)_2]^+\), is the major form and able to exert its oncological action by intercalation with DNA. This cationic molecule forms either inter- or intra-strand (between N7 atoms of nearby pairs) lesions with DNA. As a result, mono-aquated cisplatin induces the apoptosis of cancer cells by preventing DNA replication and RNA transcription.\(^3\)
The hydrolysis of cisplatin to mono-aquated species is the rate-determining step. The NMR studies, conducted by Dabrowiak, found that the concentration of the free cisplatin within cell culture media decreased with time at the same rate as that of formed by the hydrolysis of cisplatin to mono-aquated species.\textsuperscript{4}

\[
\begin{align*}
\text{H}_3\text{N} & \quad \text{Pt} & \quad \text{OH}_2^+ & \quad \text{Cl}^- \\
\text{H}_3\text{N} & \quad \text{Pt} & \quad \text{Cl} & \quad \text{OH}_2^+ \\
\text{H}_2\text{O} & \quad \text{H}_3\text{N} & \quad \text{Pt} & \quad \text{Cl} \\
\text{H}_2\text{O} & \quad \text{H}_3\text{N} & \quad \text{Pt} & \quad \text{Cl}
\end{align*}
\]

\textbf{diaquated form} \quad \textbf{mono aquated form} \quad \textbf{cisplatin}

\[
\begin{align*}
\text{H}_3\text{N} & \quad \text{Pt} & \quad \text{OH}_2 & \quad + & \quad 2\text{H}^+ \\
\text{H}_3\text{N} & \quad \text{Pt} & \quad \text{OH} & \quad + & \quad \text{H}^+ \\
\text{H}_3\text{N} & \quad \text{Pt} & \quad \text{Cl} & \quad + & \quad \text{Cl}^-
\end{align*}
\]

\textbf{Figure 1} \quad \text{Scheme of the hydrolysis equilibrium of cisplatin}

The rate of aquation is mainly dependent on the concentration of chloride ion. Since chloride ion concentration in blood is high, cisplatin is relatively stable in the circulation. In contrast, due to the low concentration of chloride ion within cells, aquation progresses quickly to generate more reactive cationic platinum species to bind to protein, DNA or RNA.\textsuperscript{5, 6} It is known that the cisplatin hydration procedure, in which patients receive vigorous hydration with saline prior to and after cisplatin doses, can reduce the renal toxicity of cisplatin since the increased chloride concentration can shift equilibriums to free cisplatin.\textsuperscript{7}
1.1.2 Drug resistance and toxicity of cisplatin

There are three primary mechanisms associated with cisplatin acquired resistance in cells, namely elevated intracellular thiol levels, decreased accumulation inside cells, and enhanced DNA repair.

a) Mechanism due to elevated intracellular concentration of thiols.\(^8\)

The two chloride ligands of cisplatin are easily replaced by water or other side chains of amino acids, such as histamine, arginine, lysine, cysteine and methionine in the systemic circulation. In general, the concentration of the S-containing tri-peptide glutathione within cells is at a millimolar level. Additionally, glutathione S-transferase π is a widespread protein and is overexpressed in drug-resistant solid tumours. Cisplatin complexation with glutathione results in cisplatin exocytosis. For this reason, the drug concentration of cisplatin in the target cancer cells is significantly reduced resulting in low biological efficacy of cisplatin. (Figure 2)\(^9,10\)

![Reaction scheme to generate glutathione cisplatin conjugated complexes.](image)

**Figure 2** Reaction scheme to generate glutathione cisplatin conjugated complexes. After the formation of the complexes, they can be removed from the cytoplasm of cells.
b) Decreased platinum accumulation within cancer cells is due to an increase in efflux transporters and binding with membrane lipid. Due to hydrophobic cell membrane barriers, most free cisplatin cannot enter cells. Cisplatin is transported through cell membranes either via passive diffusion or the major copper influx transporter, copper transport protein 1 (Ctr 1). However, two other copper efflux transporters, copper-transporting ATPase A (ATP7A) and ATP7B, play a reverse role in transporting cisplatin out of cells. In addition, several studies demonstrated that cisplatin is able to complex with phosphatidylserine lipids located in the cell membrane which also reduced the concentration of free cisplatin accumulated within the cancer cells.

c) Enhanced DNA repair. It has been shown that certain genes, which are highly expressed in tumours, play an important role in DNA repair. Therefore, these tumours acquire resistance to cisplatin after repeated doses. There are three excision repair pathways to repair single stranded DNA damage: nucleotide excision repair (NER), base excision repair (BER), and DNA mismatch repair (MMR). Cisplatin is a DNA-damaging anticancer drug, that can induce apoptosis of cells via the formation of DNA-platinum adducts. Here, NER mainly repairs bulky DNA complexes such as the ones formed by interaction with cisplatin. Excision repair cross-complementation group 1 (ERCC1) is an essential protein for the mechanism of NER. Its loss can sensitize cells to cisplatin. It was found that cisplatin treatment could downregulate the expression of ERCC1 inducing resistance to cisplatin. To counteract these mechanisms of cisplatin resistance in cells, higher drug doses are required. However, this further increases dose-limiting toxicities. Cisplatin is known to cause severe toxicities such as chronic neurotoxicity, peripheral neuropathy, gastrointestinal toxicity and ototoxicity in clinical applications. The renal accumulation of cisplatin is greater than other
organs and kidneys are the major pathway for the excretion of cisplatin. After i.v. injection of cisplatin, the drug concentration in human renal proximal tubular epithelial cells is close to 5 times the serum concentration; thus, the higher accumulation of cisplatin in kidneys contributes to nephrotoxicity. Thus the patients, who receive cisplatin-based chemotherapy, require hydration one night before intravenous (i.v.) infusion and post hydration in hospital after infusion to reduce nephron- and neurotoxicity as well as other side effects.\textsuperscript{15}

1.2 Related platinum drugs and their mechanism of drug action

Due to the toxicity of cisplatin, especially to kidneys, the second-generation of platinum drugs, such as carboplatin [\textit{cis}-diammine(1,1-cyclobutanedicarboxylato)platinum] and oxaliplatin were developed and approved for clinical use worldwide. In certain Asian countries, nedaplatin and lobaplatin are also currently used. (Figure 3)

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figures/figure3.png}
\caption{Chemical structures of cisplatin and second-generation platinum drugs}
\end{figure}

The difference between cisplatin and these second-generation platinum drugs is that the chloride ligands in cisplatin are replaced by carboxylate or alcohol groups, which have a stronger bonding with platinum (II). It is believed that the molecular mechanism of action of these DNA-damaging
anticancer drugs is through the generation of the mono-aquated form or fully aquated species within cells.\textsuperscript{16}

These carboxylate-platinum drugs were found to have a longer blood circulation period due to the relatively inactive chelation between platinum and carboxylate. The aquation rate constant (Figure 1) for cisplatin was $8 \times 10^{-5}$ s\textsuperscript{-1} for 1mM at 37°C, which is almost 100 times faster than that of carboplatin.\textsuperscript{17} Furthermore, these platinum drugs have been shown to have reduced binding to plasma proteins, which results in reduced renal and nervous system toxicity. Thus, these platinum drugs are better tolerated by cancer patients.\textsuperscript{18} Nevertheless, the down side of these second-generation platinum drugs is their lower potencies compared to cisplatin. For example, in the \textit{in vivo} experimental anti-tumor systems, the potency of carboplatin varied from 1/8 to 1/45 as effective as cisplatin depending on the cancer cell lines treated.\textsuperscript{17}

Due to the high glycolytic rate of most tumor cells, the tumor micro-environments are acidic with pH levels as low as 5.7. In addition, the endosome, a membrane-bound compartment within cells is used for the transportation of drug carriers in the lysosome, has an acidic environment (pH 5-6). Thus, these properties can be utilized to trigger the release of active mono-aquated compounds from these second generation platinum drugs.\textsuperscript{19} Robert Hay and coworker showed the carboplatin, carboxylate-platinum complex, was quite stable at a plasma chloride concentration (104 mM) at 37°C. The half-life of ligand exchange between the chloride ion and carboxylate was more than 250 h. Thus, it was expected that such ligand exchange would be even slower within cells since the chloride concentration is only 4 mM. They suggested that the formation of \textit{cis-Pt}[(H\textsubscript{2}O)(OH)NH\textsubscript{3})\textsubscript{2}]\textsuperscript{+} was due to acid-catalysed decarboxylation.\textsuperscript{20} (Figure 4)
Figure 4 Mechanism of acid-catalyzed decarboxylation of platinum-carboxylate complexes. The release of the active platinum species within cancer cells was believed to adopt this mechanism.\textsuperscript{21}

The acid-catalysed drug release mechanism was also used for the development of new platinum drugs. Recently, Lee and coworkers synthesized a carboxylate chelated platinum prodrug, \textit{cis-}[Pt\textsuperscript{II}(NH\textsubscript{3})\textsubscript{2}(N-AcLys)] (Pt-PCN), which exhibited pH-triggered platinum release. Pt-PCN can release ~ 50\% at pH 5.0 after 36 h. In contrast, only ~ 15\% of Pt was released at pH 7.4 after 72 h. The protonation of N-acetylamido ligand under acidic condition can catalyze the release of the active diaminediaquo platinum form inside the cells.\textsuperscript{21}

However, due to the indiscriminate killing of targeted cancer cells and healthy cells, these platinum drugs induce a certain degree of bone marrow toxicity. Therefore, targeted drug delivery may be a potential platform for this type of anti-cancer therapy. Utilizing drug carriers could be a potential option to enhance the efficacy of platinum drugs against multidrug-resistant
(MDR) cancer cells. The high systemic toxicity of platinum drugs could be reduced via shielding with hydrophobic polymer chains.

1.3 An introduction to drug delivery systems for cisplatin

1.3.1 Enhanced permeability and retention effect in the treatment of cancer

Tumor tissues develop from the uncontrolled proliferation of a single malignant cell. When solid tumor tissues grow to a certain size, they will form tumor vessels to deliver nutrition and oxygen. At this stage, tumor vessels present themselves as leaky and defective structures exhibiting vessel hyperplasia and vascular hyperpermeability. It is noted that the capillary endothelium in tumor tissue is much more disordered compared to normal tissues with tight capillary endothelium. It has been found that the macromolecules with particle sizes smaller than 200 nm tend to accumulate within tumor tissues due to this leaky vasculature. Moreover, tumor tissues do not possess a functional lymphatic system. Therefore, nano-scale drug carriers with the sizes ranging from 10 nm to 160 nm, tend to stay in tumor tissues and this leads to a longer retention times. All together solid tumor blood vessels present a unique property of enhanced permeability, enabling nanoparticle drug carriers to target tumor tissues. Accordingly, the concentrations of nanoparticle encapsulated chemotherapeutic agents in tumor tissues could be much higher than that of free anti-cancer drugs. Therefore, significantly improved drug efficacy and dramatically reduced systemic toxicity can be achieved.\textsuperscript{22}

To date, there are three United States Food and Drug Administration (FDA) approved nanoparticle cancer drugs, which are Doxil, Abraxane and DepoCyt. Doxil was the first FDA-approved nano-drug in 1995. It is a liposomal formulation of doxorubicin HCl.\textsuperscript{23} Abraxane, which was approved by FDA in 2005, is a paclitaxel albumin-stabilized nanoparticle
formulation. DepoCyt, a liposomal injection of cytarabine, is used for the treatment of lymphomatous meningitis, and obtained the FDA's full approval in 2007. Genexol-PM, a polymeric nanoparticle formulation of paclitaxel, has also been approved by FDA for use in patients with breast cancer. Lipoplatin, a liposomal formulation of cisplatin, finished the Phase III human clinical trials in 2011. Some nanoparticle therapeutics currently undergoing clinical trials are as follow: S-CKD602, pegylated liposomal CKD602 (topoisomerase inhibitor) (Phase I/II); CPX-1, liposomal irinotecan (Phase II); LE-SN38, liposomal SN38 (Phase II); SP1049C, glycoprotein micelle of doxorubicin (Phase II); and OSI-211, liposomal lurtotecan (Phase II).

1.3.2 Liposomal drug delivery system for cisplatin

In liposomal drug delivery systems, anti-cancer drugs are surrounded by different lipid layers which have both hydrophilic and hydrophobic moieties. Two promising platinum liposomal formulations currently in phase II clinical trials are SPI-077 and MBP-426. SPI-077 is a pegylated cisplatin liposomal formulation while MBP-426 is a transferrin targeted liposomal oxaliplatin formulation. Lipoplatin™ is a liposomal formulation of cisplatin that has completed phase I, phase II and phase III human clinical trials. In 2007, the European Medicines Agency (EMA) granted orphan drug status to Lipoplatin as a first line-treatment in pancreatic cancer. In 2009, EMA approved Lipoplatin™ as a first line anti-cancer drug for the treatment of non-squamous non-small-cell-lung cancer (NSCLC), which is mainly composed of adenocarcinomas. The formulation of Lipoplatin includes dipalmitoyl phosphatidyl glycerol (DPPG), soy phosphatidyl choline (SPC-3), cholesterol and methoxypolyethylene glycol-distearaoyl phosphatidyl-ethanolamine (mPEG2000-DSPE). T. Boulikas, the lead scientist of Regulon Inc (Greece), indicated that the anionic lipid DPPG provided unique fusogenic properties, which assisted the direct fusion between cell membranes and Lipoplatin after the drug carriers reached
the target tumor tissues. The weight composition of Lipoplatin™ is 8.9% of cisplatin and 91.1% of lipids weight by weight (w/w). Compared to the other liposomal formulations, such as SPI-77, the total amount of lipid used in Lipoplatin™ is lower; thus, less lipid would enter systemic circulation. The average particle size of Lipoplatin™ was 110 nm, ranging from 90 nm to 130 nm; additionally, due to the enhanced permeability and retention effect (EPR) effect, more Lipoplatin™ was trapped in the tumor tissues. Clinical trials have shown that the platinum concentrations inside tumor tissue to be 40- to 200-fold higher compared to the nearby healthy tissues. However, the major limitation for these liposomal platinum formulations is the poor release of the active platinum species from the nanoparticles. In addition, hand-foot syndrome occurred during the therapies with Doxil and Lipoplatin.

1.3.3 Synthetic polymer based drug delivery system for platinum drugs

The polymeric platinum delivery systems are conjugated complexes between platinum drugs and ligating groups within polymers. Gemeinhart and coworkers (University of Illinois, Chicago, IL, USA) have developed a novel micro-particle formulation to deliver cisplatin using poly(acrylic acid-co-methyl methacrylate). It was found that the burst effect (40% drug release) occurred in the first day and around 20% cisplatin was slowly released in the next five days. However, due to the micron-size of the particle, it is unable to accumulate within the solid tumor by the EPR effect.

NC-6004 nanoptin™ is a block copolymer micellar formulation of cisplatin. Currently it is in phase I clinical trial. The carboxylate group in the block copolymer of poly(glycool)-poly(glutamic acid) (PEG-P(Glu)) can form polymer-metal complex with cisplatin. The studies noted that cisplatin and PEG-P(Glu) can spontaneously form micelles after they were stirred in water. The drug loading of this micelle system was 39% (w/w) and the size distribution of the
micelles was 10 ~ 80 nm (average diameter was 28 nm). Under physiological saline condition (pH 7.4) within systemic circulation, cisplatin was released from the micelles due to inverse ligand replacement between the carboxylates in the micelle core and the chloride ions in systemic circulation. In addition, the polymer-metal complex slowly dissociated to smaller aggregates or unimers to induce the release of cisplatin. Preclinical studies showed that the blood circulation period was prolonged and more than 60% of the intravenously injected dose was detected in the plasma of tumor-bearing mice. The accumulation of cisplatin in tumor tissues was 20-fold higher than that of direct i.v. injection of cisplatin solution. This micellar formulation showed a significantly reduced acute nephrotoxicity and chronic neurotoxicity side effects via histological and functional analyses. It was believed that the reduced maximum concentration ($C_{\text{max}}$) in kidney and the decreased cumulative $cis$-diaminedichloroplatinum(II) (CDDP) concentration at the peripheral level could contribute to the reduced toxic side effects. Preclinical studies also showed that four of ten mice treated with NC-6004 obtained complete tumor regression without obvious body weight loss.35 ProLindac™ is another polymer-platinum complex and is in phase II clinical trial. The 25kDa linear hydroxypropylmethacrylamide (HPMA) polymer can form a complex with oxaliplatin via the amidomalonato groups. Poly-hydroxypropylmethacrylamide (PHPMA) is very hydrophilic, non-immunogenic and non-toxic. It is usually used to prepare biocompatible medical materials such as hydrogels. The drug carriers made from this polymer normally show longer blood circulation time. ProLindac™ is a pH-triggered drug-delivery system. Drug release studies found that the platinum drug release rate at pH 5.4 was around 7-fold higher than that at pH 7.4 after 24 h. The preclinical studies indicated that ProLindac™ was superior to oxaliplatin in human colo-26, HT-29 and HCT116 xenograft models. Other phase I and II studies showed this drug delivery system was at least equal to and likely superior to oxaliplatin.36 Currently, many other synthetic
polymer based drug delivery systems for platinum drugs are being developed and tested in laboratories. It is expected that these new platinum-based drug carriers could provide high plasma stability, low toxicity and immunogenicity, and protect platinum drugs against premature metabolism.37

1.3.4 Other drug delivery systems for platinum drugs

Miriplatin ((SP-4-2)-[(1R,2R)-cyclohexane-1,2-diamine-N,N’]bis(tetradecano-O) platinum), a hydrophobic platinum drug using myristates as chelation groups, was approved in Japan in 2009. It has been marketed under a brand name of Miripla® since January 2010. (Figure 5) This platinum drug is used to treat hepatocellular carcinoma (HCC). In east Asia, HCC accounts for around 90% of liver cancer which has a very high mortality rate. Miriplatin is encapsulated in lipiodol which mainly consists of a ethyl ester of iodized poppy seed oil. The drug is administered through the hepatic artery. The selection of lipiodol was due to its long retention time at tumor sites. In this formulation, the active platinum form, which provides the anti-cancer effect, is cyclohexan-1,2-diamineplatinum (II) dichloride (DPC). It is slowly released from the Miriplatin suspended in lipiodol. 38

Aroplatin is another liposomal formulation of a third-generation of DACH (diaminocyclohexane) platinum agent, which is [cis-bis neodecanoato-trans-R,R-1, 2 diaminocyclohexane platinum (II)]. (Figure 5) This platinum drug has two C9 long fatty acid chains, which are quite similar to Miriplatin. Currently, it is in phase II clinical trial for the treatment of colorectal cancer.
1.4 An introduction to solid lipid nanoparticles (SLNs) and polymer-lipid hybrid nanoparticles (PLNs)

Salt formation is a very effective method to enhance aqueous solubility, dissolution rates and, potentially bioavailability of acidic and basic drugs, which are classified as class II (high permeability, low solubility) and class IV (low permeability, low solubility) drugs. In the current drug market, most basic drugs are prepared as HCl salts, sulfated salts, maleic acid salts, and tartaric acid salts. A table for the FDA approved salts of drugs from 1995 to 2006 is shown as follows: 39, 40

Table 1.1 Acids used in the Food and Drug Administration (FDA) approved drugs (1995 ~ 2006) and the related information about the acids used in preparing these drugs.

<table>
<thead>
<tr>
<th>Salts of basic drugs</th>
<th>Number approved by FDA (1995 – 2006)</th>
<th>pKₐ of acid</th>
<th>acid strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrochloride</td>
<td>54</td>
<td>-6.1</td>
<td>very strong</td>
</tr>
<tr>
<td>Sulfate/bisulfate</td>
<td>3</td>
<td>-10</td>
<td>very strong</td>
</tr>
<tr>
<td>Methanesulfonate (mesylate)</td>
<td>10</td>
<td>-1.2</td>
<td>very strong</td>
</tr>
<tr>
<td>Acetate</td>
<td>5</td>
<td>4.8</td>
<td>weak</td>
</tr>
<tr>
<td>Succinate</td>
<td>3</td>
<td>4.2, 5.6</td>
<td>medium</td>
</tr>
<tr>
<td>Tartrate</td>
<td>2</td>
<td>3.0, 4.4</td>
<td>strong</td>
</tr>
</tbody>
</table>
Solid lipid nanoparticles (SLNs), which are prepared from lipids and remain as a solid state at room and body temperature, are one of the most effective drug delivery systems for controlled drug delivery. SLNs are different from colloidal drug delivery systems such as lipid emulsions and liposomes. They are submicron-sized nanoparticles (50 to 1000 nm), in which drug substances are entrapped in various matrices which are composed of solid lipids and surfactants. The outer surface of these SLNs are normally covered by a poly(ethylene glycol) (PEG) layer, which can provide the stealth property; thus, the uptake by activated macrophages in vitro will be much slower compared to the non-PEGylated SLNs. Still, SLNs have several drawbacks: 1) they mainly deliver hydrophobic drug molecules due to the poor partition of hydrophilic drugs in water-insoluble lipid matrices; 2) low drug loading capacity; and 3) high burst effect.

Recently, Wu et al. have developed a novel solid polymer-lipid hybrid nanoparticle (PLN) formulation which contains lipid, anionic polymer and one or more drug molecules which included Verapamil, GG918 (a-P-glycoprotein inhibitor), doxorubicin HCl and mitomycin C. Different from the traditional drug forms in the market, in which basic drug molecules can form ionic interactions with inorganic acids (e.g. HCl, tartrate and sulfate), the cationic anti-cancer agents in these PLNs can form the ionic interaction with the anionic polymers such as dextran sulfate. Because of the formation of these hydrophobic salts, the hydrophobic interaction between lipid matrix and the salts ensure that the anti-cancer drugs are encapsulated in the PLNs which can provide the desired drug delivery to cancer cells.

1.4.1 Verapamil- dextran sulfate PLN system

One of these novel PLN formulations can efficiently deliver the water-soluble drug, verapamil hydrochloride (VRP). The anionic polymer used in this system was dextran sulfate sodium (DS), a sulfated polysaccharide with α-1,6 glycosidic linkages between glucose molecules. Its structure
is similar to natural polysaccharides (e.g. chondroitin sulfate, dermatan sulfate) and is widely used in drug formulations as it is readily decomposed by ecological systems. In this PLN formulation, the verapamil hydrochloride (VRP)-dextran sulfate sodium (DS) ionic complex was mixed with the solid lipid of dodecanoic acid (DA) and stabilized with some surfactants. This PLN is a promising drug delivering system with a drug loading capacity of up to 36% and a loading efficiency of around 99%. The sustained drug release profile of this system can be achieved via strong drug-polymer binding and diffusion processes. The transmission electron microscopy (TEM) photographs showed this VRP-PLN was nearly spherical. However, the downside for this formulation was that the mean particle size of this PLN was 342.5 nm with a standard deviation of 54.8 nm. Furthermore, some findings showed that the polymer of dextran sulfate sodium may induce acute and chronic colitis in mice after oral administration.

1.4.2 Doxorubicin- HPESO PLN system

Since the use of dextran sulfate sodium in our PLN delivery system was quite promising, another anionic polymer, hydrolyzed polymer of epoxidized soybean oil (HPESO), which was first synthesized by Dr. Liu, was used in this PLN system. The reason to choose HEPSO was due to the easy accessibility of natural soybean oil and the potential for very low or no toxicity when used in future drug formulations. Previous experimental results showed that this new PLN (doxorubicin–HPESO) system possessed much higher in vitro cytotoxicity against the multidrug resistant (MDR) breast cancer cells, while the blank SLN/excipients showed very little cytotoxicity. The particle size of this new PLN can be as small as 80 nm. In addition, compared to Dox-solid liposphere which showed a low drug releasing effect (0.1% after 2 h), more than 90% of the trapped doxorubicin was released within 18 h. This new PLN formulation was also used to simultaneously deliver doxorubicin and GG918 (Elacridar) to target the multidrug-
resistant breast cancer cells. Recently, studies showed that the doxorubicin (Dox)-mitomycin C (MMC) co-loaded PLN had a synergistic effect against various multidrug resistant (MDR) human breast cancer cells, including MDA435/LCC6, MCF7 VP (MRP1+) and MCF7 MX (BCRP+). The drug co-encapsulated PLN against these MDR cells was 20–30 fold more potent than free drugs; plus, the co-loaded PLNs were more potent against these MDR cells than the single agent-encapsulated PLN. It is believed that the synergistic effect was associated with the enhanced induction of DNA double strand breaks which induced cancer cell apoptosis.

Microscopic fluorescent images revealed that the fluorescently labeled PLNs were mainly in the perinuclear region of the cancer cell lines. Clearly, this indicated the uptake of the PLN was via an endocytosis mechanism, which can overcome multiple types of membrane efflux pumps and deliver anti-cancer drugs to the cytoplasm of cancer cells. The in vivo study of this HPESO based PLN formulation loaded with doxorubicin showed significant tumor growth delay and tumor necrosis in a murine solid tumor model.

However, there were some limitations associated with this newly developed HPESO based polymer-lipid hybrid nanoparticle. Firstly, HPESO is a relatively weak anionic polymer with long hydrophobic fatty acid chains. Thus, even under basic conditions of the medium (pH > 8), it is only partly dissolved in aqueous solution. This property limited its drug loading capacity at physiological pH (pH = 7.4), and may partially contribute to the low drug loading capacity of the doxorubicin HCl salt. Our previous study showed that the drug loading capacity of Dox-HPESO was only around 6% with an encapsulation efficiency of 60 – 80%. Secondly, compared to the dextran sulfate (DS) PLN formulation, the HPESO based PLN showed a higher burst release (up to 48% in 2 h) in phosphate buffer solution (PBS, pH 7.4). It is possible that the precipitation of HPESO under physiological conditions induced this burst effect. Thirdly, the later slow-release phase (~ 5% from 8 h to 16 h) showed that some of the drug molecules were locked up in the
densely packed liquid crystals which were more uniform and spherical compared to the dextran sulfate based PLN system. Therefore, to achieve a better target drug delivery effect, modification of this original HPESO based PLN system was necessary.

1.5 Rationale, hypotheses and objectives
1.5.1 Rationale and hypotheses

The overall goal of this thesis is to develop novel soybean oil based polymers with better water-solubility and to apply them in the preparation of PLNs of cisplatin for cancer therapy.

To obtain a polymer with higher aqueous solubility, the introduction of either stronger anionic ions such as sulfonate groups, di-carboxylic acids or abundance of hydrophilic primary alcohols to the hydrophobic core structure of soybean oil based polymer could be the solution to overcome the problem of the previous HPESO PLNs. As discussed earlier in this introduction, due to the low aqueous solubility under physiological and/or mildly acidic conditions, novel HPESO PLNs had some drawbacks like the burst effect and low drug loading. Herein, we expect that the new polymers will have better aqueous solubility under physiological and/or mildly acidic conditions; thus, increases in drug loading of nanoparticles and reduced burst effect of PLNs are expected.

It is expected that the novel PLNs, which encapsulated platinum drugs, would have better potency against MDR cancer cells. This hypothesis is based on the following factors: a) cisplatin or other platinum drugs are DNA-damaging anticancer drugs; b) most human DNA is located inside cell nucleus; c) previous studies in our lab showed that the doxorubicin-HPESO PLNs were mainly located in the perinuclear region of cancer cell lines. It is assumed that the polymers can form complexes with cisplatin or a prodrug of cisplatin via both or one of the following mechanisms: 1) covalent bonding between carboxylate; 2) electrostatic interaction between
cationic drug molecules and anionic carboxylate or sulfonate groups. We expect that the new PLN will reach the perinuclear region of cancer cells to deliver the DNA-damaging anticancer platinum drugs such as cisplatin; thus, it is expected that the new PLNs will have higher efficacy against breast cancer cells than cisplatin. In the future, it is expected that this newly developed drug delivery system will also provide the following benefits seen for other drug delivery systems with platinum drugs: 1) longer blood circulation period; 2) reduced nephrotoxicity; 3) increased intracellular accumulation of platinum complexes via an endocytosis mechanism.

1.5.2 Objectives of this thesis

This thesis focuses on the objectives below:

- Synthesize and characterize novel soybean oil based polymers and select polymers with better water-solubility.
- Study the complexation of cisplatin with the polymers.
- Develop PLNs which have the desired nano-particle sizes (20 nm ~ 200 nm).
- Study the kinetics of platinum-release from these PLNs under physiological or mildly acidic conditions.

1.6 Organization of Thesis

In Chapter 2, in order to increase the aqueous solubility of the soybean oil based polymer such as HPESO, the synthesis of three new polymers with hydrophilic groups (primary alcohol, sulfonic acid and di-carboxylic acids) is described. In contrast to the reported method of HPESO synthesis, the alcohol-based polymer, a diblock copolymer which contained hydrophobic block of long fatty chains and hydrophilic block of primary alcohol, was synthesized using the monomers of diglycidyl 1,2-cyclohexane dicarboxylate (DGCD) and epoxy methyl soyate
(EMS). The DGCD can bring hydrophilic primary alcohols to the di-block copolymer after the copolymerization. Also, EMS, a different type of soybean oil based monomer, was used to avoid the competitive intra-molecular polymerization which would form HPESO solely. Aspartic acid-based polymer and taurine-based polymer were prepared through the modification of HPESO: a) the first step was the activation of the carboxylic acid groups of HPESO using isopropyl chloroformate; b) the second step was addition reactions using aspartic acid or taurine to give the desired polymers. Because these polymers contained stronger anionic moieties such as sulfonic acid and di-carboxylic acid, the aqueous solubility of the polymers was increased due to their lower pKa values.

In Chapter 3, the structures of the new polymers were confirmed using $^1$HNMR, $^{13}$CNMR and heteronuclear single quantum coherence (HSQC) NMR. The gel permeation chromatography (GPC) analysis showed that the polymers had similar molecular weights as HPESO. Various solubility tests showed that the newly synthesized polymers had higher aqueous solubility compared with HPESO. An HPLC method was developed to determine the ratios between the polymers and cisplatin. It was found that the actual chelation ratios were close to the theoretical calculation.

In Chapter 4, in-depth studies on the PLN encapsulating cisplatin showed that the mechanism of trapping cisplatin was through the covalent bonds between the chelation groups (e.g. carboxylate, sulfonate) of the polymers and platinum. In addition, higher temperatures and neutral conditions favored the formation of the carboxylate-platinum complex. The drug release studies elucidated that the complex between carboxylate and platinum was stronger than that between sulfonate groups and platinum. Because of the rapid drug release in physiological saline solution, cisplatin was not a good payload for these new PLN formulations. The rapid drug release was mainly due to the poor affinity of cisplatin to the lipid matrix. Nevertheless, detailed
studies on the PLN formulations of cisplatin provided a solid foundation for the development of another novel PLN formulation in which a prodrug of cisplatin was encapsulated. The prodrug used had two stearate chains attached to platinum atom. Compared to the approved formulation of Miriplatin, this PLN formulation has shown a sustained drug release profile due to the nano-size of particles as well as an ionic interaction between the taurine-based polymer and the cationic prodrug. In the appendix, *in vitro* cytotoxicity studies are described which showed that the formulation was four-fold more potent than free cisplatin against human MDA-MB-231 breast cancer cells. The cellular uptake kinetics indicated that the internalization of the PLN was via an endocytosis mechanism.

A summary of the thesis conclusions follows in chapter 5, identifying the main results of this study. Future directions evolving from this work are also recommended.
Chapter 2

Synthesis of the novel soybean oil based polymers

2.1 Abstract

The preparation of three new soybean based polymers exhibiting better aqueous solubility compared with HPESO is presented in this chapter. The polymers can be synthesized either from the ring opening copolymerization with different monomers or the functional group modification from HPESO.

The alcohol-based polymer was a di-block copolymer which was synthesized through the copolymerization between monomers of epoxy methyl soyate (EMS) and diglycidyl 1,2-cyclohexane dicarboxylate (DGCD). The monomer of DGCD can provide an abundance of the hydrophilic primary alcohols, while the use of EMS to replace epoxidized soybean oil (ESO) can avoid the unwanted intra-molecular polymerization after the mixing of ESO and DGCD.

Another method was to introduce stronger anionic moieties (e.g. sulfonic acid and di-carboxylic acids), which have lower pKa values, to soybean oil based polymer through the modification of HPESO. The aspartic acid-based polymer and the taurine-based polymer were prepared through the activation of the carboxylic acid groups of HPESO using isopropyl chloroformate as a coupling reagent. The active intermediate adduct of mixed anhydride was reacted with either aspartic acid or taurine. This was followed by purification using dialysis to give the desired aspartic acid-based polymer and taurine-based polymers.
2.2 Introduction

Soybean oil had an annual production of 30.6 million tons worldwide in the 2002 – 2003 growing season, and it is the second largest crop plant in the United States. Soybean oil is mainly a mixture of triacylglycerides, which contain triesters of glycerol and three long fatty-acid chains. The chain length of these fatty acids varies from 14 to 22, and there are 0 to 3 double bonds per fatty acid. There are four advantages in using soybean oil based polymers in drug formulations, namely cost effectiveness, renewable resource, biocompatibility, and biodegradability. Khot et al. have described the general development and composites of soybean oil based polymers. The four methods for preparing soybean oil based polymers are presented in Figure 6:

**Figure 6** General chemical reaction routes in preparing polymers from triglyceride
Hydrolyzed polymer of epoxidized soybean oil (HPESO), one of the soybean oil based polymers, was used in the novel PLN formulation developed in our lab. The number average molecular weight (Mn) and molecular weight (Mw) were determined by gel permeation chromatography (GPC) and they were found to be Mn = 3160 and Mw = 4866, respectively. Moreover, different molecular weights of HEPSO can be prepared via variation of reaction temperature and amount of Lewis acid added. As discussed in Chapter 1, HPESO has been used in the novel PLNs to selectively deliver drugs of verapamil, GG918 (Elacridar), mitomycin C (MMC) and doxorubicin. However, due to its poor aqueous solubility under neutral or mildly acidic conditions, there were some limitations associated with this type of PLN. In this work, three new soybean oil based polymers were synthesized to resolve these issues.

One strategy for increasing the water solubility and reducing the pKa of soybean oil–based polymers involves the introduction sulfonic acid groups to the HPESO. This may be achieved by diblock copolymerization of epoxidized soybean oil (ESO) with another monomer such as 2-propene-1-sulfonic acid sodium salt. (Figure 7, top scheme) While this method appeared quite straightforward at first glance, findings by Meyer et al. indicated that the specific catalysis was required to undergo the polymerization to prepare this type of copolymer between an epoxy monomer and an olefin. In addition, Khot et al found that acrylic acid can also react with the epoxy part of ESO via a nucleophilic addition. A similar reaction may also occur for a sulfonate group. (Figure 7, bottom scheme) For these reasons, the expected copolymer with sulfonate group cannot be synthesized from the copolymerization using the monomers of 2-propene-1-sulfonic acid sodium salt and ESO.
A copolymer with an abundance of the hydrophilic primary alcohols can also increase the aqueous solubility of soybean oil based polymer. Carboxylic acid glycidyl ester appeared to be another good monomer to prepare a copolymer with ESO. Unfortunately, this carboxylate glycidyl used as a monomer was not described in detail in the literature. Only one US patent described the preparation of a copolymer using glycidyl carboxylate and required a specific catalyst derived from dibutyltin oxide (or tributyltin chloride) and tributyl phosphate. After an
extensive search for the potential monomer to prepare the desired di-copolymer with hydrophilic groups, diglycidyl 1,2-cyclohexane dicarboxylate (DGCD) was found to be quite promising. Each molecule of this monomer contains two epoxy groups for copolymerization and the expected primary alcohol groups can be obtained via a alkaline hydrolysis reaction. (Figure 11) Another method to bring a polar sulfonate group or a di-carboxylic acid group to the core structure of HPESO was via modification of HPESO. The end functional group of HPESO, a carboxylic acid group, can form an amide bond to link taurine or aspartic acid. Taurine, 2-aminoethanesulfonic acid, is naturally present in the tissues of many mammals including humans.60 One report showed that the annual production of taurine in 1993 was around 5000 – 6000 tons, of which 50% was used for the preparation of pet foods while the rest went to pharmaceutical applications.61 Taurine has also been used as a functional food additive in many energy products such as energy drinks.62 Given this information, taurine is biocompatible and should have low toxicity like soybean oil. Aspartic acid is an acidic amino acid with two carboxylic acids. Through the addition of aspartic acid moiety via an amide bond, the new polymer not only can have increased aqueous solubility, but also may form a typical bi-dentate chelating complex with cisplatin. For example, Tauro and coworker synthesized a novel hydrogel matrix, in which cisplatin was conjugated with the peptide complex through the bi-dentate chelation from the aspartic acid part.63

2.2.1 Reported procedure in preparing HPESO polymer

Hydrolyzed polymerized epoxidized soybean oil (HPESO) was synthesized via a typical two steps reactions: a) the first step was a Lewis acid (BF₃·Et₂O) catalytic ring-opening polymerization with ESO to give glycerol ester of the cross-linked polymer; b) the second step
was a basic hydrolysis reaction to remove the glycerol and obtain sodium salt of HPESO. (Figure 8)

![Reaction Scheme](image)

**Figure 8**  Reported reaction scheme in preparing HPESO (see text).53

### 2.3 Material and Methods

#### 2.3.1 Chemicals

ESO was obtained from AKSci (Union City, CA, US) and used as received. Other chemical reagents, such as (C$_2$H$_5$)$_2$O·BF$_3$, methylene chloride, methanol, acetone, DGCD, taurine, sodium methoxide, triethylamine, isopropyl chloroformate and aspartic acid were purchased from Aldrich Canada (Oakville, ON, Canada) and used without further purification. Distilled and deionized (DDI) water was prepared with a Milli-Q water system (Milli-Pore, Etobicoke, ON, Canada).
2.3.2 Synthesis of the EMS/DGCD polymer

The monomer EMS was synthesized according to the reported procedure with some modification.\textsuperscript{64}

The monomer ESO (50 g), pre-dissolved in methanol (25 mL), was treated with sodium methoxide (0.5 g) as catalyst. The reaction mixture was then heated to 50 °C and stirred at this temperature for 2 h. The mixture was cooled to room temperature and centrifuged. The viscous bottom layer was glycerol and the top layer was EMS monomer which can be directly transferred. (Figure 9) The structure of EMS was further confirmed by \textsuperscript{1}H NMR. (Figure 10)

\begin{center}
\textbf{Figure 9} Reaction scheme in preparing EMS monomer. The desired EMS monomer can be separated by the centrifugation due to the viscous glycerol.
\end{center}
(EMS)

Figure 10  Proton NMR of EMS monomer. The $^1$H NMR spectrum of EMS showed the peaks of the glycerol moiety of ESO at around 4.5 ppm$^{53}$ were replaced by the peak of methoxy group at around 3.7 ppm. See text for details.

The new di-block copolymer was synthesized from the monomers of EMS and DGCD using the following procedure, which was similar to the reported procedure in preparing HPESO: $^{53}$ The monomers EMS (13 g) and DGCD (9 g) were treated with anhydrous dichloromethane (50 mL) at room temperature under nitrogen blanket, followed by the addition of catalyst (C$_2$H$_5$)$_2$O-BF$_3$ (0.5 mL). The reaction mixture was stirred at room temperature for 16 h, and the mixture was then stored in a refrigerator overnight to obtain a gel-like solid. The solid was further hydrolyzed in 1.6 N NaOH solution (200 mL) at 85 °C for 48 h. The hot solution was filtered and then cooled in an ice-bath. The pH of the solution was adjusted to 6 using 1.0N HCl solution. The precipitate was washed with 3 × 50 mL of DDI water and air-dried.

2.3.3 Synthesis of the soybean oil based polymer possessing aspartic acid groups

HPESO was prepared according to the reported procedure$^{53}$ and treated with acetone (400 mL). After stirring at room temperature for 30 min, the solution was cooled in an ice-bath for 10 min followed by the addition of triethylamine (20 mL). Isobutyl chloroformate (IBCF) (12 ml) was dropwise added to the reaction mixture, which was then stirred in an ice-bath for 2 h and slowly warmed up to room temperature. The mixture was stirred at this temperature for 2 days. After the
removal of the salt of triethylamine \( \cdot \)HCl through the centrifugation of the suspension, the separated supernatant was blow dried using nitrogen flow to obtain the mixed anhydride (7 g). To synthesize aspartic acid-based polymer, L-aspartic acid (9 g) was dissolved in 1.35N NaOH solution (100 mL) and the solution was heated at 70 °C for 1 h. The intermediate of the mixed anhydride described above was added to this aspartic sodium salt solution. The mixture was stirred at room temperature for 48 h. The organic solvent of acetone in the reaction mixture was removed using a constant nitrogen flow, followed by the addition of DDI water (100 mL). The pH of the solution was adjusted to 2 using a diluted HCl solution. The precipitate was washed with DDI water (3 \( \times \) 50 mL) and air-dried.

2.3.4 Synthesis of the soybean oil based polymer possessing taurine acid groups

HPESO acetone solution was prepared as described in section 2.3.3. IBCF (12 ml) was then added dropwise to the mixture. The reaction mixture was stored in a 4 °C cold room for 24 h. The suspension was centrifuged to remove the solid of triethylamine-HCl. After the removal of the salt of triethylamine-HCl through the centrifugation of the suspension, the separated supernatant was blow dried using nitrogen flow to obtain the mixed anhydride (~ 18 g).

To synthesize taurine-based polymer, taurine was treated with 0.9 N NaOH solution (150 mL) and then heated at 70 °C for 30 min. This resultant solution of sodium taurinate was cooled to room temperature and added to the above intermediate adduct of mixed anhydride. The mixture was stirred at room temperature for another 4 days and the pH of solution was adjusted to 2 using 1.0N HCl solution. The suspension was centrifuged to obtain a solid sample. The solid sample was then dissolved in methanol and the excess of sodium taurinate, which precipitated in
methanol, filtered off. The solution was blow dried using a constant nitrogen flow to obtain the sticky light brown solid.

2.3.5 Purification of the polymers

A suspension of the crude product (~ 0.8 g) of the aspartic acid-based polymer or the taurine-based polymer (~ 2 g) was treated with 20 ml or 50 ml of DDI water, respectively. Then, they were transferred to a dialysis bag (Spectra/Por 6, molecular weight cut off (MWCO): 2000 dalton, Spectrum Labs, Rancho Dominguez, CA, USA). The dialysis bag with polymer was stirred in 1L or 4L of DDI water, respectively, for 48 h. The external aqueous solution was changed and replaced with fresh DDI water after 24 h. The pH of the solution inside the dialysis bag was adjusted to 6.0 or 4.0, respectively, using 1.0 N HCl solution. The solid was isolated by centrifugation (3150 × g for 20 min), and then washed with DDI water (150 mL×3) and air-dried at room temperature.

2.4 Results and discussion

2.4.1 Polymerization of ESO and DGCD monomers

It was expected that the di-block copolymer with abundance of hydrophilic primary alcohol groups could be synthesized through the copolymerization of ESO and DGCD. To prepare the desired copolymer, ESO and DGCD were mixed and followed a similar reaction scheme which was used to prepare HPESO (Figure 8).\textsuperscript{53} Interestingly, the $^1$H and $^{13}$C-NMR spectra indicated that the newly synthesized polymer was HPESO. It showed that the monomer of DGCD did not participate in the copolymerization process with ESO under this reaction condition. One report showed that the cationic polymerization, which was catalyzed by boron trifluoride in halogen solvent using epoxy monomer (allyl glycidyl ether), can only yield a linear polymer with a
degree of polymerization of 4-6. On the basis of this information, it was speculated that an unwanted intra-molecular cationic epoxy ring opening occurred because the epoxy moieties of ESO were linked with a glycerol; thus, the intra-molecular reaction excluded the DGCD monomer to participate in the desired copolymerization.

2.4.2 Preparation of the EMS and DGCD copolymer

The previous study showed that ESO was not a good monomer candidate to prepare the desired copolymer with DGCD due to the unwanted intra-molecular reaction. To avoid this competitive intra-molecular cationic polymerization, the glycerol esters of ESO were broken down to another soybean oil based monomer which was epoxy methyl soyate (EMS). The proton NMR of EMS was shown in Figure 10. EMS can be prepared through a transesterification catalyzed by sodium methoxide with an abundance of methanol. The transesterification can convert the esters of glycerol from ESO to a mono-methyl ester of EMS. It has also been shown that EMS was an alternative bio-based monomer to be used in preparing polymers with improved physical properties. (Figure 9)

Here, the alcohol-based polymer, a di-block copolymer, was prepared via a two-step reaction route. The first step was the Lewis acid catalyzed ring opening copolymerization between EMS and DGCD, both of these monomers contained the desired epoxy rings for polymerization. The second reaction step was an alkaline hydrolysis of esters to give free carboxylic acids and primary alcohols in a one-step reaction. (Figure 11) Here, EMS provided the hydrophobic soybean oil core structure to the new copolymer, while the monomer of DGCD brings abundance of the primary alcohol groups which can increase the aqueous solubility of the soybean oil based polymer.
Figure 11  Reaction scheme in preparing the alcohol-based polymer. This new copolymer was prepared using the monomers of EMS and DGCD. The synthesis of the polymer was a simple two-steps reaction, the two steps were polymerization and hydrolysis reactions. See text for details.

2.4.3 Preparation of the soybean oil based polymer possessing di-carboxylic acids /sulfonic acid

As discussed above, another method to increase the aqueous solubility of soybean oil based polymers was to add sulfonate group or di-carboxylic acid groups to the hydrophobic core structure of a soybean oil based polymer. (Figure 12) The end-functional groups on HPESO, carboxylic acid groups, can be converted to an active intermediate adduct such as mixed carboxylic–carbonic anhydride using a coupling reagent such as IBCF. Next, the active mixed anhydride can either react with taurine or aspartic acid to prepare the soybean oil based polymers with either sulfonic acid group or di-carboxylic acids group. Due to the lower pKa values of these functional groups, these two polymers should have higher aqueous solubility compared to HPESO which has weak carboxylic acid groups.
2.5 Conclusions

Three synthetic procedures have been developed to prepare the alcohol-based polymer, the aspartic based polymer and the taurine-based polymer. Compared with HPESO, which is a hydrophobic polymer due to its long fatty acid chains and weak carboxylic acid group, a hydrophilic groups such as primary alcohol, di-carboxylic acid groups and sulfonic acid groups were brought to the hydrophobic core structure of soybean oil based polymer to increase the aqueous solubility of the polymers.
Chapter 3

Characterization of the novel soybean oil based polymers and their polymer-cisplatin complexes

3.1 Abstract

This chapter focuses on the characterization of the newly synthesized soybean oil based polymers. The $^1$H NMR, $^{13}$C NMR and HSQC confirmed that these new polymers possessed the expected hydroxyl groups (alcohol-based polymer), di-carboxylic acids group (aspartic acid-based polymer) and sulfonate groups (taurine-based polymer). In addition, the GPC analysis indicated that these polymers had similar molecular weights as the standard HPESO sample. The polymers were also converted to hydrophobic triethylamine salts, which were expected to provide better solubilities of polymers and stability for PLN formulations. Various solubility tests showed that the addition of hydrophilic groups, such as primary alcohol, di-carboxylic acids group and sulfonate group, can increase the aqueous solubility of these new soybean oil based polymers compared with HPESO. A HPLC method has been developed to measure the chelation ratios between cisplatin and these three new polymers. The HPLC tests showed that the chelation ratios of polymer-cisplatin conjugates were close to theoretical calculation.

3.2 Introduction

3.2.1 Structural identification of HPESO

The structure of HPESO has been characterized using NMR, FTIR and GPS analysis. The FTIR spectrum of HPESO showed the disappearance of the oxirane absorption, which is the typical absorption of ESO, at 823.3 cm$^{-1}$ due to the ring-opening polymerization. In addition, the absorption shift of C=O bonds was also observed after the conversion of the tri-ester of glycerol
to the sodium salt of carboxylic acids. The $^1$H-NMR showed that the alpha $\text{CH}_2$ protons to >C=O at 2.2 – 2.4 ppm. The $^{13}$C-NMR of HPESO noted the disappearance of the epoxy carbons peaks and glycerol peaks from the ESO starting material. The number average molecular weights and molecular weight of HPESO were found to be $M_n = 3160$ and $M_w = 4866$, respectively, as determined by gel permeation chromatography (GPC) in which the calibration curve of the GPC analysis was prepared using a polystyrene mixture of different molecular weights (1700, 2450, 5050, 7000, 9200 and 10,665).$^{53}$

3.2.2 Formation of the triethylamine salt of the polymers

It was expected that the new polymers would have better aqueous solubility and higher drug loading of cationic drugs due to the formation of stable hydrophobic triethylamine salts. Triethylamine is a bulky organic base and its pKa value is 10.65. In general organic reactions, it is used to trap free protons. Triethylammonium acetate, a salt between acetic acid and triethylamine, is widely used as a buffering reagent for pH about 7. Although the preparation of triethylamine salts of active pharmaceutical ingredients is not a very common practice in the pharmaceutical industry, the triethylamine salts were still reported in some patents. For example, the triethylamine salt was used in a second generation injectable cephalosporin.$^{66, 67}$ In addition, some reports showed that the triethylamine salts of lipopolysaccharides, which contained an acid, a lipid and a polysaccharide, had better solubility compared with other inorganic salt forms, such as sodium salt.$^{68, 69}$ Moreover, Schwab and coworkers found that the triethylamine salt of vegetable oil acid was a suitable surfactant to prepare a micro-emulsion between vegetable oil and aqueous alcohol.$^{70}$ In contrast, inorganic cations, such as $\text{Na}^+$, $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$, tended to form colloidal aggregation with supra-molecules of natural organic matter in an aqueous environment.$^{71}$ Recently, Pinkerton and coworkers have developed a new hydrophobic salt
forming technique to entrap hydrophobic, weak basic drug molecules such as cinnarizine and clozapine. These drug molecules form an ionic interaction with counter acidic ions (e.g. camphor-10-sulfonic acid, pamoic acid and oleic acid), and these hydrophobic salts were then encapsulated in a PLA-b-PEG block copolymer. Without the formation of the hydrophobic salts, this study noted that it would be harder to obtain a stable nano-carrier formulation. Here, the key for the formation of a stable hydrophobic salt is that the pKa difference between an acid and a base should be larger than 2, and this is also referred to the rule of two. Since the new polymers contained sulfonic acid group and di-carboxylic acids groups which had lower pKa values compared to carboxylic acid, they can form stable hydrophobic triethylamine salts and provide good stability for PLN formulations.

3.2.3 High-performance liquid chromatography (HPLC) methods for cisplatin

In general, platinum content in polymer-platinum complexes is analyzed by atomic absorption spectroscopy (AAS) or inductively coupled plasma - optical emission spectrometer (ICP-OES). HPLC methods can be used for the analysis of small molecules of platinum drugs (e.g. cisplatin) and their aqua species.

Using a HPLC method, Brandsteterova and coworkers discovered that carboxylate-platinum complex was the most stable form in an aqueous environment, in which the original 1:1 mixture of cisplatin and carboplatin would slowly change to 87% carboplatin and 13% aqua-platinum complex after 24 h at 37 °C. Since cisplatin can form reactive aqua species, which were vulnerable to various nucleophiles, such as amino acids, acetonitrile, phosphate salts and carboxylic acids, Appleton and coworkers used various sulfonic acids, which were claimed to be
unreactive to platinum species, to develop an HPLC method for the analysis of aqua species of cisplatin.  

3.3 Material and methods

3.3.1 Chemicals

Cisplatin was a gift from Johnson Matthey Pharmaceutical Research (West Deptford, NJ, US), and ethyl arachidate was purchased from TCI-America (Portland, OR, US). Both reagents were used as received. Deuterium oxide (D$_2$O) and deuterated chloroform (CDCl$_3$) were purchased from Cambridge Isotopes Laboratories, Inc. (Andover, MA, US). Other chemical reagents, such as triethylamine and polyoxyethylene 40 stearate were purchased from Aldrich Canada (Oakville, Ont, Canada). Methanol was purchased from EMD Chemicals Canada (Mississauga, ON, Canada). Distilled and deionized (DDI) water was obtained with a Milli-Q water system (Milli-Pore, Etobicoke, ON, Canada)

3.3.2 Analytical methods

3.3.2.1 Nuclear magnetic resonance

$^1$H NMR and $^{13}$C NMR spectra of the products were acquired using a Varian Mercury-400B MHz (399.5 MHz and 100.5 MHz, respectively) NMR spectrometer (Agilent, Palo Alto, CA, US). Five mm NMR probes were used and $^1$HNMR was referenced to MeOH-d$_4$ (3.35 ppm), CDCl$_3$ (7.24 ppm), DMSO-d$_6$ (2.49 ppm), and acetone-d$_6$ (2.04 ppm) (Cambridge Isotopes Laboratories, Inc. (Andover, MA, US)).
3.3.2.2 Gel Permeation Chromatography (GPC)

The GPC analysis was performed with a Waters system equipped with a waters quaternary pump, a waters 600 controller, a 2707 autosampler, a 2998 photodiode array detector, and software of Empower (Waters, Mississauga, ON, Canada). Separations were performed on an ultrahydrogel 120 column (Molecular weight range: 100 – 5000 dalton). The polymer samples were prepared by dissolving 2 mg of the polymer in 10 mL of A/B mixture (A/B = 8 mL/2 mL; A: 25 mM pH 7.76 phosphate buffer solution; and B: acetonitrile). The polymer solution (5 μL) was injected into the column at 50 °C and eluted with a mixture of two mobile phases (A/B = 80/20) at a flow rate of 1.0 mL/min. The elution lasted for 10 min and the polymer concentration was detected by a (refractive index) RI detector at 50 °C.

3.3.2.3 Solubility tests of the new soybean oil based polymers

To determine if the newly synthesized polymers have better aqueous solubility compared to HPESO, three solubility tests were conducted as follow:

A) Solubility test using a mixture of a true solvent and a diluent:

An alcohol, such as ethanol, was a true solvent to dissolve the polymers. Water was a diluent, which would induce the precipitation of the polymers. To the polymers, which were first dissolved in different volumes of ethanol in different vials, water was added dropwise until the polymer solutions changed to cloudy emulsions.

B) Solubility test using a buffer solution:

To the polymers, which were placed in different vials, was added dropwise 0.1 mM of pH = 7.74 phosphate buffer solution at 60 °C until the polymer emulsions changed to clear solutions.

C) pH endpoint determination of the polymers:
To the polymers, which were suspended in DDI H$_2$O kept at 40 °C in a water bath, was added dropwise triethylamine until the polymer emulsions changed to clear solutions. At this point, the pH of solutions was measured by an Accumet® AB15 pH Meter (VWR Canada, Mississauga, ON, Canada).

3.2.2.4 HPLC instrument and method

The HPLC analysis was performed with a Waters system equipped with a waters quaternary pump, a waters 600 controller, a 2707 autosampler, a 2998 photodiode array detector, and software of Empower. Separations were performed on a column of Sunfire C-18 (4.6×50 mm, 5μm particle size). Sample preparation: to a solution of a triethylamine salt of the soybean oil based polymer (50 mg polymer/ 0.5 mL Et$_3$N) was added cisplatin solution (2.4 mg/mL, 25 mL) and the solution was agitated at room temperature for 72 h before the HPLC analysis. The sample solution (5 μL) was injected into the column at 25 °C and eluted with a mixture of two mobile phases (A/B = 97/3; A: DDI water or 50 mM pH = 2.5 hexanesulfonic acid buffer solution; B: MeOH) at a flow rate of 1.0 mL/min. The elution lasted for 10 min and the cisplatin concentration was detected by a UV detector at 210 nm or 305 nm.

3.4 Results and discussion

3.4.1 NMR spectral characterization of the EMS/DGCD polymer

The structure of the alcohol-based soybean oil polymer was identified by $^1$H, $^{13}$C and HSQC spectra (Mercury-400B, 399.5 MHz, using a 5 mm NMR probe in CDCl$_3$). Compared to the proton NMR spectrum of HPESO, the peak at 3.08 ppm by $^1$H NMR showed the methylene signal of $-\text{CH}_2\text{-O-}$. (Figure 13) The peak at 45 ppm by $^{13}$CNMR also proved the addition of methylene ($-\text{CH}_2\text{-O-}$) groups to this new soybean oil based polymer. (Figure 14) The 2D $^1$H–$^{13}$C
HSQC (Heteronuclear Single-Quantum Correlation) experiment showed the correlation between the proton (–CH₂-O-) and the carbon of the primary alcohol (–CH₂-O-). (Figure 15)

Figure 13 ¹H NMR spectrum of the alcohol-based soybean oil polymer. Compared to the proton NMR of HPESO, the ¹HNMR of the alcohol-based polymer showed the new peak (3.08 ppm) which was the methylene signal of –CH₂-O-.

Figure 14 ¹³C NMR spectrum of the alcohol-based soybean oil polymer. Compared to the ¹³C NMR of HPESO, the spectrum of the alcohol-based polymer showed the new peak (45 ppm) which was the methylene signal of –CH₂-O-.
3.4.2 \( ^1\text{H} \) NMR spectral characterization of the soybean oil based polymer possessing aspartic acid group

The structure of the aspartic acid-based soybean oil polymer was identified by \( ^1\text{H} \) NMR (Mercury-400B, 399.5 MHz, using a 5 mm NMR probe in CDCl\(_3\)). Compared to the \( ^1\text{H} \) NMR spectrum of HPESO\(^3\), the peak at 3.85 ppm showed the methine signal of >CH-NH-, which is part of the aspartic acid group. (Figure 16)
3.4.3 $^1$H NMR spectral characterization of the soybean oil based polymer possessing taurine group

The structure of the taurine-based soybean oil polymer was identified by $^1$H NMR (Mercury-400B, 399.5 MHz, using a 5 mm NMR probe in CD$_3$OD). Compared to the spectrum of HPESO,$^{53}$ the peak at 3.83 ppm showed the protons of methene ($-\text{CH}_2\text{-N}$-) and the peak at 3.25 ppm showed the protons of methene ($-\text{CH}_2\text{-S}$-). These signals showed that the taurine moiety was added to this new soybean oil polymer. (Figure 17)

![Figure 17 $^1$H NMR spectrum of the taurine-based soybean oil polymer.](image)

Figure 17 $^1$H NMR spectrum of the taurine-based soybean oil polymer. The peak at 3.83 ppm, 3.25 ppm showed the protons of methylene ($-\text{CH}_2\text{-N}$-) and ($-\text{CH}_2\text{-S}$-), respectively. Both of them were derived from the taurine moiety.

3.4.4 Molecular weight of the polymer

GPC analysis can determine the molecular weight of polymers. Herein, this HPESO sample was used as an internal standard to determine if the newly synthesized polymers had molecular weights similar to HPESO. The number and weight average molecular weight of the standard sample of HPESO, which was a gift from Dr. Liu (Food and Industrial Oil Research, Peoria, Illinois, USA) were measured to be $\text{Mn} = 3160$ and $\text{Mw} = 4866$ in a conventional calibration.$^{41}$
The polymer samples, which were measured by GPC, were the HPESO sample from Dr. Liu (HPESO-std), HPESO prepared in our lab (HPESO-JW), the alcohol-based polymer (Soy-Alcohol), the aspartic acid-based polymer (Soy-Aspartic) and the taurine-based polymer (Soy-Taurine) derived from HPESO-JW. The tests showed that the eluting time of these polymer samples were very close to the HPESO standard sample, the time difference was within 0.1 min. Thus, the new soybean oil based polymers synthesized in our lab should have the similar molecular weights as the standard HPESO sample.

![GPC spectra of the HPESO and the new polymers](image)

**Figure 18**  **GPC spectra of the HPESO and the new polymers.** The eluting time of these polymer samples was as follow: a) Test A: HPESO-std (13.666 min), alcohol-based polymer (13.591 min), and taurine-based polymer (13.622 min); b) Test B: HPESO-std (13.550 min), HPESO-JW (13.590 min), and aspartic acid-based polymer (13.583 min). All the eluting time were very close (± 0.1 min).

### 3.4.5 Formation of the triethylamine salts of the polymers

After the addition of triethylamine to the polymers, which were suspended in DDI water, the polymer suspensions converted to clear solutions. The pH of these polymer solutions was
between 8 to 9. Next, these solutions were used to develop the PLNs loaded with cisplatin. The use of the triethylamine salts of the polymers has three benefits: first, the formation of the hydrophobic triethylamine salt of the polymers can increase the solubility of the polymers in aqueous solution; thus, the drug loading of hydrophilic drug molecules may increase; secondly, since cisplatin is known to be vulnerable to nucleophiles such as thiols, primary or secondary amines and cyanide, triethylamine would not react with cisplatin as a bulky base and a tertiary amine; lastly, the excess of triethylamine could be easily removed either via evaporation or lyophilization as compared with other solid form of organic base.

3.4.6 Solubility of the newly synthesized polymers

The solubility tests indicated that the taurine-based soybean oil polymer had the best aqueous solubility among the tested polymers due to the addition of the sulfonate group, which had the lowest pKa value among the these functional groups. (Table 3.1) Also, the aqueous solubility of the aspartic acid-based and the alcohol-based polymers was increased compared to HPESO due to the addition of di-carboxylic acids group and primary alcohol group. The detailed test results are listed as follows:
Table 3.1  **Results of the solubility test for the polymers in ethanol/H2O mixture.** The polymers were dissolved in different volumes of ethanol which was the true solvent for the polymers, and then distilled, deionized (DDI) water, which was the diluent for the polymer, was added dropwise till a cloudy emulsion appeared.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>amount (mg)</th>
<th>Ethanol (ml)</th>
<th>H2O (ml)</th>
<th>ethanol/H2O/per 10 mg of polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol Based</td>
<td>18</td>
<td>4.8</td>
<td>1.5</td>
<td>2.7/0.8</td>
</tr>
<tr>
<td>Aspartic Acid based</td>
<td>18</td>
<td>4.3</td>
<td>1</td>
<td>2.4/0.6</td>
</tr>
<tr>
<td>Taurine Based</td>
<td>34</td>
<td>4.2</td>
<td>1.5</td>
<td>1.2/0.4</td>
</tr>
<tr>
<td>HPESO</td>
<td>24</td>
<td>&gt; 6 (cloudy)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2  **Results of solubility test in pH = 7.74 buffer solution at 60 °C.** The preheated PBS buffer solution (25 mM) was dropwise added to different amount of the polymers with stirring till a clear aqueous solution appeared.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Amount (mg)</th>
<th>Buffer solution (ml)</th>
<th>solubility per mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol Based</td>
<td>30</td>
<td>12</td>
<td>2.5 mg/mL</td>
</tr>
<tr>
<td>Aspartic Acid based</td>
<td>11.6</td>
<td>19</td>
<td>0.6 mg/mL</td>
</tr>
<tr>
<td>Taurine Based</td>
<td>13</td>
<td>3</td>
<td>4.3 mg/mL</td>
</tr>
<tr>
<td>HPESO</td>
<td>18</td>
<td>28</td>
<td>0.6 mg/mL</td>
</tr>
</tbody>
</table>
Table 3.3 pH endpoint determination for the mixture of polymers and triethylamine at 40 °C. Triethylamine was added dropwise to the suspension of the polymers (45 mg) in DDI water (3 mL) with stirring. The pH was measured using a pH meter when the suspension fully converted to a homogeneous solution.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Amount (mg) of polymer in 3 mL of DDI H₂O</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid based polymer</td>
<td>45</td>
<td>~ 8.1</td>
</tr>
<tr>
<td>Taurine based polymer</td>
<td>45</td>
<td>~ 8.5</td>
</tr>
</tbody>
</table>

3.4.7 Solubility of the polymer-cisplatin conjugates

The solubility of the polymer-cisplatin conjugate may have an effect on the preparation of the PLNs; thus, several experiments were conducted to investigate the solubility of the polymer-cisplatin complex.

The experimental results showed that the pH dropped (from pH 9.4 to 8.1) after mixing aspartic acid-based polymer and cisplatin over 72 h. The pH drop was due to the release of hydrogen chloride (HCl) which was derived from the formation of the carboxylate-platinum conjugate.

This aggregation could not be re-dissolved even when the pH was adjusted back to 10. In another experiment, the addition of trace amounts of CaCl₂ could also induce more aggregation and a pH drop (from 8.1 to 7.7). All these tests indicated that the aqueous solubility of the polymer-platinum conjugate, in which the polymer part may still have some free carboxylic acids, was poor under neutral conditions. Furthermore, the aqueous solubility of the calcium salt of this polymer-platinum conjugate was even poorer. Therefore, it was assumed that the formation of the triethylamine salt of the polymer-platinum conjugate, in which the polymer part may still have some free carboxylic acids, that can increase the aqueous solubility of the polymer-cisplatin complex.
In another experiment, the solution of polymer/platinum/triethylamine conjugate was prepared under strong basic condition (pH >11). It was found that there was no aggregation observed after the addition of CaCl$_2$ to these solutions and the solution stayed at room temperature for 7 days. In addition, no nanoparticles were detected by DLS in this solution. This indicated that Pt[[(OH)$_2$NH$_3$)$_2$] was the major form under a strong basic condition (Figure 1) and this induces the slow formation of carboxylate-platinum. The in depth study of the platinum-polymer conjugate will be further discussed in Chapter 4.

### 3.4.8 Theoretical chelation ratio of the HPESO-cisplatin conjugate

In order to load the right amount of cisplatin to the PLN formulations, it was necessary to calculate the theoretical chelation of the HPESO-cisplatin conjugate.

Soybean oil includes different percentages of fatty acids, which are palmitic acid (10%), stearic acid (5%), oleic acid (26%), linoleic acid (52%), and linolenic acid (7%). Among these long chain fatty acids, oleic acid has one double bond, linoleic acid has two double bonds and linolenic acid has three double bonds. Because epoxidized soybean oil (ESO) can only be synthesized from unsaturated fatty acids due to the formation of oxirane rings, monomer ESO should only have oleic acid, linoleic acid and linolenic acid moieties.

The molecular weights of epoxidized oleic acid, epoxidized linoleic acid and epoxidized linolenic acid are 298.46, 312.45 and 326.43, respectively. Thus, the average molecular weight of each fatty acid chain is calculated as follow:

$$\frac{(298.46 \times 0.26 + 312.45 \times 0.52 + 326.43 \times 0.07)}{(0.26 + 0.52 + 0.07)} = 309.4$$

Each fatty acid chain has one carboxylic acid as a binding site within HPESO. As the molecular weight of HPESO is around 3219, each molecule of HPESO should have around 10 carboxylic acid groups ($3219/309 \approx 10$). Because each molecule of cisplatin can theoretically form two
covalent bonds with carboxylate groups, the maximum equivalent molar ratio between cisplatin and HPESO is \(10/2 = 5\) to 1. The molecular weight of cisplatin is 301.1. In theory, 50 mg of HPESO (0.0155 mmol) can encapsulate 23.5 mg \((0.0155 \times 5 \times 301.1)\) of CDDP. In the next section, the results of using the HPLC method to identify if there were differences between the theoretical chelation ratio and the actual loading of cisplatin are reported.

### 3.4.9 HPLC method to determine the ratios of the polymer-cisplatin conjugates

Before determining the ratios of the polymer-cisplatin conjugates using HPLC, it was necessary to ensure the formation of the polymer-cisplatin complexes. In general, platinum-carboxylate complexes were synthesized via an intermediate adduct of diaminedi(hydroxo) nitrate. However, Miller and House found that cisplatin can convert to di-(hydroxo) species at physiological pH or under mild basic conditions. The hydrolysis period of cisplatin under basic condition was 48 h at room temperature. One of the aqua species, cis-Pt(NH\(_3\))\(_2\)(OH)Cl, was found to be 98% in a mild basic solution. (Figure 1)\(^{75,76}\)

On the basis of this information, before the HPLC analysis, the triethylamine salts of the polymers were mixed with cisplatin in an aqueous solution for 3 days at room temperature to ensure the formation of polymer-platinum conjugates. Since cisplatin and its aquated species can be detected by a UV detector at 205 nm or 305 nm, the chelation ratios of the polymer-cisplatin complexes can be calculated based on the intensity of reduction compared to a standard solution of cisplatin. In the equilibrium between cisplatin and its aquo species, the cisplatin solution is a mixture of platinum species; thus, the standard solution of cisplatin was stored at room temperature for 24 h before the HPLC tests. Burger and coworkers found that the pH of a 5 mM cisplatin solution (1.5 mg/mL) was 5.5 and contained around 10% of diaquated (aquo/hydroxo)
species. Yachnin et al. also reported that a mono-aquated platinum complex was the major form which was about 80% under a mild acidic condition (pH 6.0). Therefore, it was expected that all cisplatin would be converted to highly hydrophilic aquo-platinum species after the 24 hours of storage at room temperature. The gradient conditions, in which the percentage of MeOH was increased from 1% to 100%, has been used for the HPLC analysis. The HPLC chromatography showed that there was no other platinum species detected at a high percentage of methanol (> 20%) as cisplatin was converted to hydrophilic aquated species. Accordingly, the eluting condition of HPLC was set to an isocratic condition using DDI H$_2$O: MeOH = 97: 3, in which all platinum species should be eluted.

The HPLC study showed that the actual loading of cisplatin to HPESO polymer (21 mg cisplatin per 50 mg of HPESO) was very close to the theoretical calculation, which was 23.5 mg of CDDP per 50 mg of HPESO. The HPLC analysis showed that the aspartic acid-based polymer can encapsulate higher amounts of cisplatin (~ 44 mg). Because each fatty chain of the aspartic acid-based polymer contained three chelation moieties, which were two carboxylic acids and one amide bond, it was expected that the loading of cisplatin to this polymer would be almost double that of HPESO. As to the taurine-based polymer, each chain of the polymer contained one amide bond and one sulfonate group. Thus, it was expected that the drug loading (40 mg) would be almost twice of that of HPESO.

### Table 3.4  Ratios between the polymers and cisplatin measured by the HPLC method.

The loaded amount of cisplatin was determined spectroscopically by comparing to a standard absorption curve of a free cisplatin solution.

<table>
<thead>
<tr>
<th>Aspartic acid-based polymer (50 mg)</th>
<th>Taurine-based polymer (50 mg)</th>
<th>HPESO (50 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>44 mg of cisplatin was loaded</td>
<td>40 mg of cisplatin was loaded</td>
<td>21 mg of cisplatin was loaded</td>
</tr>
</tbody>
</table>
3.5 Conclusions

In Chapter 3, NMR characterization showed that the three synthetic polymers contained the desired hydrophilic groups, which were the primary alcohol group, the di-carboxylic acid group and the sulfonate group. The GPC analysis also showed that the polymers had similar molecular weights as the standard HPESO sample. Various solubility tests elucidated that these new polymers had increased aqueous solubility related to HPESO. The HPLC tests showed that the chelation ratios between cisplatin and the polymers were very close to the theoretical calculation. In the next Chapter, we use these new polymers to prepare PLN formulations to deliver cisplatin or other platinum drugs.
Chapter 4

PLN formulation development using the novel polymers to deliver platinum drugs

4.1 Abstract

In this chapter, the work focuses mainly on formulation development of PLN with trapped platinum drugs, synthesis of the prodrug of cisplatin, and drug release studies under different conditions.

In contrast to the previous PLN formulations of our laboratory, in which the anticancer drugs doxorubicin and mitomycin C were physically trapped in the PLNs, cisplatin is the payload of the present PLNs. In aqueous solution, cisplatin has an aquo equilibrium of different platinum species; in addition, cisplatin can form covalent bonds with chelation groups (e.g. carboxylate group and sulfonate group) of the polymers. Thus, formulation development of the PLN loaded with cisplatin was found to be very challenging. After the optimization of the process parameters e.g. different types of surfactants, different ratios of polymer/lipid ester/surfactant, the Et$_3$N removal technique, addition volume of aqueous phase and power of sonication, PLNs with the desired nanoparticle size range (50 nm ~ 120 nm) were obtained using the aspartic acid-based polymer (formulation A) and the taurine-based polymer (formulation B). The mechanism studies on the correlation between polymer-platinum conjugates and PLN proved that higher temperature and neutral condition favor the formation of carboxylate-platinum conjugates. The in-vitro drug release of the PLNs with aspartic acid-based polymer and taurine-based polymer (formulation A and B) showed that more than 90% of the trapped cisplatin was released in 7 days and 3 h in DDI H$_2$O, respectively. It was elucidated that the mechanism of loading cisplatin in these two formulations was via a covalent bond conjugation. In addition, the covalent bond of
carboxylate-platinum conjugate was shown to be stronger than that of sulfonate-platinum conjugate. However, the drug release profiles of the PLNs under physiological saline conditions showed that most of the trapped cisplatin was released within 30 min. This suggested that cisplatin was not a good payload for this type of PLN because the hydrophilic monohydrate of cisplatin (cis-Pt[(H$_2$O)(OH)NH$_3$)$_2]^+$ had very low affinity to the lipid matrix of PLNs; thus, the encapsulated platinum species tended to move from the lipid core to an outer aqueous environment.

On the basis of the in-depth studies on the PLNs loaded with cisplatin, a prodrug of cisplatin, which had two covalent conjugated stearate chains, was synthesized. Because of the hydrophobic interaction between a prodrug and lipid matrix as well as the ionic interaction between a cationic prodrug and an anionic taurine-based polymer, this novel PLN formulation (formulation C) provided a sustained drug release profile similar to another micro-particle formulation of oxaliplatin, Miriplatin. The drug release studies found that around 95% of platinum species were released from the nanoparticle in saline either at pH 7.4 or 5.6 after 24 h or 48 h, respectively. The particle sizes measured by DLS were within the desired range (60 ~ 120 nm).

4.2 Introduction

4.2.1 Rationale for replacing fatty acids with fatty acid esters in new PLN formulation

Fatty acids (FA) are crucial building blocks for a synthesis of membrane phospholipid. Dietary triacylglycerol is one source of FA, it can also be converted to fatty acids or mono-acylglycerols in human beings. Both of these metabolites are transported through the human body using vascular and lymphatic systems. However, due to their highly hydrophobic property in an aqueous medium, certain carriers are necessary for the delivery of the fatty acids. Human albumin is a 66 kDa single-chain protein, which has about 585 amino acid moieties and 17
disulfide bridges.\textsuperscript{79} Albumin can bind water, cations, fatty acids, hormones, bilirubin and hydrophobic drugs; thus, it is also referred as a molecular "taxi".\textsuperscript{80} The concentration of albumin in plasma is approximately 0.6 mM. Under physiological conditions, each mole of albumin can transport around 1.5 moles of fatty acids.\textsuperscript{81} Albumin has several hydrophobic pockets, which have a distinct chemical environment and a conformation, to bind various fatty acids or small molecules like cisplatin. The residues His 67 and His 247 in human albumin can also chelate with cisplatin. Several studies noted that 65 to 98\% of cisplatin was bound to plasma protein after \textit{i.v.} injection; thus, the binding sites of albumin for an essential element of Zinc were blocked due the formation of the albumin-cisplatin complex. In addition, the crosslinking between cisplatin and albumin triggered a conformational change and this also inhibited the hydrophobic binding between albumin and other fatty acids.\textsuperscript{82} Recently, Garmann and coworkers developed some novel albumin-binding platinum drugs, which included small molecule carboplatin analogues and macromolecular platinum complexes. The study showed that the endocytotic pathway was likely a mechanism to deliver the macromolecular platinum drugs.\textsuperscript{83}

Interestingly, fatty acid ethyl esters (FAEEs), which are neutral hydrophobic compounds, can bind to albumin due to their similar molecular structure to FAs. The affinity between albumin and FAs is stronger than that between albumin and FAEEs due to a combination of electrostatic and hydrophobic interactions. One study found that FAEEs were easily replaced by FAs from albumin \textit{in vitro}. This specific property will promote the delivery of FAEEs, which can act as drug carriers, to tissues and cells.\textsuperscript{84} The solid lipid matrix used in this study was ethyl arachidate, a FAEE, which would not form a carboxylate-platinum complex with free cisplatin. It was expected that the PLN loaded with cisplatin or a prodrug of cisplatin may avoid a direct contact between the platinum species and albumin. Also, due to the specific binding property of albumin
and fatty acid ethyl esters (e.g. ethyl arachidate), it may assist the drug delivery of platinum drug(s). Future studies will focus on this specific area.

4.2.2 Physical-chemical properties of the components making up the PLNs

The physical-chemical properties of the components in the PLNs play a crucial role in the formulation development of the PLNs. There were three major components in the PLNs: a) soybean oil based polymers; b) solid lipid acids or lipid acid ester; 3) PEG type surfactants with long fatty acid chains. All these components contained different hydrophobic fatty acids or their derivatives.

As described in Chapter 3, aspartic acid-based polymer, alcohol-based polymer and taurine-based polymer have better aqueous solubility compared to HPESO; still, they cannot be fully dissolved in DDI water. To dissolve these polymers in an aqueous solution, they were converted to the hydrophobic triethylamine salts. The melting points of polyoxyethylene 40 stearate and ethyl arachidate are 37–44 °C, 41–45 °C, respectively. Therefore, PLNs prepared from these two components were expected to be solid at normal human body temperature (37 °C). During the development and optimization of PLN formulations, these two ingredients would be melted at higher temperatures (e.g. 60–80 °C) to give a homogeneous liquid lipid matrix. As to the payload of PLNs, cisplatin, due to its low water-solubility at room temperature (~ 2.5 mg/mL), to encapsulate 4.5 mg of cisplatin in 10 mg of polymer, it was necessary to prepare cisplatin solution using 3 mL of DDI water before the preparation of PLNs. After the addition of a cisplatin/polymer/Et3N solution to the above melted lipid ester/surfactant lipid matrix, the biphasic water/oil (w/o) emulsion would be broken down into nano-scale droplets using an
ultrasonicator. Then, these liquid droplets would form a solid form of PLNs at room temperature or lower temperature.

4.3 Material and methods

4.3.1 Chemicals

Cisplatin was a gift from Johnson Matthey Pharmaceutical Research, and ethyl arachidate was purchased from TCI-America. Poly(ethylene oxide)-40-stearate (Myrj52©), methanol, triethylamine and stearic acid were purchased from Sigma-Aldrich and used without further purification. Pluronic F-68 was purchased from BASF Canada (Mississauga, ON, Canada). Distilled and deionized (DDI) water was obtained with a Milli-Q water system (Milli-Pore).

4.3.2 Characterization of the PLNs

Particle size and zeta potential were measured by dynamic light scattering and electrophoretic mobility, respectively, using a NICOMP™ 380ZLS (PSSNICOMP, Santa Barbara, CA, US) apparatus and/or Malvern Zetasizer Nano ZS (Worcestershire, UK).

4.3.3 Method for the drug release study

To quantify the release kinetics of cisplatin from the PLNs, the cisplatin-loaded PLNs or the prodrug-loaded PLN (~ 30 mg of nanoparticles) were treated with 10 mL of PBS solution or DDI H₂O and was transferred into a micro-dialysis bag (Spectra/Por 6, MWCO: 2000 dalton). The dialysis bag was stirred either in 70 mL of phosphate buffered saline (PBS) solution or in 70 mL of DDI H₂O. The dialysis bags in releasing media were placed in an orbital water bath at 37 °C. The released platinum species, which outside the dialysis bags, were sampled at a defined time
periods. The amount of platinum species was calculated by measuring the absorbance at 205 nm by Agilent 8453 UV-Vis spectrophotometer.

4.3.4 Synthesis of the stearate prodrug of cisplatin

To a 250 mL, one-necked, round bottle flask was added stearic acid (540 mg, 1.9 mmol, 2.2 equiv.) and 0.1N NaOH solution (20 mL). The suspension was heated at 70 °C until a clear solution was formed. To another 100 mL, one-necked, round bottle flask was added cisplatin (259 mg, 0.86 mmol, 1.0 equiv.) and DDI H₂O (100 mL). The cisplatin suspension was heated at 70 °C until a clear solution was formed. These two solutions were mixed and stirred at 70 °C for 5 h. A white solid formed during the heating period. The solids were isolated by the centrifugation (3150 × g for 20 min), and were washed with DDI water (150 mL × 3) and air-dried at room temperature. The proton NMR and FTIR spectra were acquired at the Department of Chemistry of the University of Toronto. ¹H NMR spectra were acquired using a Varian Mercury 400 MHz (CA, US). FTIR spectra were recorded on a Perkin Elmer Spectrum 1000 series spectrometer (Waltham, MA, US). Spectra were taken with a resolution of 4 cm⁻¹ and average over 32 scans.

4.3.5 PLN Formulation loaded with the prodrug (Formulation C)

To a 20 mL glass vial was added ethyl arachidate (27 mg), polyoxyethylene 40 stearate (12 mg), the taurine-based polymer (13 mg) and the prodrug of cisplatin (12 mg). The vial was heated in a circulating water bath at 80 °C for 10 min; then, two mL of DDI H₂O, which was pre-heated to 80 °C, was added to the melted lipid matrix. The emulsion was vigorously stirred at this temperature for 1.5 h in a closed vial. The mixture was then sonicated using a Hielscher UP100H probe ultrasonicator (Hielscher USA, Inc., Ringwood NJ, USA) at 40% peak amplitude and 5
mm probe depth in solution for 3 ~ 5 min. After the sonication, the emulsion was charged with 5 mL of the pre-cooled DDI H₂O and stirred in an ice bath for 1 h. Particle size and zeta potential were measured by dynamic light scattering using a NICOMPTM 380ZLS (PSSNICOMP, Santa Barbara, CA, USA) apparatus and / or Malvern Zetasizer Nano ZS (Worcestershire, UK).

### 4.4 Results and discussion

#### 4.4.1 Initial flow chart for the PLN using the aspartic acid-based polymer

Based on the physical-chemical properties of the components discussed in the Introduction, the following flow chart was designed to prepare the PLNs using the aspartic acid-based polymer:

**Figure 19**

**Initial flow chart for preparing the PLNs**

- **cisplatin + polymer + triethylamine in DDI H₂O**
- **heated at 60 – 80 °C for X min**
- **mixed and heated at 80 °C for Y min**
- **ultrasonicated at 80% peak amplitude for 3 min**
- **added DDI H₂O and adjusted pH**
- **cooled in ice-water and checked particle size**
The drug-loaded PLNs using the aspartic based polymer were prepared as previously reported, with some modification.\(^{48}\)

To a 20 mL glass vial was added ethyl arachidate (25 mg), polyoxyethylene 40 stearate (10 mg). The vial was heated in a circulating water bath at 80 °C until the lipid ester and surfactant were melted. At the same time, to another 20 mL glass vial, cisplatin (4.5 mg) in DDI H\(_2\)O (3 mL) was heated in a circulating water bath at 40 °C until all the cisplatin dissolved. Then, the polymer (10 mg) and triethylamine (25 \(\mu\)L) were added to this cisplatin solution; the mixture was stirred at 80 °C for certain period (from 50 min to 2 h). The solution of CDDP/polymer/Et\(_3\)N was added to the melted lipid-surfactant mixture described above and stirred for 5 min. The mixture was sonicated using a Hielscher UP100H probe ultrasonicator (Hielscher USA, Inc., Ringwood, NJ, USA) at different peak amplitudes and a 5mm probe depth in solution for 3 ~ 5 min. After the sonication, the emulsion was treated with 5 mL of DDI H\(_2\)O and stirred in an ice bath. The pH of emulsion was adjusted to 7.2 ~ 6.8 using 2-(N-morpholino) ethanesulfonic acid (MES) or 0.1N NaOH solution and stirred in an ice bath for 10 min. Particle size was measured by dynamic light scattering using a NICOMPTM 380ZLS (PSSNICOMP, Santa Barbara, CA, USA) apparatus.

### 4.4.2 Studies on the PLN formulation

To prepare a stable PLN formulation with the desired nanoparticle size range, different combinations of the aspartic acid-based polymer, ethyl arachidonate (lipid ester), polyoxyethylene 40 stearate (surfactant) and triethylamine were tested. In addition, the heating period, different types of surfactants such as Pluronic F-68 and Myrj 59, different types of lipid esters such as glyceryl behenate, pH of the solutions and sonication at different peak amplitudes were investigated as well. However, the particle sizes measured by dynamic light scattering
(DLS) showed that the sizes of PLNs were not consistent, and the sizes varied from 10 nm to 80 nm. Interestingly, the blank nanoparticles, prepared using the same formulations, had sizes measured of 80 ± 10 nm; in addition, the blank PLNs were quite stable after they were stored at room temperature for several weeks. This finding showed that the formation of polymer-platinum conjugates had effects on the formation of PLNs.

As discussed in Chapter 1, cisplatin can form different aquo species and they were in equilibrium with each other. (Figure 1) These platinum aquo species are reactive intermediates which can form covalent bonds with different nucleophiles (e.g. carboxylate group). Presumably, the carboxylate groups of the aspartic acid-based polymer and cisplatin can either form monodentated platinum complexes or bidentated platinum complexes. The structure of the bidentated platinum complex should be very similar to the Nα-acetylamido ligand, which was used to prepare the [PtII(NH3)2(Nα-AcLys)]. According to the reported procedure, it took more than 24 h to form a five member bidentated complex. As to the aspartic acid-based polymer, its molecular weight was more than 3,000 daltons and it was much bigger than the small molecule of Nα-AcLys whose molecular weight was 188 daltons. Thus, the formation rate of the bidentated platinum complex would be unlike the aspartic acid-based polymer and it was presumed that the monodentated platinum complex was more likely to form. Since the formation of the platinum-polymer conjugate releases free protons, the aqueous emulsions became more acidic; thus, the surface charge of PLNs was not stable. As a result, the PLNs which were freshly prepared may not be very stable since the pH of the outer aqueous media kept changing.

4.4.3 Mechanism studies of the effects of the polymer-platinum conjugate on the PLN formulation
Previous studies on the formulation development of PLNs showed that the particle sizes of PLNs varied from batch to batch. It was presumed that the formation of polymer-platinum conjugate induced a pH change which would have effects on the formation of PLNs. Therefore, different experiments were conducted to test this hypothesis and the experiments are described below:

Test A: The study of the pH of cisplatin solution. In this study, the pH of cisplatin solution was measured before the formation of the polymer-platinum conjugate and the PLN loaded with other ingredients.

Cisplatin (4.56 mg) was dissolved in DDI water (3 mL). The pH measured by the pH meter was 5.65. This acidic solution was attributed to the formation of platinum aquo species (Figure 1) and the slightly acidic DDI water with dissolved CO₂ from the environment. Burger and coworkers also reported a similar result. They found that the pH of a 5 mM cisplatin solution (1.5 mg/mL) was 5.5 and it contained around 10% of the diaquated (aquo/hydroxo) species.\(^{86}\) Moreover, Yachnin et al. reported that a mono-aquated platinum complex was the major form which was about 80% under mildly acidic conditions (pH 6.0).\(^{87}\)

Test B: The study on the pH change of cisplatin/polymer mixture under strong basic conditions. The study can determine if the formation of polymer-platinum complex occurred at high pH.

Cisplatin (9.1 mg) was dissolved in DDI water (6 mL). The solution was then treated with the aspartic acid-based polymer (20 mg) and triethylamine (50 µL). The mixture was agitated at room temperature. The pH values were measured and plotted against time. (Figure 20) The results showed that the pH variation of the solution was very small, between 11.86 and 11.22. This suggested that the formation rate of the polymer-platinum conjugate under strong basic condition was very slow. This result was consistent with the observation in Chapter 3.
Test C: The study on the encapsulation of cisplatin using the aspartic acid-based polymer only. This study was to identify the optimum conditions to prepare carboxylate-platinum conjugates.

The cisplatin/polymer/Et$_3$N solution (3 mL, 1.5 mg/mL) from test B (above) was further analyzed by HPLC to determine the drug encapsulation (Table 4.1, entry 1, 2). In another experiment, a cisplatin solution (6 mL, 1.0 mg/mL) was treated with the aspartic acid-based polymer (20 mg) and triethylamine (40 µL). Then, the mixture was further heated at 60 °C for 18 h (Table 4.1, entry 3). The results showed that the formation of the polymer-platinum conjugate under basic condition (pH ≈ 11) at room temperature was very slow. However, higher temperature tended to push the formation of the carboxylate-platinum complex.

**Table 4.1** Cisplatin encapsulation with the aspartic acid-based polymer under various conditions. The loaded amount of cisplatin was determined spectroscopically by comparing with a standard absorption curve of a free cisplatin solution.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>Cisplatin encapsulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mixed at room temperature for 13 days (pH = 11.22)</td>
<td>38%</td>
</tr>
<tr>
<td>2</td>
<td>Mixed at room temperature for 13 days (condition 1) and heated at 80 °C for 1 h</td>
<td>49%</td>
</tr>
<tr>
<td>3</td>
<td>Mixed at room temperature for 2 days and then heated at 60 °C for 18 h</td>
<td>82%</td>
</tr>
</tbody>
</table>
Test D: The study on the encapsulation of cisplatin within the PLNs. This study was to further identify if other ingredients within PLN had effects on the formation of polymer-platinum complex.

Another cisplatin/polymer/Et$_3$N solution (3 mL, 1.5 mg/mL) from test B (above) was further used to prepare the PLN using the typical formulation (see 4.1.2 flow chart). Then, the free cisplatin concentration was measured by HPLC to determine the encapsulation of cisplatin. The results showed that most of the cisplatin was not trapped in PLN at room temperature after 3 days.

In another experiment, the cisplatin/polymer/Et$_3$N solution (2 mL, 1.0 mg/mL) from test C (Table 4.1, entry 3), was used to prepare the PLN using the same formulation (see 4.1.2 flow chart). The HPLC analysis showed that the encapsulation of cisplatin was 94%. This study showed that other components such as lipid ester and surfactant did not have an important role on the formation of the polymer-platinum conjugate.

Test E: The study on the correlation between the pH change and the formation of the polymer-platinum conjugate. This test can identify if the mechanism of loading cisplatin was through a covalent bond formation or via physical encapsulation.

The PLN was prepared using the typical formulation (see 4.1.2 flow chart). The only difference was that the triethylamine was replaced by a 1.0N NaOH solution. The pH was adjusted back to a neutral condition using a diluted NaOH solution after the pH of the mixture dropped to 5 ~ 6. (Figure 21) It was observed that the pH kept dropping during the formation of the polymer-platinum conjugate at higher temperature (e.g. 80 °C). In another experiment, it was noted that the pH of the cisplatin/polymer/Et$_3$N mixture slowly dropped from 10.5 to 8.8 at room temperature during a 16 days of storage at room temperature. This data indicated that the
mechanism of loading cisplatin was through covalent bond formation and not via physical encapsulation due to the continuous drop in pH which was induced by the release of HCl.

![Figure 21](image.png)

**Figure 21** pH drops during the formation of the polymer-platinum conjugate. The pH was adjusted to a neutral condition using a base solution (NaOH or Et₃N) after the pH dropped below 7.

Test F: The effect of pH on the compatibility of lipid ester and the polymer/cisplatin/Et₃N solution. This study was to investigate the formation of bigger particles during the PLN formulation development.

The tests of the above formulation development showed that the pH of the PLN emulsions varied from 8.8 to 10.5 before the pH adjustment using 2-(N-morpholino)ethanesulfonic acid (MES). Since the mixing of the ingredients occurred in a closed system, it was presumed that the high pH was due to the excess triethylamine left in the PLN emulsions. In addition, the previous experiments in Chapter 3 showed that the aspartic acid-based polymer was quite soluble at pH ≥ 8.5. Thus, during the preparation of some batches of the PLNs, it was observed that some melted lipid ester still floating on top of the aqueous solution and was not compatible to the polymer/cisplatin/Et₃N solution. After the emulsions were cooled in an ice-bath, some big particles formed. Thus, this may also contribute to the size variation of the PLNs.

4.4.4 Modified formulation for the aspartic acid-based polymer (Formulation A)
On the basis of the above studies on the effects of the polymer-platinum conjugate on the PLN formulations, it was suggested that higher temperature and a neutral condition would favor the formation of the carboxylate-platinum complex. (Figure 22)

\[
\begin{align*}
\text{pH} &= 5 \sim 7 \\
\text{higher temperature} \ (60 \sim 80^\circ C) &\quad \rightarrow \\
\text{high pH} &\quad \times \\
\text{polymer} &\quad + \quad H^+ \\
\text{mono_dentated structure}
\end{align*}
\]

**Figure 22** Proposed mechanism for the formation of platinum-carboxylate complex with the polymer. The formation of the monodentated polymer-platinum conjugate leads to the release of free HCl which induces a drop in pH.

Therefore, the flow chart for the preparation of the PLN was modified as follow (Formulation A): (Figure 23)

After a mixing of the formulation ingredients, the vial cap was removed and the mixture was kept heated at 80 °C; therefore, the excess triethylamine was removed via evaporation. Without pH adjustment using 2-(N-morpholino)ethanesulfonic acid (MES), the pH of PLN emulsions was found to be around 7. In addition, the particle sizes were measured 7 days later by DLS since the PLNs would be stable until the full formation of the carboxylate-platinum complex. The particle sizes of some PLNs (entry 1 – 3; Table 4.2) measured by DLS were around 100 nm. In two of the PLN formulations (entry 5 and 6; Table 4.2), triethylamine was replaced by a diluted NaOH
solution to adjust pH. However, bigger sizes of particles were found after two weeks of storage. This suggested that the use of triethylamine can stabilize this type of the PLN formulation due to the formation of the stable hydrophobic salt. The modified formulation was also applied to the alcohol-based polymer and the taurine-based polymer (entry 6 - 8). However, a darker solution which could be the decomposition of cisplatin or bigger particles was found after several weeks of storage. Therefore, other optimized formulations needed to be developed for these two new polymers.

**Figure 23** Modified flow chart for preparing the PLN using the aspartic acid-based polymer (Formulation A)
A typical particle size of PLN was determined by a Nicomptm 380ZLS apparatus. Parameters for the formulations of PLNs studied. Particle diameter was the number-weighted diameter of readings averaged over 3 min. The diameter value was the mean (standard deviation) of two measurements.

<table>
<thead>
<tr>
<th>Formulation Entries</th>
<th>Cisplatin (mg)</th>
<th>PEG40S (mg)</th>
<th>lipid ester (mg)</th>
<th>Et3N (μL)</th>
<th>Aspartic acid polymer (mg)</th>
<th>heating period Y (min)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>15</td>
<td>24</td>
<td>20</td>
<td>10</td>
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<td>7.2</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>15</td>
<td>25</td>
<td>25</td>
<td>10</td>
<td>55</td>
<td>7.1</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>15</td>
<td>35</td>
<td>25</td>
<td>10</td>
<td>55</td>
<td>6.9</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>20</td>
<td>25</td>
<td>25</td>
<td>10</td>
<td>55</td>
<td>6.7</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>15</td>
<td>20</td>
<td>0</td>
<td>10</td>
<td>120</td>
<td>7.0</td>
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<tr>
<td>Taurine-based polymer (mg)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>15</td>
<td>20</td>
<td>0</td>
<td>10</td>
<td>90</td>
<td>7.4</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>20</td>
<td>25</td>
<td>15</td>
<td>10</td>
<td>30</td>
<td>6.6</td>
</tr>
<tr>
<td>Alcohol-based Polymer (mg)</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>8</td>
<td>4.5</td>
<td>15</td>
<td>25</td>
<td>15</td>
<td>10</td>
<td>30</td>
<td>3.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Formulation Entries</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95 +/- 5</td>
</tr>
<tr>
<td>2</td>
<td>105 +/- 5</td>
</tr>
<tr>
<td>3</td>
<td>112 +/- 3</td>
</tr>
<tr>
<td>4</td>
<td>67 +/- 8</td>
</tr>
<tr>
<td>5</td>
<td>precipitated, not applicable</td>
</tr>
</tbody>
</table>

4.4.5 Modified PLN formulation for the taurine-based polymer (Formulation B)

As described above, using the aspartic acid-based polymer, the PLNs with an average particle size of approximately 100 nm can be prepared. However, the taurine-based polymer cannot be prepared by formulation A. Therefore, another formulation (formulation B) was proposed for the taurine-based polymer or the alcohol-based polymer.
In this formulation, blank PLNs were first prepared and cisplatin was added to the blank PLNs. The proposed mechanism is described as follows:

After the formation of the blank nanoparticles, the hydrophilic moieties (e.g. carboxylic acids or sulfonic acids) would be on the outer layers of PLNs in aqueous solution. Presumably, the sulfonate or the carboxylate parts can form a complex with platinum atoms; thus, the complex should be the final outer layer of the new PLNs. As to the taurine-based polymer, there were two possible mechanisms to encapsulate the aquo species of cisplatin: 1) electrostatic interaction between the negatively charged polymer(s) and the cationic aquo species of cisplatin. A similar mechanism has been reported by Burger and coworkers. They have prepared the cisplatin nano-capsules, in which the cationic aquo species of cisplatin formed the ionic interaction with the negatively charged liposomes; 2) formation of the conjugated sulfonate-platinum complex via covalent bonding.

The flow chart of the new formulation (formulation B) was as follows:
Figure 24  Modified flow chart for preparing the PLN using the taurine-based polymer (Formulation B)
Table 4.3  Parameters for the formulation development of the PLNs (Formulation B)

<table>
<thead>
<tr>
<th>Formulation Entries</th>
<th>Cisplatin (mg)</th>
<th>PEG40S lipid ester (mg)</th>
<th>Et$_3$N (µL)</th>
<th>polymer (mg)</th>
<th>heating period Y (min)</th>
<th>pH (before CDDP addition)</th>
</tr>
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<tr>
<td>1</td>
<td>4.0</td>
<td>15</td>
<td>25</td>
<td>15</td>
<td>11</td>
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<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>20</td>
<td>25</td>
<td>15</td>
<td>11</td>
<td>7.60</td>
</tr>
<tr>
<td></td>
<td>(alcohol-based)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4.0</td>
<td>20</td>
<td>25</td>
<td>15</td>
<td>10</td>
<td>7.83</td>
</tr>
<tr>
<td></td>
<td>(aspartic acid)</td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>4.0</td>
<td>20</td>
<td>25</td>
<td>15</td>
<td>10</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>(taurine-based)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.6</td>
<td>15</td>
<td>25</td>
<td>25</td>
<td>10</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>(alcohol-based)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4.7</td>
<td>15</td>
<td>27</td>
<td>15</td>
<td>13</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>(taurine-based)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4.0</td>
<td>11</td>
<td>25</td>
<td>0</td>
<td>11</td>
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<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

As expected (Table 4.3; entry 1, 3), due to the formation of the platinum-carboxylate complex, a drop in pH of the PLN emulsions occurred with the aspartic acid-based polymer. The pH of the emulsions was adjusted to neutral condition using a diluted NaOH solution (~ 0.03 N) when the pH of emulsions dropped to 6.5. Black precipitation, due to the decomposition of cisplatin, formed from the PLNs with the aspartic acid-based polymer occurred in one of the two tested formulations after two weeks of storage. Therefore, as to this new formulation (formulation B), the aspartic acid-based polymer was not a good option due to the extremely slow formation rate of carboxylate-platinum conjugate at room temperature.

As discussed in Chapter 3, one HPLC study claimed that cisplatin would not react with the buffer reagents with sulfonic acid groups such as triflic acid, hexanesulfonic acid and sodium dodecyl sulfate (SDS). However, after extensive literature review, several updated publications reported that the preparation of sulfonate-platinum (II) complexes was possible under some specific conditions. Interestingly, the drop in pH was also noticed for the PLNs with the
taurine-based polymer (Table 4.3; entry 4, 6, 7); thus, it was presumed that the sulfonate-platinum conjugate was formed as well. As the formation rate of a sulfonate-platinum complex at room temperature was slow, it was observed that the drop in pH of this PLN emulsion lasted for 3 days. (Figure 25)

**Figure 25** Proposed mechanism for the formation of platinum-sulfonate complex with the taurine-based polymer.

In another experiment (Table 4.3; entry 7), the organic solvent of dichloromethane was used to replace triethylamine to dissolve the lipid matrix which consisted of polymer, lipid ester and surfactant. Here, the purpose using a water immiscible organic solvent with low boiling point (e.g. dichloromethane) was to prepare cisplatin-loaded PLNs using o/w emulsion method. Before the addition of cisplatin, the pH measured was 5.4. Under this acidic condition, the taurine-based polymer should present itself as the free sulfonic acid form. After four days of storage, the black precipitation, which was the decomposition of cisplatin, was observed as well. To avoid the formation of the black precipitate, the pH adjustment to a neutral condition was necessary. This indicated that a neutral or slightly basic condition was necessary to prepare stable sulfonate-
platinum complexes at room temperature. The particle sizes of the PLNs were measured 7 days later used DLS and transmission electron micrographs (TEM) (TEM data see appendix). The range of particle sizes was from 70 nm to 160 nm. The wide range of the nanoparticle sizes may be due to the inter-molecular chelation between two parts of the blank PLN and cisplatin or the intra-molecular chelation between one part of the blank PLN and cisplatin. (Figure 27) A similar result was reported by Khoee and coworkers as well.91 Therefore, it was hypothesized that a prodrug of cisplatin maybe a better choice as a payload for this type of PLN formulation.

As shown in Table 4.4, the PLN, which was prepared following formulation B, had the desired nano-size with an average size of 84 nm which was measured seven days after PLN preparation. The zeta potential for this formulation was – 56 mV, which can be compared to the PLNs using HPESO, which had zeta potentials ranging from – 10 to – 20 mV; the PLN with the lower zeta potential was due to the use of a taurine based polymer with sulfonate group which has lower pKa value compared with HPESO. Drug loading content (DLC) (w/w%) = weight of cisplatin in PLN / weight of the PLN (excluded the weight of surfactant) × 100%.

**Table 4.4** A typical particle size and zeta potential of PLN were determined by Malvern Zetasizer Nano ZS (Worcestershire, UK). Particle diameter was the number-weighted diameter of readings averaged over 3 min. Drug loading was the percent of cisplatin. The diameter value was the mean (standard deviation) of two measurements.

<table>
<thead>
<tr>
<th>PLN Formulation</th>
<th>Diameter (nm)</th>
<th>Zeta Potential (mV)</th>
<th>Drug Loading (w/w%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5 mg cisplatin</td>
<td>84 (4)</td>
<td>-56</td>
<td>7.34</td>
</tr>
<tr>
<td>+ Taurine-based polymer</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 26  Proposed chelated structures of the taurine-based polymer and cisplatin. Route A showed the intra-molecular interaction of one part of the blank PLN with cisplatin. Route B showed the intermolecular interaction of two parts of the blank PLNs with cisplatin. As to the PLN formed from route A, its particle size should be smaller than the one from route B.

4.4.6 Drug release profiles for the PLNs loaded with cisplatin

The drug release profiles for the PLNs trapped with cisplatin can help identify if cisplatin would be a suitable payload in this PLN formulation; also, it can further elucidate if the mechanism of loading platinum drug was through covalent bond chelation or ionic interaction between the cationic drug and anionic polymers. In the drug release studies, the drug release conditions were in physiological saline [20 mM phosphate buffer (pH 7.4) + 150 mM NaCl] or DDI H₂O at 37 °C. Rapid drug release was found for the PLNs with either the aspartic acid-based polymer or the taurine-based polymer; most trapped cisplatin was released within 30 min for formulation A and B. (Table 4.5) The fast drug release of the two formulations in physiological saline at 37 °C showed that the bonds between polymers and platinum were not very strong. Since the soybean oil based polymers were cross-linking polymers, it was very likely that the monodentate
complexes were formed between the polymers and cisplatin due to the lack of flexibility of the chelation chains of the polymers. Therefore, the monodentate platinum-polymer complexes were easily converted to free cisplatin due to the high concentration of chloride ion (150 mM).

**Table 4.5** Drug release kinetics from cisplatin-loaded PLN formulations. Drug release was measured in 70 mL of pH 7.4 PBS solution (20 mM phosphate + 150 mM NaCl) at 37 °C, two formulations were analyzed for the release kinetics: (A) Aspartic acid-based PLN (B) Taurine-based PLN. The platinum content was determined spectroscopically by an Agilent 8453 UV-Vis spectrophotometer.

<table>
<thead>
<tr>
<th>PLN Formulation (Formulation A and B)</th>
<th>95% Drug release in pH 7.4 PBS saline (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulation A (aspartic acid based PLN)</td>
<td>30</td>
</tr>
<tr>
<td>Formulation B (taurine based PLN)</td>
<td>30</td>
</tr>
</tbody>
</table>

In contrast, the drug release of these two PLN formulations (formulation A and B) was longer in DDI H₂O. More than 90% of platinum species was released in 3 h from the formulation B using the taurine-based polymer. Furthermore, the drug release from the formulation A with the aspartic based polymer was much slower; more than 90% of platinum species was released in 7 days. (Figure 27) These results proved that cisplatin was entrapped in the PLNs via a covalent bonds chelation, and not through an ionic interaction. Otherwise, the stronger ionic interaction between an anionic sulfonate group and cationic platinum species should have a slower drug release profile which is contrary to experimental results. Moreover, the drug release study showed that the covalent bond between a platinum atom and a carboxylate group was stronger than that between a platinum atom and a sulfonate group. As to formulation B with the taurine-based polymer, cisplatin was added after the formation of a blank PLN; therefore, most sulfonate-platinum complexing was in the final outer layer of PLN and this complex was more vulnerable to water as compared with the PLN using the aspartic acid-based polymer.
Drug release kinetics from cisplatin-loaded PLN formulations. Drug release was measured in 70 mL/140 mL of DDI H₂O at 37 °C, two formulations were analyzed for the release kinetics: (A) Taurine-based PLN (B) Aspartic acid-based PLN. The platinum content was determined spectroscopically by an Agilent 8453 UV-Vis spectrophotometer.

Physical entrapment of cisplatin was tested through a direct addition of cisplatin solid or a cisplatin solution (in DMSO) to a melted lipid ester/polymer/surfactant matrix; still, it was found that free cisplatin was released within 15 min from the PLNs. All together the drug release studies suggested that cisplatin was not an ideal drug candidate for this type of PLN formulation due to its low aqueous solubility and lipophilicity of cisplatin. Thus, a prodrug of cisplatin may be a better potential payload for the PLNs with the newly synthesized polymers.
4.4.7 Synthesis of the prodrug of cisplatin

As discussed above, the PLNs with the newly synthesized soybean oil based polymers can encapsulate cisplatin via covalent chelation, and they have the desired nano-particle sizes (70 nm ~ 150 nm). The drawback for these formulations was their fast drug release under physiological conditions at a high NaCl concentration (150 mM). To overcome this problem, a prodrug of cisplatin, with better compatibility with lipid matrix, would be an alternative option. Miriplatin, which has two C14 myristate chelation groups, is a prodrug of oxaliplatin. It is highly aqueous insoluble when suspended in hydrophobic iodinated poppy seed oil. Due to the high affinity between poppy seed oil and the two long chains of fatty acid, this formulation provides a slow sustained drug release profile. The downside for this formulation is the extremely slow drug release in the in vitro drug release tests. Studies showed that only around 4% of platinum was released under physiological saline condition; in addition, around 50% of platinum was released in 14 days using the rat serum containing 0.1% of polysorbate 80. For this reason, it requires intra-hepatic arterial administration to deliver Miriplatin to hepatic tumors. The studies on this specific formulation also showed that the smaller the particle size, the faster the platinum drug was released.38

SPI-077 was the first liposomal platinum formulation evaluated clinically. Preclinical studies found high levels of platinum were present in tumors. However, due to the low release of the platinum species from the liposomes within the tumor, SPI-077 showed poor clinical efficacy. All together it shows that drug release rates play an important role in the treatment of solid tumors: a) without drug carriers, the free cisplatin therapy would lead to high concentration of the chemical agent in plasma and low accumulated concentration at tumor sites; b) with a drug carrier such as the liposomal formulations with an extremely slow drug release profile, the anti-
cancer therapy may find the drug concentration at the tumor site is below the therapeutic threshold.\textsuperscript{92}

In the present work, a prodrug of cisplatin was prepared using stearic acid. Some clinical studies found that diets high in stearic acid had beneficial effects of lowering LDL cholesterol; thus, stearic acid is healthier than other saturated fatty acids.\textsuperscript{93} Here, it is assumed that the PLN loaded with this prodrug, which has two hydrophobic fatty acid chains, should have a sustained drug release profile under physiological saline condition due to the following three factors:

1) The high affinity between lipid matrix (lipid ester and anionic soybean oil based polymer) and the two hydrophobic fatty acid moieties from the prodrug should reduce the platinum release rate as compared to the previous formulations with cisplatin (Formulation A and B).

2) The nano-particle size of the PLN should result in shorter drug release profile as compared to a micro-particle size formulation of Miriplatin.

3) The ionic interaction between an anionic taurine-based polymer and a cationic prodrug should increase the solubility of the hydrophobic prodrug under physiological conditions; this should lead to a relatively faster drug release profile as compared to Miriplatin.

The reaction route to synthesize this prodrug of cisplatin was quite similar to the reported procedure in preparing Miriplatin.\textsuperscript{94} (Figure 28) The reaction medium was an aqueous solution and excluded silver nitrate; thus, the prodrug will not bring any residue of toxic organic solvents or silver residue to the PLN formulation. The proton NMR of the prodrug showed that the line broadening compared with starting material of stearic acid. This prodrug with platinum was a paramagnetic molecule which can decrease the relaxation time of the protons of the prodrug; thus, it induced the line broadening of these proton signals. (Figure 29) An absorption shift of C=O bonds was observed after the formation of the prodrug with stearate chains. The typical
carbonyl absorption of stearic acid is at around 1700 cm\(^{-1}\), and the absorption moved to two close carbonyl absorptions at 1619 cm\(^{-1}\) and 1594 cm\(^{-1}\) due to the formation of platinum-stearates complex. (Figure 30) Both of the NMR and IR spectra proved the formation of a prodrug of cisplatin.

![Reaction scheme](image)

**Figure 28** Reaction scheme in preparing the prodrug of cisplatin and possible formation of the hydrophobic salt of the prodrug. The preparation of the prodrug was in aqueous medium without toxic metallic ions such as Ag\(^+\).
Figure 29  $^1$H NMR spectra of stearic acid (top) and the prodrug of cisplatin (bottom). Due to the paramagnetic platinum atom, the relaxation time of the protons was decreased and it induced the line broadening of the proton signals of the stearate moiety.

Figure 30  FTIR spectra of stearic acid and prodrug of cisplatin. An absorption shift of C=O bonds occurred which was from 1700 cm$^{-1}$ (stearic acid) to 1616 cm$^{-1}$ and 1594 cm$^{-1}$ (prodrug of cisplatin).
4.4.8 Characterization of the prodrug-loaded PLN formulation (Formulation C)

As shown in Table 4.5, the PLN, which were prepared following formulation C, had the desired nano-size with an average size of 74 nm which was measured two hours after PLN preparation; in addition, the sizes of nanoparticles were quite consistent. The difference between this formulation and the previous two formulations (A and B) was due to the use of the prodrug, which could not undergo the same aqueous equilibrium as cisplatin and would not form the covalent chelation with the anionic polymer. The zeta potential for the PLNs prepared from formulation B and C showed that they were $-55 (+/- 2 \text{ mV})$, which can be compared to the PLNs using HPESO, which had zeta potentials ranging from $-10$ to $-20 \text{ mV}$, the PLNs with the lower zeta potential should have the better stability. Some reports also found that the colloids with high negative zeta potential ranged from $-40$ to $-60 \text{ mV}$ had a much better stability compared to the ones ranging from $0$ to $-30 \text{ mV}$.

\textbf{Table 4.6} A typical particle size and zeta potential of PLN were determined by Malvern Zetasizer Nano ZS (Worcestershire, UK). Particle diameter was the number-weighted diameter of readings averaged over 3 min. Drug loading was the percent of cisplatin converted from the prodrug. The diameter value was the mean (standard deviation) of two measurements.

<table>
<thead>
<tr>
<th>PLN Formulation</th>
<th>Diameter</th>
<th>Zeta Potential</th>
<th>Prodrug Loading</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 mg Prodrug + Taurine-based polymer</td>
<td>74 (1)</td>
<td>-55</td>
<td>8.86</td>
</tr>
</tbody>
</table>

Note: 12 mg of prodrug (Mw: 796.08 dalton) of cisplatin was equal to 4.5 mg of free cisplatin (Mw: 301.1 dalton)
4.4.9 Drug release profile for the PLN loaded with the prodrug (Formulation C)

The drug release study for the PLN loaded with the prodrug (Formulation C) tested the previous hypothesis that the prodrug with stearate chains can provide a sustained drug release profile in physiological saline: a) the drug release of the PLN should be much slower than the PLNs with cisplatin; b) the drug release of this PLN should be faster than the similar microparticle formulation of oxaliplatin, Miriplatin.

In the drug release study for the PLN loaded with the prodrug of cisplatin, the drug release conditions were in physiological saline [20 mM phosphate buffer (pH 7.4) + 150 mM NaCl] or [20 mM phosphate buffer (pH 5.6) + 150 mM NaCl] at 37°C. Different from the formulation A and B, in which all trapped cisplatin was released within 30 min, the drug release profile for this formulation C showed that it had a much slower drug release profile. More than 90% of cisplatin, which was converted from the prodrug of cisplatin, was released after 20 h in the pH 7.4 PBS buffer. (Figure 32) Also, due to the formation of the hydrophobic salt of polymer and mono-aquated form of prodrug during the PLN preparation, the aqueous solubility of the prodrug under physiological condition increased. (Figure 31) This led to the faster drug release of cisplatin compared with Miriplatin formulation, in which only 4% of free cisplatin was released after 3 months in physiological saline solution.
Figure 31  Proposed mechanism for the formation of the hydrophobic salt between the prodrug and the taurine-based polymer.

Figure 32  Drug release kinetics from prodrug-loaded polymer-lipid hybrid nanoparticle formulations. Drug release was measured in 70 mL of pH7.4 PBS solution (20 mM phosphate + 150 mM NaCl) at 37 °C. The platinum content was determined spectroscopically by comparing with a standard absorption curve of blank PBS solutions by an Agilent 8453 UV-Vis spectrophotometer.
4.5 Conclusions

From the in-depth studies on the PLNs loaded with cisplatin (formulations A and B), the following conclusions can be drawn:

1) The interaction between the new polymers, which were the aspartic acid-based polymer and the taurine-based polymers, and cisplatin was via covalent bond chelation.

2) The desired nano-sized particles, whose sizes ranged from 60 nm ~ 150 nm, can be prepared using the polymers, ethyl arachidate (lipid ester) and poly(ethylene oxide)-40-stearate (surfactant).

3) Higher temperature and a neutral condition favor the formation of carboxylate-platinum complex.

4) The drug release study in DDI H₂O showed that a covalent bond between carboxylate group and platinum was stronger than the one between a sulfonate group and a platinum atom.

5) The fast drug release profiles of the PLNs, in which cisplatin was loaded through either covalent bond chelation or physically encapsulation, in physiological saline solution showed that cisplatin was not an excellent payload for this type of PLN.

On the basis of these findings, a prodrug of cisplatin was synthesized as a payload to overcome the hurdle of the fast drug release of cisplatin in physiological saline solution. This prodrug has two long stearate chelation arms which could provide the desired high affinity to the lipid matrix of the PLN. It was presumed that the prodrug would tend to stay in the lipid core and would not easily move to the outer aqueous medium. The in vitro drug release study in saline showed that the release of cisplatin, which was converted from the prodrug of cisplatin, in formulation C was much slower than the previous PLN formulations (formulation A and B) at pH 7.4. Around 95%
of platinum species were slowly released within 24 h. Also, due to an ionic interaction between the cationic prodrug and anionic taurine-based polymer, together with the nano-size of particle (< 100 nm), formulation C can provide a sustained drug release profile which is more complete than another microparticle formulation of a prodrug of oxaliplatin, Miriplatin, in which only around 4% of platinum species was released in 3 months.
Chapter 5

Conclusion and future perspectives

5.1 Summary of findings

In conclusion, this M.Sc. thesis has focused on the preparation of new soybean oil based polymers for use on PLN and the formulation development for the delivery of the anticancer drug of cisplatin or a prodrug of cisplatin. Progress made in this study was as follows:

- The design and synthesis of new soybean oil based polymers (aspartic acid-based polymer, taurine-based polymer and alcohol-based polymer).
- Identification of the structures of the polymers using NMR (\(^1\)H, \(^{13}\)C, COSY and HSQC) and GPC analysis.
- Solubility tests showed that new polymers had better aqueous solubility than HPESO.
- The development of the HPLC/UV methods used to measure the release of encapsulated platinum species.
- The measurement of the cisplatin loading ratios of the polymers by HPLC.
- The development of cisplatin-loaded nanoparticles with the desired nano-sizes.
- The completion of drug release studies under physiological saline and DDI H\(_2\)O conditions using the PLNs loaded with cisplatin and a prodrug of cisplatin.
- The development of a new PLN formulation with a prodrug of cisplatin. The in vitro cytotoxicity showed that this PLN against MDA-MB-231 cancer cells was four-fold more potent than free cisplatin (Appendix 1). (Summary of this PLN formulation see Table 5.1)
Table 5.1  Summary of the PLN loaded with the prodrug of cisplatin (Formulation C)

<table>
<thead>
<tr>
<th>PLN Formulation (Formulation C)</th>
<th>Diameter (nm)</th>
<th>Zeta Potential (mV)</th>
<th>Encapsulation efficiency (%)</th>
<th>Prodrug Loading (w/w%)</th>
<th>Drug release in saline 95% released</th>
<th>Drug release in saline 24 h (pH 7.4)</th>
<th>MTT assay IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prodrug + lipid ester + taurine-based Polymer</td>
<td>74 (±1)</td>
<td>-55</td>
<td>95 (± 3)</td>
<td>8.86</td>
<td>24 h (pH 7.4)</td>
<td>16 µM</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a,b</sup>: The experiments of encapsulation efficiency and MTT assay were conducted by Lucy Lin and Jason Li.

5.2  Suggested future work

This work has resulted in a promising platinum drug formulation for cancer therapy. As a next step to further develop this novel formulation, the following areas should be focused on as well:

1) Increase drug loading of the prodrug.

Since the prodrug of cisplatin was compatible with the lipid matrix, which included the soybean based polymer and the lipid ester, it is assumed that the drug loading percentage can be increased as well. Therefore, the influence of process parameters such as the ratio of polymer/surfactant/lipid ester and the conditions of sonication should be investigated to ensure the desired nanoparticle sizes and good stability can be obtained.

2) Study the effect of PLN loaded with cisplatin prodrug on different cisplatin resistant tumor cell lines.

Cellular uptake kinetics showed that the novel PLN (formulation C) can be delivered to the perinuclear area of MDA-MB 231 cancer cells via an endocytosis mechanism. This allows the PLN to overcome multiple types of membrane efflux pumps and deliver anti-cancer drugs to the cytoplasm of cancer cells. Thus, other cisplatin resistant tumor cell lines (e.g. androgen-sensitive human prostate adenocarcinoma LNCaP cell, OVCAR-3
ovarian cancer, and colon cancer cell lines LY294005) should be screened as well to
determine if this PLN is also potent against them.

3) Evaluate the possible synergistic effects of other anti-cancer drug(s) (e.g. doxorubicin,
paclitaxel) in multidrug resistance cancer cell lines.

The PLN formulation C contained the taurine-based polymer which possesses strong
anionic sulfonate groups. It can also form ionic interactions with other cationic drugs
molecules such as doxorubicin. Thus, drugs co-loaded PLN may have synergistic effects
on multidrug resistance cancer cell lines.

4) Evaluate other anionic polymers for the PLN formulation with the cisplatin prodrug.

So far, only the taurine-based polymer in formulation C was investigated due to the
timeline of this study. This anionic polymer has two key functions in this PLN
formulation. Firstly, it formed ionic bonds with cationic prodrugs; thus, the hydrophobic
salt can increase the aqueous solubility of the prodrug. Secondly, similar to anionic lipid
DPPG in the Lipoplatin formulation, which provides unique fusogenic properties and
assists the direct fusion between the particle and the cell membrane, it is hypothesized
that this newly synthesized anionic polymer can also assist PLN to enter cancer cells via
an endocytosis mechanism. Thus, other anionic polymers such as the aspartic acid-based
polymer, the alcohol-based polymer or the other novel anionic polymers prepared in this
lab can be tested in this novel formulation (formulation C).

5) Other biological and stability studies.

Other biological and stability studies are currently underway by other coworkers in Dr.
Wu’s lab as follows:

a) Clonogenic assays for drug toxicity with MDA-MB-231 cells; b) colloidal stability of
NPs as a function of time: incubation of PLN in culture medium and plasma at 4 °C and
37 °C. Measurement of particle size and zeta potential for up to 7 days. c) *In vivo* bio-distribution. Use breast tumor bearing mice for time-course bio-distribution (whole body and organs) studies.

Currently, liver cancer has tripled in men and doubled in women since the 1970s in Canada. It is the only cancer in Canada for which mortality is increasing.\(^9\) In addition, due to widespread distributions of chronic hepatitis B and C, which are two infectious diseases of the liver, in East Asian countries, liver cancer is one of the fastest-rising forms of cancer in this region. Since the successful application of a similar formulation (Miriplatin) for the treatment of hepatocellular carcinoma, formulation C developed in this lab, might also be used to target liver cancer. It is expected that PLN with a prodrug of cisplatin might be given i.v and avoid the need for direct injection through hepatic artery used for Miriplatin, which has a very long drug release profile under physiological saline conditions.
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Appendix. Evaluation of Cytotoxicity and Cellular Uptake of a Cisplatin PLN Formulation

Abstract

*In vitro* cytotoxicity studies found that both polymers (aspartic acid-based polymer and taurine-based polymer) were nontoxic to human breast cancer cells MDA-MB-231. Moreover, *in vitro* toxicity studies noted that the formulation with the taurine-based polymer (formulation B) was slightly more potent compared to free cisplatin. The *in vitro* toxicity of the PLN formulation (formulation C) against MDA-MB-231 cancer cells was found to be four-fold more potent than free cisplatin. The cellular uptake kinetics indicated that the uptake of PLN was through an endocytosis mechanism, which may overcome multiple types of membrane efflux pumps and deliver anti-cancer platinum species to the cytoplasm of drug resistant cancer cells.

1. Methods

1.1 Cytotoxicity determined by MTT assay

MTT assays were conducted to evaluate the relative effectiveness of cisplatin free drug and nanoparticle formulation in the inhibition of human cancer cell proliferation. Triple negative human breast cancer cells MDA-MB-231 were seeded at a density of 10,000 cells per well of 96-well plate with complete medium (a-MEM + 5% FBS) (Princess Margaret Hospital Media Lab,
Toronto, ON, Canada) 24 h prior to treatment. Cells were treated with drug (e.g. free cisplatin, or platinum drug-loaded PLNs) for 24 h and then drug was removed by washing with PBS three times. MTT reagent (Sigma-Aldrich, Inc. Oakville, ON) dissolved in complete medium (0.5 mg/mL) was added and incubated with cells for 4 h at 37 °C. 10% SDS-HCl solution was added to dissolve the insoluble formazan overnight and absorbance was read at 570 nm. Percent survival was determined based on a no drug control.

1.2 Cellular uptake

Seven thousand MDA-MB 231 cells were seeded in 96-well plates and incubated for 24 h. After the incubation, cell nuclei were stained with Hoechst 3342 for 20 min and rinsed with culture medium. Cells were then incubated with the prodrug of cisplatin loaded nanoparticles (equivalent to 0.66µg/mL of cisplatin solution, 3µL per well) for 0.5 – 60 min. Prior to the fluorescent imaging, cells were rinsed 3 × with PBS solution. The fluorescent images were obtained using the DAPI and RFP filter set to image Hoechst-stained cell nuclei (blue) and the fluorescent labeled- prodrug-loaded PLN (red). The fluorescence intensities were analyzed using ImageJ.

1.3 Transmission electron micrographs (TEM)

Transmission electron micrographs were acquired on a Hitachi H7000 electron microscope (Hitachi Canada, Ltd., Mississauga, ON, Canada) following drying and phosphotungstic acid staining of the particle suspension.
2. Results and discussion

2.1 Cytotoxicity of PLN loaded with cisplatin

The MTT assay can elucidate if the polymers and blank PLNs are toxic to cells; furthermore, it can indicate if PLN loaded with drug are more potent than free cisplatin solution against cancer cells. The in vitro cytotoxicity results showed that both the polymers (aspartic acid-based polymer and taurine-based polymer) were nontoxic towards MBA-231 cells (Figure A) and the blank PLN with the taurine-based polymer (formulation B) showed no or only minimal toxicity except at the highest concentration. Also, this PLN formulation against human breast cancer cells MDA-MB-231 was slightly more potent as compared to free cisplatin. The medium used for the in vitro cytotoxicity was a culture medium (a-MEM + 5% FBS), in which the chloride concentration was 123 mM; thus, the drug release from the PLN should be similar to the drug release studies under pH 7.4 PBS saline condition. Even the cisplatin release was fast under this condition, due to the quick PLN internalization which was discussed in section 2.2. It was expected that some PLN entered the cells and this resulted in slightly better potency as compared to free cisplatin. As discussed in Chapter 1, the cellular uptake of the PLN with HPESO was via an endocytosis mechanism, which can overcome multiple types of membrane efflux pumps and deliver the anti-cancer drugs to cytoplasm of cancer cells. The images of microscopic fluorescence showed that the fluorescently labeled PLNs were mainly located in the perinuclear region of the cell lines. It was assumed that the soybean oil based polymers, which have long fatty acid chains and anionic groups (e.g. carboxylic acids and sulfonic acid),
would play an important role in the endocytosis mechanism. A similar mechanism was also described for Lipoplatin™, which is a liposomal formulation of cisplatin. The anionic lipid DPPG within this formulation provides the unique fusogenic properties, which assist a direct fusion between cancer cell membrane and Lipoplatin after drug carriers reach target tumor tissues.\textsuperscript{9}

In some MTT assays, the blank PLNs, in which Et\textsubscript{3}N was used to dissolve either the aspartic acid-based polymer or the taurine-based polymer, showed some cytotoxicity to human breast cancer cells MDA-MB-468. Further investigation will be conducted to investigate if this cytotoxicity could be attributed to trace amounts of Et\textsubscript{3}N. The Et\textsubscript{3}N within the PLNs (formulation B), which were used for the MTT assays described above, was replaced by (bis(2-hydroxyethyl)-amo\textsubscript{no-tris(hydroxymethyl)-methane) (bis-tris). In general, bis-tris is used as a buffering reagent in biochemistry tests and is nontoxic to cell lines. Its structure is quite similar to Et\textsubscript{3}N and can also act as a counter base to increase aqueous solubility of the soybean oil based polymers. Thus, it was expected that the bis-tris in the PLN (formulation B) did not induce any cytotoxicity to the cancer cells MDA-MB-468.
**Figure A**  An *in vitro* cytotoxicity assessment of the polymers against human breast cancer cells MDA-MB-231 (Depositor: Cailleau; Organism: Human; Ethnicity: Caucasian; Age/Stage: 51 years of age; Gender: female; Tissue: Breast; mammary gland (pleural effusion); Celltype: epithelial (adenocarcinoma); Growth Properties: monolayer. Error bars represent the standard error of the mean (SEM) for N = 2 independent experiments.

**Figure B**  An *in vitro* cytotoxicity assessment of the blank PLN, free cisplatin and the taurine-based PLN loaded with cisplatin against human breast cancer cells MDA-MB-231. Error bars represent the SEM for N = 2 independent experiments.
2.2 Cytotoxicity and cellular uptake of PLN loaded with the prodrug of cisplatin

The MTT assays for the PLNs loaded with prodrug of cisplatin can elucidate if the PLN formulation against MDR cancer cells would be more potent than the previous PLN loaded with cisplatin. The cellular uptake kinetics study can determine if the PLN had the same uptake kinetics as the previous PLN which contained HPESO, doxorubicin and mitomycin C. The MTT assay showed that the IC$_{50}$ of prodrug-loaded PLNs was 16 µM, which was four-fold more potent against MDA-MB 231 cells than free cisplatin (IC$_{50}$ = 63 µM). (Figure C a) The typical fluorescence microscopy images indicated that the cellular uptake kinetics of the novel PLN appeared to be endocytosis mediated, and most PLN were delivered to the perinuclear region of the cancer cells within 60 min. (Figure C b and Figure D) As a result, this PLN can bypass the efflux pumps of free cisplatin (e.g. copper efflux transporters, ATP7A and ATP7B) and avoid platinum (II) complex formation with intracellular substrates such as glutathione and methionine-containing proteins/peptides. This is very likely to be at least part of the explanations of why the new PLN were more potent than free cisplatin.
Figure C  a) Relative toxicity of MDA-MB-231 cells by MTT assay. Cells were treated with prodrug loaded SLN and free drug. IC$_{50}$ prodrug-NP = 16 µM, IC$_{50}$ free cisplatin = 63 µM). Error bars represent the SEM for N = 2 independent experiments for prodrug-loaded NP and the SEM for N = 3 independent experiments for prodrug-loaded NP.
b) Intracellular localization of fluorescent PLN in breast cancer cell lines MDA-MB-231. Cells were incubated with nile red-loaded PLNs (red) and visualized with a red fluorescence protein (RFP) filter (PLNs). Nuclei were stained with Hoescht 3342 (blue) and visualized with a DAPI filter (Nucleus) and PLN/Nucleus images overlayed (Merge). Images were acquired with a 20× objective lens.
Figure D  Uptake of the prodrug-loaded PLN in MDA-MB-231 cells. Treatment times varied between 0.5 and 60 min at 37 °C, 5% CO₂. Cell nuclei stained in blue, nanoparticles shown in red. Error bars represent the SEM for N = 3 independent experiments.

2.3 Transmission electron micrograph (TEM)

TEM showed the shape of the particles was nearly spherical, and the size ranged from 67 nm to 160 nm.

Figure E  TEM examination of PLN using the taurine-based polymer. Transmission electron micrographs were acquired on a Hitachi H7000 electron microscope following the drying and phosphotungstic acid staining of the particle suspension. The PLN particles were spherical and their diameters were determined by TEM at the Microscopy Imaging Laboratory (MIL) at the University of Toronto. The nano-particles had an average particle size of about 112 nm.