On the use of liposomal delivery systems for oxaliplatin and in dual drug delivery in combination with chemo-sensitizing and chemotherapeutic agents

Ayat Zein-elabedeen Mohamed Ibrahim

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School of Science and Engineering

On the use of liposomal delivery systems for oxaliplatin and in dual drug delivery in combination with chemo-sensitizing and chemotherapeutic agents

A Thesis Submitted to

The Chemistry Graduate Program

In partial fulfillment of the requirements for the degree of Master of Science

By Ayat Zein-elabedeen Mohamed Ibrahim

Under the supervision of Dr. Tamer Shoeib

Spring 2016
TO MY DEAR PARENTS
Acknowledgments

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ABSTRACT

The American University in Cairo

On the use of liposomal delivery systems for oxaliplatin and in dual drug delivery in combination with chemo-sensitizing and chemotherapeutic agents

BY: Ayat Zein-elabedeen Mohamed Ibrahim

Under the supervision of Dr. Tamer Shoeib

Cancer is one of the rapidly growing leading causes of death worldwide where combinational chemotherapy is the most used strategy to control it. Drug delivery constitutes a major segment in chemotherapeutics including liposomes as a drug delivery system enabling the encapsulation of both hydrophilic and hydrophobic drugs. Recently, liposomes have been recognized as an efficient means for drug delivery of combinational chemotherapy. The aims of this study were to prepare stable liposomal formulations with particle sizes of less than 200 nm and surface negative potentials that offer high encapsulation efficiency for oxaliplatin. Subsequently, the use of the developed liposomal system in dual drug loading was investigated using two approaches, oxaliplatin and ascorbic acid as a chemo-sensitizing agent (LP-Ox-AA), and oxaliplatin combined with satraplatin as a chemotherapeutic agent (LP-Ox-Stp). In addition, the effect of dual drug loading on liposome characterization, in-vitro release profile and cytotoxic efficiency were investigated. In this study, all prepared liposomal formulations were highly stable for at least 6 month at 4°C maintaining its size unchanged associated with minimal fluctuation in particles surface charge. The prepared liposomal formulations (LP-Ox) and its dual drug loaded analogues were compared to un-encapsulated oxaliplatin and the commercial liposomal formulation of oxaliplatin, Lipoxal™. The encapsulation efficiency (EE%) of oxaliplatin in LP-Ox was found to be 23.7% with a particle size of 149.5 nm compared to 54% in Lipoxal encapsulated in liposome particles with a diameter of 118.5 nm. Both LP-Ox and Lipoxal offered higher cytotoxicity in cancer cells relative to free un-encapsulated oxaliplatin. The EE% in LP-Ox-AA was shown to be 28.5% for oxaliplatin and 97.8% for ascorbic acid. The additional loading of ascorbic acid was found to enhance the EE% for oxaliplatin, the stability of ascorbic acid for up to 6 month when stored at 4°C, as well as the cytotoxicity profile in cancer cell lines. While in LP-Ox-Stp the EE% for oxaliplatin was reduced to 16.8 %, and 49.3 % for satraplatin.
The additional loading of satraplatin was shown to have a negative influence on the EE% of oxaliplatin, while it enhanced the retention of oxaliplatin under simulated physiological conditions. The LP-Ox-Stp showed the most efficient controlled release profile compared to other formulations, mainly due to the incorporation of hydrophobic satraplatin within the lipid bilayer. In addition, LP-Ox-Stp was the most potent in DNA damage induction. It was shown that liposomes can act as successful drug delivery systems for single and combinational chemotherapy enhancing the cytotoxic efficiency and minimizing the associated toxicity of the delivered drugs.
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<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ataxia telangiectasia mutated and Rad3-related</td>
</tr>
<tr>
<td>CTR1</td>
<td>copper transporter 1</td>
</tr>
<tr>
<td>DACH</td>
<td>1,2-diaminocyclohexane group</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPPC</td>
<td>dipalmitoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DPPE</td>
<td>dipalmitoyl phosphatidylethanolamine</td>
</tr>
<tr>
<td>DPPG</td>
<td>dipalmitoyl phosphatidylglycerol</td>
</tr>
<tr>
<td>DSB</td>
<td>double stranded DNA breaks</td>
</tr>
<tr>
<td>DSPC</td>
<td>Distearoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DSPE</td>
<td>Distearoyl phosphoethanolamine</td>
</tr>
<tr>
<td>DSPE-PEG 2000</td>
<td>Distearoyl phosphatidylethanolamine-polyethylene glycol 2000</td>
</tr>
<tr>
<td>DSPG</td>
<td>Distearoyl phosphatidylglycerol</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin-3-gallate</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>HII</td>
<td>Hexagonal phase</td>
</tr>
<tr>
<td>IC50</td>
<td>The half maximal inhibitory concentration</td>
</tr>
<tr>
<td>LC</td>
<td>Lipid concentration</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicles</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen- activated protein kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drug resistance</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MPS</td>
<td>Mononuclear phagocytic system</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>MVV</td>
<td>Multivesicular vesicles</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>OCT</td>
<td>Organic cation transporters</td>
</tr>
<tr>
<td>Oxpt</td>
<td>Oxaliplatin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>pKa</td>
<td>Acid dissociation constant</td>
</tr>
<tr>
<td>PP</td>
<td>Pellet permeabilization</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>R²</td>
<td>Coefficient of determination</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>SSB</td>
<td>Single stranded DNA breaks</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>UC</td>
<td>Ultracentrifugation</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>ζ potential</td>
<td>Zeta potential</td>
</tr>
</tbody>
</table>
1. Introduction

Cancer is reported by the world health organization (WHO) to be one of the rapidly growing leading causes of death worldwide, with an estimate of 8.2 million cancer-related deaths, and around 14.1 million new cancer cases in 2012, compared with 12.7 million new case in 2008 (Globocan 2012) [1]–[3]. Progression of cancer can be controlled using several interventions such as, surgery, radiation, immunotherapy, suicide gene therapy, and chemotherapy; where most of these interventions induce their anticancer effect by inhibiting cancer cell proliferation, that might lead to senescence or activation of cell death pathways through apoptosis, necrosis, and mitotic catastrophe in tumor cells [4]–[6]. Chemotherapeutic agents are used widely post-surgery and radiotherapy as an adjuvant therapy to eradicate residual cancer cells, also used as a palliative treatment where it aids in reducing tumor size, or for complete cure of cancer [7].

1.1 Platinum-based complexes

Platinum-based complexes are among the most active broad spectrum chemotherapeutic agents, justifying their extensive usage in treatment in an estimate of 50-70% of all patients with cancer [8]–[10]. The first generation platinum complex cisplatin, cis-diammine(dichloro)platinum(II), (Figure 1-1 (a)) is a square planar, Pt(II) complex, synthesized in 1844 by Michele Peyrone; however, its cytotoxic properties were discovered coincidentally by Barnett Rosenberg in 1965 [11]–[14]. Studies have proven cisplatin to be a successful anticancer drug, capable of curing 90% of cases diagnosed with metastatic testicular germ-cell cancer [13], [14]. In addition, cisplatin expressed a significant therapeutic efficiency in a broad spectrum of solid tumors, including ovarian, bladder, lung, head and neck, esophageal, cervical, and uterine cancers [13], [15], [16]. Consequently, cisplatin was granted the US Food and Drug Administration (FDA) approval in 1978 [12]–[15]. However, the administration of cisplatin has been limited by severe toxic adverse reactions, such as nephrotoxicity, peripheral neuropathy (neurotoxicity), ototoxicity, and emetogenesis [8], [10]–[19]. Another limitation for cisplatin therapy is intrinsic resistance exhibited by various cancer cells, and acquired resistance developed during repeated treatment courses [8], [10], [13], [15], [17]–[19]. This has triggered the development of other platinum complexes in order to overcome the drawbacks of cisplatin [18], [19].
Carboplatin, cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II), (Figure 1-1 (b)) was developed as a second generation platinum complex [11], [13]–[15]. Carboplatin is considered to be a safer alternative to cisplatin affording a more tolerable toxicological profile in terms of ototoxicity and neurotoxicity, as well as diminished nephrotoxicity and emetogenesis [11]–[15], [17], [19], [20]. However, carboplatin usage is limited by the development of severe myelosuppression and thrombocytopenia at high doses [13]. In addition, the resulting similarity in chemical structure between carboplatin and cisplatin did not broaden its spectrum of activity, particularly in cisplatin-resistant tumors [13]–[15], [17], [19], [20]. This complete cross-resistance of carboplatin in cisplatin-resistant tumors renders it ineffective in treatment of irresponsive cases to cisplatin therapy [13], [14], [17], [19]. This has led to the development of the third generation platinum complexes with an altered chemical structure, where a cyclohexane ring is attached to the nitrogen atoms forming a 1,2-diaminocyclohexane group (DACH) [13], [16]–[18], [21]–[23]. The revolutionary third generation member oxaliplatin, (trans-R,R-1,2-diaminocyclohexane)oxalatoplatinum(II), (Figure 1-1 (c)) was found to overcome the issue of cross-resistance with both cisplatin and carboplatin, thus broadening the spectrum of activity of platinum-based complexes; where oxaliplatin was found to be cytotoxic to colorectal cancer, an intrinsically cisplatin-resistant type of cancer [11], [13]–[15], [17], [19], [20], [22]. However, oxaliplatin has a dose limiting neurotoxicity i.e. peripheral sensory neuropathy [11], [13], [14], [16], [17], [21]. Cisplatin, carboplatin, and oxaliplatin are the only platinum-based chemotherapeutic agents with US FDA approval for clinical use in cancer treatment [8]–[11], [14], [17]–[19]. The following sections justify the difference in potency and efficacy between the three clinically approved platinum-based complexes, and the ability of oxaliplatin to exert its cytotoxic activity in cis- carboplatin resistant tumors.
1.1.1 Mode of Cytotoxicity

A. Bioactivation

The structure of platinum-based complexes is composed of a “leaving group” (X) and a “carrier ligand” (Y) attached to the platinum center, forming \([X \text{Pt}Y]_2\) complex [13], [14], [21]. The differences observed in potency and reactivity between the three generations of platinum-based complexes is attributed to having different leaving groups. As shown in table 1-1 both cisplatin and carboplatin have diamine as carrier ligands, yet differ in their leaving groups, these being dichloride, and bidentate cyclobutane dicarboxylate for cisplatin and carboplatin respectively; resulting in a reduced reactivity of carboplatin, and thus lower toxicity profile [14], [17]. On the contrary, oxaliplatin has a relatively more inert oxalate leaving group, and a lipophilic DACH carrier ligand [11], [14]. The aquation of the leaving group is the rate limiting step in the drug bioactivation leading to cytotoxic activity exerted by platinum-based complexes, producing the active forms of platinum-based complexes mono-aqua \([(X)(\text{H}_2\text{O})\text{Pt}(Y)]^+\) and di-aqua species \([(\text{H}_2\text{O})_2\text{Pt}(Y)]^{2+}\) [18].

Table 1-1 Platinum-based complexes leaving groups and carrier ligands

<table>
<thead>
<tr>
<th>Platinum-based complex</th>
<th>Leaving group (X)</th>
<th>Carrier ligand (Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>2 Chlorides</td>
<td>2 Amine groups</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>Cyclobutane dicarboxylate</td>
<td>2 Amine groups</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>Oxalate group</td>
<td>DACH</td>
</tr>
</tbody>
</table>

i) Extracellular fluids

Extracellular fluids are characterized by a high chloride ion concentration (100 mM) [14], [15], [23]. This influences the bioactivation of platinum-based complexes differently depending on the nature and reactivity of the leaving groups. In the case of cisplatin, having chlorides as the leaving groups, the high chloride ion concentration in extracellular fluids suppresses its hydrolytic activation [13]–[15]. Carboplatin has a slower rate of hydrolytic activation due to the low reactivity of the cyclobutane dicarboxylate leaving group [24]. On the other hand, oxaliplatin is transformed into other compounds in extracellular fluids, most of which are
pharmacologically inactive with the exception of dichloro-(DACH) platinum complexes showing higher cytotoxic activity than oxaliplatin [13], [21]. Moreover, it was found that both cisplatin and oxaliplatin have a high tendency to bind to plasma proteins and red blood cells (RBCs), reducing the pharmacological activity of these drugs [13], [14]. As for carboplatin, it exists in its unbound form in plasma having the least tendency to bind plasma proteins due to its low reactivity [24].

**ii) Intracellular fluids**

Intracellular fluids have a significantly lower chloride ion concentration (4-23 mM), and do not suppress the hydrolytic activation of cisplatin thus allowing for the generation of the active aqua species intracellularly [9], [13]–[15]. Whereas in the case of carboplatin this activation reaction occurs at a significantly slower rate, where bioactivation involves the displacement of the bidentate cyclobutane dicarboxylate group [24]. For oxaliplatin, the low intracellular chloride ion concentration results in the aquation of the dichloro-(DACH) platinum complex into mono- and di-aqua species [15], [21], [23]. The highly reactive aqua complexes are capable of interacting with biomolecules [13], [14].

**B. Pt-based complexes intracellular interaction**

All anticancer platinum-based complexes are believed to exert their cytotoxic activity through the interaction of their activated species with deoxyribonucleic acid (DNA) forming Pt-DNA adducts, refer to figure 1-2 [9], [11], [13]–[16], [21]. The active aqua species are highly reactive towards biomolecules containing oxygen in the form of carbonyl or hydroxide groups, nitrogen from amino acids and nucleic acids in the form of amine groups or N-heterocycles, and sulfur in the form of thioether or thiol as in methionine and cysteine amino acids [11], [13]–[15], [23]. However, interaction with nitrogen containing biomolecules tend to form the most thermodynamically stable adduct, therefore the ultimate target for platinum-based complexes would be the electron rich N7-position of the guanosine nucleotide within the DNA helix [11], [13]–[16], [18], [21]. There are several forms of Platinum-DNA adducts, that could be either mono-functional \[(H_2O)(DNA)Pt(Y)_2]^+ showing an insignificant cytotoxic activity, or in the form of the significantly cytotoxic bi-functional adduct \[(DNA)Pt(Y)_2]^2^2 [15], [21]. The bi-functional DNA-Pt adducts could involve the crosslinking of two adjacent guanosine nucleotide
bases on the same DNA strand i.e. 1,2 Pt-d(GpG) intrastrand adduct; exhibited by 60% of total platinum-DNA adducts [11], [13]–[16], [22]. While 30% of total platinum-DNA adducts are in the form of crosslinked adjacent adenosine and guanosine nucleotide bases forming 1,2 Pt-d(ApG) intrastrand adduct [11], [13], [16], [22]. Other less frequent forms of Pt-DNA bifunctional adducts include, interstrand adducts being the crosslinking of two nucleotide bases on complementary DNA strands; and DNA-biomolecule crosslinks such as, protein and RNA [11], [13], [15], [16], [21]. The 1,2-intrastrand Pt-DNA adducts results in structural modifications within the DNA double helix; thus blocking DNA replication and transcription ultimately leading to induction of apoptosis [9], [11], [14], [15], [17], [21], [22]. Cisplatin, carboplatin, and oxaliplatin vary in their cytotoxic efficacies and toxicological profiles. Although oxaliplatin is associated with the formation of the same type of Pt-DNA adducts produced by cisplatin; oxaliplatin is found to be more cytotoxic than cisplatin and carboplatin [11], [17], [18], [21], [23].

![Figure 1-2 Simplified scheme for the reaction of oxaliplatin with DNA](image)

**1.1.2 Mechanisms of resistance**

The clinical efficacy of anticancer platinum-based complexes is limited by inherent or acquired resistance [14], [20], [26]. There are several mechanisms involved in aggravating the development of resistance such as, decreased intracellular accumulation of the drug either through enhanced efflux, reduced influx, increased detoxification of the drug by thiol containing biomolecules, stimulation of DNA repair resulting in excision of the DNA regions involved in Pt-DNA adducts, modulation of downstream signaling pathways, and defective apoptosis [11], [13]–[15], [17], [18], [21], [27]. Cisplatin and carboplatin both are susceptible to the same mechanisms of resistance resulting in cross-resistance, while the different structure of oxaliplatin
renders it capable of overcoming cross-resistance exhibited by cis- and carboplatin through bypassing the mechanisms of resistance described in this section [14], [17].

A. Intracellular accumulation

The reduction in intracellular accumulation of the platinum-based complexes is thought to have a significant influence on the development of resistance [26]. The cellular influx of platinum-based complexes is initially mediated by passive diffusion, followed by cell-line specific facilitated and active transport [26]. There is a hypothesis that cisplatin, carboplatin, and oxaliplatin accumulate within the cells mainly by passive diffusion, while the activated aqua species influx is achieved through active transport with the aid of copper transporter 1 (CTR1) [13]–[15]. [26]. However, exposure of the cell to platinum-based complexes results in the down-regulation of CTR1, thus limiting intracellular accumulation of the platinum-based complexes to passive diffusion only [26], [28]–[30]. Homozygous CTR1 mutant mice showed up to 70% reduction in cisplatin cellular influx[31]. Unlike cisplatin and carboplatin, oxaliplatin happens to be a substrate for organic cation transporters 1 and 2 (OCT) which is another active transport system, due to the organic nature of the cyclohexane function in DACH carrier ligand, thus the intracellular accumulation of oxaliplatin is not markedly affected by CTR1 down-regulation [17], [19], [26], [32].

B. Impaired DNA repair

Pt-DNA adducts can be repaired by several cellular processes concerned with repairing DNA damaged sequences [11]. The nucleotide excision repair (NER) process involves the excision of the damaged DNA sequence ~ 30 nucleotide bases; to be replaced with a repaired DNA sequence aided by DNA polymerases δ, and ε, refer to figure 1-3 [11], [15]. NER is capable of efficiently removing cisplatin, carboplatin, and oxaliplatin DNA adducts, thus aids in decreasing sensitivity towards these drugs [11], [14], [17]. This is in addition to the mismatch repair (MMR) process that tends to amend base mispairs and small strand loops that occur during DNA replication, as illustrated in figure 1-3 [11]. However, the MMR process is not as efficient in the removal of oxaliplatin-DNA adducts as compared to those of cis- and carboplatin, due to its unique conformational distortion, where the bulky DACH carrier ligand prevents the binding of the MMR protein complex by steric hindrance [11], [14], [16], [17], [20], [21], [23]. Therefore,
oxaliplatin retains activity in cis- and carboplatin-resistant tumors due to the formation of a relatively irreversible Pt-DNA adduct [11], [14], [17], [20], [21], [23]. However, in the case of oxaliplatin the NER is not capable of compensating for the defect in the MMR process. This is mainly attributed to the ability of key polymerases involved in translesion synthesis in bypassing oxaliplatin-GG adducts more efficiently than cisplatin-GG adducts [33].

Figure 1-3 Various DNA repair pathways

C. Intracellular detoxification

The activated aqua species of platinum complexes are susceptible to interactions with other non-DNA ligands such as, cytosolic and nucleic biomolecules; which may result in detoxification of the drug, increased side effects and chemo-resistance reducing the cytotoxic activity of the drug [15], [16], [23]. In addition, these non-DNA ligands interactions might be associated in the enhancement of the cytotoxic activity of the drug [15], [16], [23]. These are partially attributed to
the interaction of the activated aqua species with cytosolic proteins/peptides, RNA and lipids [9], [11], [17]. In this context, oxaliplatin could interact with enzymes and protein complexes responsible for the DNA repair process, which can concomitantly hinder the production of new proteins by preventing transcription of the damaged DNA, thus leading to the saturation of the cellular capacity to repair Pt-DNA adducts and eventually enhance oxaliplatin cytotoxic activity [11]. In addition, thiol containing biomolecules, such as reduced glutathione (GSH) and metallothionein, act as cytoprotective complexing agents for platinum-based complexes, leading to their detoxification [15]. It is thus interesting to note that prolonged exposure to platinum-based complexes was reported to result in an increase in GSH intracellular concentration [27].

1.1.3 Novel platinum-based complexes

Novel platinum-based complexes are developed in order to broaden the spectrum of anticancer activity including cisplatin-resistant tumors, and enhance the toxicological profile of current platinum-based complexes [10], [13], [14], [17]. To selectively accumulate drug in tumor tissues, the following approaches are typically employed to prolong the drug half-life in the blood stream, to target the accumulation of the drug within the tumor cells either passively or actively, to intracellularly activate the drug or release the drug within the tumor cells, and to decrease the drug interaction with non-DNA ligands [13], [14]. To accomplish these goals three main routes are followed: (i) synthesis of platinum-based complexes with altered structure activity relationship such as, multinuclear structures, (ii) development of prodrugs for current platinum-based complexes, and (iii) utilization of drug delivery systems [8], [10]. However, the majority of newly synthesized forms of platinum-based complexes are associated with a high toxicity profile limiting their usage in anticancer therapy [10]. Thus, the main approach of recent research is the improvement of current platinum-based complexes.

A. Platinum (IV) prodrug

Platinum (IV) complexes are considered as prodrugs of platinum (II) complexes, with two axial anionic leaving groups (Z) in addition to the equatorial leaving groups (X) and carrier ligand (Y) attached to a platinum cation center forming an octahedral structure i.e. [(X)2 (Z)2 Pt (Y)2] [13], [14]. The axial leaving groups render the platinum(IV)-based complex relatively inert compared to platinum (II)-based complexes, resulting in a reduced toxicity profile, reduced cytotoxic
activity, and increased kinetic stability [10], [12], [13]. Upon administration, the relatively inert platinum (IV)-based complexes circulate around the body, where they lose their axial leaving groups by reduction within hypoxic tumor cells to form the substantially more active cytotoxic platinum (II)-based complexes [8], [10], [12]–[14]. The first orally active platinum (IV)-based complex, currently in phase III clinical trials is satraplatin (JM-216), bis-(acetato)-amminedichloro(cyclohexylamine)-platinum(IV), (Figure 1-4) having two acetate groups as the axial ligand, and a cyclohexylamine as one of the carrier ligands; thus increasing the drug lipophilicity [8], [10], [13], [17], [18], [20]. In addition, the kinetic inertness of platinum(IV)-based complexes, and their stability in gastrointestinal tract support the oral administration of satraplatin [8], [13], [18], [20]. Satraplatin demonstrates a similar cytotoxic activity to carboplatin and a lower toxicity profile, specifically lower nephrotoxicity, neurotoxicity, and ototoxicity [10], [13]. Satraplatin was found to be active in hormone-refractory prostate cancer, and in cisplatin-resistant tumors. It is suggested that satraplatin retains cytotoxic activity in platinum resistance resulting from decreased intracellular accumulation [8], [10], [17], [20]. However, satraplatin tends to biotransform rapidly in human red blood cells, generating cis-amminedichloro(cyclohexylamine)platinum(II) complex [(Cl)₂ Pt (NH₃) (cyclohexylamine)] as the major metabolite [13], [18]. Thus, satraplatin administration is associated with the dose limiting side effects thrombocytopenia, and leukopenia [13].

![Chemical structure of satraplatin](image)

**Figure 1-4 Chemical structure of satraplatin**

**B. Drug delivery**

Drug delivery systems constitutes a major segment of drug design, with the objective of limiting side effects and improving drug efficacy [10], [17], [34]–[36]. The use of nanotechnology in designing nanoparticle delivery systems, sized 1-100 nm, had a significant impact on the efficiency of drug delivery; where they efficiently target tumors, and protect the encapsulated drug from plasma proteins and other non-DNA ligands [17], [34]–[37]. There are several types
of nanoscale delivery systems including liposomes, polymer-based micelles, dendrimers, niosomes, and nanotubes [10], [14], [17], [34], [37]. To the best of our knowledge liposomes are the most extensively studied delivery systems with twelve commercially available parenteral formulations; thus entitled as the encapsulation system with utmost capabilities [34], [38], [39]. Platinum-based complexes have three drug delivery systems currently in clinical trials, two liposomal formulations LipoPlatin™, and LipOxal™ for cisplatin and oxaliplatin respectively; and ProLindac an oxaliplatin loaded to polymer carrier [8], [14], [17]. Compared to polymer-based conjugates, liposomes arose as an evident delivery system for platinum-based complexes offering higher encapsulation capacity, reduced toxicity profile, and more efficient protection from plasma protein interactions and enzymatic degradation [14], [34]. Lipoxal was reported to minimize the toxicological profile of oxaliplatin even at high doses with 300 mg/m² as the maximum tolerated dose (MTD) relative to un-encapsulated oxaliplatin having a toxicity limited dose of 20 – 180 mg/m² [16], [17], [21], [40], [41].

1.2 Liposome

Liposomes are the first discovered nanoscale drug delivery systems [37], [39]. Amphiphiles constitute the building structure of liposomes, as a result of their characteristic structure possessing a hydrophilic head and a hydrophobic tail, and with the aid of hydrophobic interaction, they self-assemble in aqueous environment forming a closed structure composed of one or more lipid bilayer, i.e. lamella, having the hydrophilic heads oriented towards the inner and outer surfaces of the lamella, and the hydrophobic tails constituting the lamella interiors; resulting in the formation of an aqueous core within the liposomal vesicle [34], [37], [39], [42].

![Figure 1-5 Structure of liposomes and possible functionalization](image)
This unique structure of liposomes (Figure 1-5) enables the use of liposomes as a delivery system for both hydrophilic and hydrophobic drugs, where hydrophilic drugs can be encapsulated in the aqueous core, while hydrophobic ones can reside within the hydrophobic bilayer of the lamella [37], [39]. The physicochemical properties of liposomes can be manipulated to control their size within the wide range of 20-1000 nm, lamellarity, surface charge, and functionality; mainly by integrating different lipids within their structures [34], [37]–[39]. Most of the lipids used in liposome preparations are biocompatible, biodegradable and FDA approved [37], [39]. The application of liposomes in drug delivery presents a wide range of advantages, such as, biocompatibility and the ability to deliver hydrophilic and hydrophobic drugs simultaneously for synergistic effect [10], [38], [42]. In addition, liposomes are kinetically stabilized delivery systems, that unlike thermodynamically stabilized systems, are capable of adapting to changes in their surrounding environment such as dilution [39].

Liposomes are found to be highly efficient in targeted delivery to tumor cells [14], [42]. Generally, there are two approaches in drug delivery being the passive and active approaches [14], [42]. Active targeting involve the addition of a targeting functionality that acts as a substrate to overexpressed receptors in tumor cells such as, estrogen, folate, and galactose receptors. Immuno-targeting, another form of active approach, involves the use of antibodies as a targeting functionality [14], [34], [38], [42]. Passive targeting is based on the enhanced permeability and retention (EPR) effect exhibited by tumor tissues [38], [42].

1.2.1 Enhanced Permeability and Retention effect

A major obstacle for liposomal drug delivery is crossing the blood vasculature, which is composed of a continuous tightly packed layer of endothelial cells; thus preventing the extravasation of liposomes [13], [39]. Nevertheless, tumor tissues tend to grow at a relatively fast rate and are thus associated with rapid uncontrolled angiogenesis, producing defective leaky vasculature [8], [13], [14], [34], [35], [39], [42]. In addition, lymphatic drainage is impaired in tumor tissues, and permeability mediators such as bradykinin, nitric oxide, and prostaglandins are overexpressed; resulting in an enhanced permeability and retention of macromolecules such as albumin, immunoglobulin G, and transferrin [8], [10], [13], [14], [35], [42]. Consequently, liposomes within the optimal size range (100 - 200 nm) capable of efficiently protruding through the leaky tumor vasculature and selectively accumulate in tumor tissues due to the EPR effect,
serving as the foundation for passive targeting [13], [14], [34]–[36], [39]. However, efficient targeting with the aid of EPR effect requires the capability of prolonged circulation of liposomes [14], [34], [39]. Conventional liposomes circulating in the blood are susceptible to rapid clearance by opsonization through the mononuclear phagocytic system (MPS), also known as the reticuloendothelial system (RES) [14], [35], [44]. The interaction between circulating liposomes and opsonins is highly dependent on surface features of liposomes, where hydrophobic and charged liposomal surfaces have a higher potential for hydrophobic and ionic interaction with opsonins, and consequently rapid clearance by the MPS [35], [44]. Nevertheless, this rapid clearance can be prevented by the incorporation of stealth properties to the liposomes [35].

1.2.2 Stealth liposomes

In order to prolong the liposomes residence time in blood circulation, the surface features of liposomes need to be altered [35]. This can be achieved with the aid of hydrophilic polymers, such as polyethylene glycol (PEG), thus preventing opsonization [34], [35]. Moreover, hydrophilic polymers are capable of sterically stabilizing liposomes preventing phagocytosis by the MPS [10], [35], [38]. This form of liposomes is referred to as stealth liposomes [10], [34], [35], [37]–[39], [42]. Stealth liposomes show a prolonged half-life in the blood stream (> 40 hours), thus enabling for more efficient accumulation within the tumor tissues, through passive targeting [10], [39]. PEG was found to be the most effective polymer to produce stealth liposomes, having a direct relationship between PEG-chain length and the circulation half-life of liposomes [35], [38].
1.3 Statement of purpose

This research aims at investigating the use of liposomes as a dual delivery system for oxaliplatin and satraplatin; as well as for oxaliplatin and ascorbic acid aiming for the synergistic effect of these combinations and reducing their toxicity profiles. The following experiments were conducted for this purpose:

- The preparation and characterization of stable liposomal systems and the identification of the best system for our purpose with particles size less than 200 nm and a surface negative potential.
- Use the developed liposomal system in dual drug loading of hydrophobic and hydrophilic drugs.
- Determination of the encapsulation efficiencies of the total amount of drugs loaded.
- Studies of the release patterns of the platinum-based drugs from the liposomal systems to determine the ability of the liposomal system used in delivering the loaded drugs to tumor cells.
- Examination of the cytotoxicity and DNA damage induction for the prepared liposomal formulations relative to free drug preparation and the commercial liposomal formulation Lipoxal™.
2. Literature review

The evaluation of oxaliplatin stability in human plasma has revealed complete transformation of oxaliplatin to PtCl$_2$-(DACH) within 2 hours, and that 85 – 88% of oxaliplatin gets bound to plasma proteins [16]. In addition, oxaliplatin was reported to accumulate in erythrocytes [16]. The main advantage of oxaliplatin over other platinum drugs is its lack of cross-resistance; however, clinical trials have revealed that oxaliplatin may be used in combination with other cytotoxic agents for more efficient treatment of cisplatin refractory and resistant cancers [45]. These drawbacks of oxaliplatin can be reduced by its encapsulation in a liposomal delivery system. Liposomal formulations of oxaliplatin were proven to minimize partitioning of oxaliplatin into erythrocytes with more than 90% retained in plasma, relative to non-formulated, free oxaliplatin < 20% [46].

There are several methods reported for the preparation of liposomes. However, the majority of reported studies utilize reverse-phase vaporization technique for the preparation of oxaliplatin liposomes [46]–[52]. It is worth noting that Zalba et al. have conducted a study comparing the efficacy of three different preparation methods in the production of oxaliplatin loaded stealth liposomes, thin film hydration, reverse-phase vaporization, and the heating method. The conclusion of this study was that the thin film hydration method associated with membrane extrusion is the simplest technique with the highest reproducibility and homogeneity of resulting liposomal formulations [53].

There are numerous studies on liposomal encapsulation of oxaliplatin [46]–[52]. Recently, a comparative study between stealth cationic liposomes, and stealth neutral liposomes, showed that loaded stealth cationic liposomes significantly suppressed tumor angiogenesis by selectively targeting tumor angiogenic blood vessels more efficiently than stealth neutral liposomes [46], [47]. This was mainly attributed to the electrostatic interaction between the liposomal cationic surface and the anionic surface of endothelial cells plasma membrane [46]. In addition, stealth cationic liposomes were found to efficiently suppress tumor growth; which may be attributed to facilitated inra-tumor accumulation by electrostatic interaction, followed by extravasation into interstitial spaces by EPR effect targeting tumor cells upon saturation of tumor vascular
endothelial cells [47]. Stealth cationic liposomes are therefore capable of targeting tumors, through endothelial cells in angiogenic vessels and tumor cells [46], [47].

However, upon studying the use of combination therapy of a chemotherapeutic with the two developed oxaliplatin loaded liposomes, cationic and neutral stealth liposomes, their respective cytotoxic efficacy was found to vary [49], [50]. A metronomic dosing system was used for the chemotherapeutic drug S-1, an oral fluoropyrimidine derivative, that exerts a potent anti-angiogenic effect by targeting endothelial cells in tumor vasculature, and a cytotoxic effect on viable tumors and stroma cells [49], [50]. Upon co-administration with the neutral stealth liposomal formulation of oxaliplatin, a synergistic effect was observed as S-1 facilitates EPR effect of neutral liposome by damaging endothelial cells of tumor vasculature, and resulting in a decrease in tumor interstitial pressure [49], [50]. On the other hand, the combination of S-1 with the cationic stealth liposomal oxaliplatin formulation was found to have an antagonistic effect, where S-1 impairs the delivery of cationic liposomes to tumor tissue by depriving them of their binding sites [50].

Several factors that affect the therapeutic efficacy of oxaliplatin loaded liposomal formulations were the subject of recent studies [48], [51], [52]. It was reported that dividing the therapeutic regimen of oxaliplatin loaded neutral and cationic liposomes over two or more sequential doses for in-vivo experiments enhances the therapeutic efficacy of the subsequent dose administered [48], [51]. This was mainly attributed to the potent apoptotic response of the initial treatments, resulting in a cumulative cytotoxic effect in both endothelial and tumor cells [48], [51]. The resulting decrease in tumor interstitial pressure due to the reduction in number of tumor cells facilitates the penetration of the subsequent liposomal oxaliplatin dose [48]. The effect of tumor type on the anti-tumor efficacy of oxaliplatin loaded stealth liposomes was recently investigated [52]. Tumor types having a high growth rate showed a higher tendency to exert mechanical stress over angiogenic blood vessels, compromising their vascular permeability where the high growth rate is associated with an increase in the tumor interstitial pressure that limits the effective diffusion of liposomes by the EPR effect. This results in a decrease in the local oxaliplatin concentration to levels insufficient to induce a potent apoptotic effect in some cell types [52].

A commercial oxaliplatin liposomal formulation, Lipoxal has successfully reached phase II clinical trials and was proven to reduce oxaliplatin side effects at doses of 100-250 mg/m²,
however at doses of 300-350 mg/m² myelotoxicity, nausea, and peripheral neuropathy were observed [14], [17], [40], [41]. Upon the examination of possible combined treatment of Lipoxal and radiotherapy, it was reported that a relatively low combined effect with lipoxal on delaying tumor growth compared to free oxaliplatin was observed. This was mainly attributed to the low level of oxaliplatin-DNA adducts associated with lipoxal administration [54], [55]. However, unlike Lipoplatin, a commercial liposomal formulation of cisplatin, Lipoxal was proven to have a potent cytotoxic activity in MLH1-deficient cells, where MLH1 is a protein associated with the mismatch repair system [56]. This is mainly attributed to the bulky structure of oxaliplatin, which sterically hinder the binding of MLH1 protein, thus oxaliplatin-DNA adducts are not susceptible to repair by MMR system [56].

The use of combinational chemotherapy is associated with multiple benefits such as providing synergy between the two drugs, or the two therapeutic approaches, thus reducing the amount of drugs administered to the patient and their associated toxicities [57], [58]. In addition, combinational chemotherapies are capable of efficiently overcoming multi-drug resistance (MDR) mechanisms developed by some types of cancer [57], [59]. There are many approaches utilized to achieve successful combinational chemotherapy, these could include the co-delivery of chemosensitizing, pro-apoptotic agents along with the cytotoxic drugs or the co-delivery of multiple cytotoxic drugs [59]. Attempts to co-deliver cyclosporine A, as a chemosensitizer P-glycoprotein inhibitor, and doxorubicin in polymeric nanoparticles were recently reported [59], [60]. These resulted in a 2-fold increase in cytotoxicity against a doxorubicin resistant leukemia cell line compared to doxorubicin nanoparticles administered on their own [59], [60]. Liposomes used in the co-delivery of anticancer drugs and P-glycoprotein modulators all shared the capability of overcoming multi-drug resistance where active targeting of a liposomal delivery system was found to further enhance the cytotoxicity towards MDR cells [61]–[64]. The use of delivery systems for proapoptotic agents such as cytotoxic drug combinational therapy was reported using polymeric micelles in ceramide and paclitaxel combination [59], [65]. This resulted in 100-fold increase in cytotoxic activity relative to paclitaxel nanoparticles administered on their own [59], [65].

The combination of multiple cytotoxic drugs with different mechanisms of action within the same delivery system enables efficient targeting and containment of cancer cells. However, there
is always an encountered difficulty in the co-encapsulation of hydrophilic and hydrophobic drugs [59]. The successful encapsulation of hydrophilic doxorubicin by its intercalation to RNA aptamer conjugated to the surface of docetaxel loaded polymeric micelles was recently reported [66]. The formulation of polymeric nanoparticles co-delivering a hydrophilic platinum (IV) prodrug and docetaxel to prostate cancer cells demonstrating superior cytotoxic activity over nanoparticles with a single drug, was also the subject of a recent study [67]. A novel approach of dual cytotoxic drug loading was also recently presented which involved conjugation of hydrophilic drug to the hydrophobic drug with a hydrolysable linker and the subsequent loading of the drug conjugate into the polymeric nanoparticles. The dual drug loaded nanoparticle was reported to exhibit efficient cytotoxicity against a human pancreatic cancer cell line, with the drug conjugate being readily hydrolysable in mild acidic conditions [68].

The application of combinational therapy can be efficiently achieved using liposomes as dual drug delivery systems. This is due to their capability of carrying more than one drug. Several methods were reported for dual drug loading of liposomes such as active extrusion and passive diffusion. Active extrusion involves the addition of drugs during the preparation phase of liposomes followed by membrane extrusion whereas in passive diffusion the drug to be encapsulated is incubated with a previously prepared liposomal suspension, which facilitates diffusion of drug into the liposomes [59]. Several dual drug loaded liposomal formulations have made their way to clinical trials. These include CPX-351 and CPX-1, the first is a dual drug loaded liposomal formulation of hydrophilic cytarabine loaded by active extrusion and hydrophobic daunorubicin loaded by passive diffusion in the ratio of 5:1 targeting the treatment of acute leukemia; while the latter is a liposomal formulation loaded with hydrophobic irinotecan and hydrophilic floxuridine in the ratio of 1:1 for treatment of colorectal cancer [69]–[72].

Several innovative liposomal systems were reported for dual drug delivery. It was reported that the formulation of double liposomes, multivesicular vesicles (MVV), is capable of efficiently treating peptic ulcer by oral administration in comparison with treatment using a combination of drug solution [73]. A similar double liposome system was the subject of a report evaluating the capability of loading hydrophilic versus hydrophobic drugs [74]. This report has shown that the double liposome system exhibited higher encapsulation efficiency for hydrophobic drugs [74]. In addition, the effect of different surface charge of liposomes on the release pattern of drug were
examined and it was reported that double liposomes containing cationic inner liposomes had the least cumulative release percentage of the entrapped lipophilic drug. This suggest a possible relation between the in-vitro drug release profile and electrical charge interaction between lipid membrane and the encapsulated drugs [74], [75]. This particular system was loaded with insulin in combination with aprotinin yielding an effective hypoglycemic effect, and upon loading of salmon calcitonin yielded an effective hypocalceimic effect [75]. However, the main drawback of such double liposomes is the large particle size which was reported to be in the micrometer range limiting its suitability for parenteral applications [75]. Very recently, a relatively small sized double liposomal system in the range of 200 nm was designed using unsaturated lipids with short carbon chains and anionic lipids, in addition to the utilization of probe sonication and membrane extrusion unlike the previously reported thin-film hydration methods [76].

One of the most widely debated additions in combinational chemotherapy involves the use of ascorbic acid in combination with cytotoxic drugs for cancer treatment. Several research groups have reported the cytotoxic efficiency of pharmacological doses of ascorbic acid, achievable only through intravenous administration [58], [77]–[79]. The main reported mechanism for ascorbic acid cytotoxicity at pharmacological doses, is the production of hydrogen peroxide (H₂O₂) and ascorbate radical in the extracellular environment [80], [81]. It was reported that ascorbate treatment induces G0/G1 cell cycle arrest in DU-145 prostate cancer cells [58]. However, the pharmacologic use of ascorbic acid as a single treatment in clinical studies was not curative, thus it was suggested to use ascorbic acid in combinational therapy with other cytotoxic drugs as a chemo-sensitizing agent [82]. Ascorbic acid varied in its cytotoxic effect upon combination with different cytotoxic drugs. As previously reported, pre-incubation of DU-145 cells with ascorbic acid resulted in an increase in sensitivity towards docetaxel, epirubicin, irinotecan, and fluorouracil; whereas no substantial increase in oxaliplatin, or vinorelbine cytotoxicity was observed [58]. Other reports investigated the possible synergy of ascorbic acid with a set of cytotoxic drugs including cisplatin, gemcitabine, paclitaxel, and epigallocatechin-3-gallate (EGCG) and reported synergy of ascorbic acid only with gemcitabine and EGCG in REN cells [77]. As for combination with oxaliplatin, an individual case study was recently reported in a human trial of intravenous vitamin C, where the patient received 3 week cycles of intravenous
oxaliplatin and oral capecitabine [83]. This was reported to be sufficient in maintaining long-lasting stable disease [83].

As for satraplatin, a study was conducted to evaluate the kinetic parameters for satraplatin stability in plasma relative to its active metabolite, JM118[84]. Despite their instability in human plasma, satraplatin was reported to be more stable retaining up to 40% of total added drug intact relative to 25% for JM118 [84], [85]. Satraplatin offered slower rate of protein binding, where 25% satraplatin was bound irreversibly to plasma proteins compared to 65% for JM118 [84], [85]. Satraplatin was reported to degrade more extensively than JM118 producing 6 platinum-containing degradation products constituting 30% of total added drug, relative to 3 products for JM118 accounting for 10% of total drug added [84], [85]. The hydrolytic degradation of satraplatin involves a stepwise displacement of the chloride ligands by hydroxyl ligands, with a preceding aquation step [85]. It was reported that satraplatin accumulates at higher levels by colorectal cancer cells compared to oxaliplatin, which was mainly attributed to its lipophilic nature and consequently associated with an enhanced cytotoxic activity of satraplatin over oxaliplatin by inducing G2/M cell cycle arrest [86]. Despite the high stability of satraplatin in plasma, and its passive diffusion into cells facilitated by its lipophilic nature satraplatin failed to maintain progression free survival superior to that of cisplatin in clinical trials [85].
3. Materials and Methods

3.1 Material

3.1.1 Chemicals and reagents

L-(+)-Ascorbic acid
Sucrose 98%
Dimethylthiazol diphenyl tetrazolium bromide
Sodium dodecylsulphate (SDS)
Dichloromethane, HPLC grade, stabilized with ethanol
JT Baker® Acetonitrile HPLC Far UV / Gradient Grade
JT Baker® Methanol (ultra) Gradient HPLC grade
Dimethyl sulfoxide (DMSO)
Biowhittaker® Phosphate Buffered Saline 0.0067 M (PO₄) without Ca and Mg
Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) and 5% Penicillin-Streptomycin mixture
Milli-Q ultrapure water

Platinum Complexes

Oxaliplatin
Satraplatin

NenTech Ltd., Northants, UK
Aldrich chemical company Inc., Wisconsin, USA
Serva, Germany
Sigma-Aldrich, USA
Scharlau, Barcelona, Spain
Avantor performance materials, Pennsylvania, USA
Avantor performance materials, Pennsylvania, USA
Sigma-Aldrich, USA
Lonza, Basel, Switzerland
Lonza, Basel, Switzerland

Sanofi-Synthelabo Limited and Shandong Boyuan Pharmaceutical Co. Ltd.
Sanofi-Synthelabo Limited and Shandong Boyuan Pharmaceutical Co. Ltd.,
Lipoxal™

**Lipid**

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC)  
Corden Pharma, Plankstadt, Germany

1,2-Distearoyl-sn-glycer-3-phosphoethanolamine (DSPE)  
Corden Pharma, Plankstadt, Germany

1,2-Distearoyl-sn-glycero-3-phospho-rac-glycerol, sodium salt, (DSPG-Na)  
Lipoid, Steinhausen, Switzerland

N-(Carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt  
(MPEG-2000-DSPE)  
Corden Pharma, Plankstadt, Germany

Cholesterol  
Corden Pharma, Plankstadt, Germany

An overview of the lipid structures is presented in table 2-1.
<table>
<thead>
<tr>
<th>Lipid</th>
<th>LIPID MAPS</th>
<th>IUPAC</th>
<th>Structure</th>
<th>Charge</th>
<th>Transition Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSPC</td>
<td>18:0 PC</td>
<td>(2R)-2,3-Bis(stearoyloxy)propyl 2-</td>
<td>Zwitterion</td>
<td></td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(trimethylammonio)ethyl phosphate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSPE</td>
<td>18:0 PE</td>
<td>3-{[(2-Aminoethoxy)(hydroxy)phosphoryl]oxy}-2-(stearoyloxy)propyl stearate</td>
<td>Zwitterion</td>
<td></td>
<td>74</td>
</tr>
<tr>
<td>DSPG</td>
<td>18:0 PG</td>
<td>3-{[(2,3-Dihydroxypropoxy)(hydroxy)phosphoryl]oxy}-2-(stearoyloxy)propyl stearate</td>
<td>Anionic</td>
<td></td>
<td>55</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Cholesterol</td>
<td>(3β)-Cholest-5-en-3-ol</td>
<td>Neutral</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>MPEG-2000-DSPE</td>
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<td>1,2-distearoyl-sn-glycero-3-</td>
<td>Anionic</td>
<td></td>
<td>NA</td>
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<tr>
<td></td>
<td>PEG2000 PE</td>
<td>phosphoethanolamine-N-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[methoxy(polyethylene glycol)-2000] (sodium</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>salt)</td>
<td></td>
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</tr>
</tbody>
</table>
3.1.2 Solutions

Oxaliplatin solution at 7.55 mM concentration, a 7.52 mM Ascorbic acid, and 19.28 mM Sodium dodecylsulphate (SDS) solutions were prepared in ultrapure water.

3.1.3 Cell lines

The human mammary gland adenocarcinoma cell line MCF-7, human liver hepatocellular carcinoma HepG2, and human kidney normal cells, BHK-21 (kindly provided by Dr. Sameh Saad Ali, Zewail City of Science and Technology, Egypt) were used in the study. MCF-7 is reported as a relatively resistant cell line to cisplatin compared to other breast cancer cell lines, however it does not develop resistance to oxaliplatin [87], [88].

3.1.4 Consumable materials

Pippette tips
Nanoceq® MF centrifugal devices with GHP membrane
Visking® dialysis tubing (MWCO 12-14 KDa)
Eppendorf microcentrifuge tubes – Safe lock (2 ml)
Falcon™ tubes – Conical centrifuge (15 ml)
Parafilm M®
Clear glass screw thread vials
Tissue culture plates, 96 wells

3.1.5 Equipment

Rotavapor®
Cooling centrifuge
Grant Bio PV-1 Vortex mixer
Analytical balance
Hot plate

Socorex, Ecublens, Switzerland
Pall Life Sciences, USA
SERVA, Germany
Fisher Scientific, UK
Fisher Scientific, UK
Parafilm - Bemis, USA
Thermo Fisher Scientific Inc., USA
Greiner Laborteknik, Germany
BÜCHI, Flawil, Switzerland
HERMLE LABORTECHNIK, Wehingen, Germany
Grant instruments (Cambridge) Ltd., UK
Mettler Toledo, USA
VWR, USA
10, 200, 1000 µl Micropipettes
Shaking water bath
Microplate reader
Countess® Automated cell counter, Invitrogen

**Zetasizer**
Zetasizer
Disposable capillary cells (DTS1070)

**Ultra High Performance Liquid Chromatography**
Ultimate UHPLC dionex
BDS Hypersil C18 column (250x4.6 mm)
Photodiode array detector

**Transmission electron microscopy**
Transmission electron microscope
Carbon coated copper grid

**Membrane extrusion**
Avanti® Mini-extruder
Extruder set with heating block
Polycarbonate membrane 0.1 µm
Filter supports
Gas tight syringe

Eppendorf, Germany
Grant instruments (Cambridge) Ltd., UK
FLUOstar OPTIMA (BMG LabTech, Germany)
Thermo Fisher Scientific Inc., USA
Malvern Instruments Ltd., UK
Malvern Instruments Ltd., UK
Thermo Fisher Scientific Inc., USA
Thermo Fisher Scientific Inc., USA
Thermo Fisher Scientific Inc., USA
JEOL, Massachusetts, USA
Polysciences Inc., Germany
Avanti polar lipids Inc., USA
Avanti polar lipids Inc., USA
Avanti polar lipids Inc., USA
Avanti polar lipids Inc., USA
Avanti polar lipids Inc., USA
3.1.6 Software

Chromeleon™ Software
Thermo Fisher Scientific Inc., USA

Malvern
Malvern Instruments Ltd., UK

Controller for JEM-2100/HT
JEOL, Massachusetts, USA

3.2 Preparation of liposomes

Stealth liposomes were prepared using the thin-film hydration method followed by membrane extrusion to control the particle diameter as previously described by Nallamothu et al. [89]. For the preparation of oxaliplatin loaded liposomes, lipids were dissolved in a 250 ml round bottomed flask containing a sufficient amount of dichloromethane forming a lipid mixture. This was followed by the removal of the organic phase by rotary evaporation under reduced pressure at 60 °C, a temperature equivalent to the lipids transition temperature (Tm), to obtain a continuous thin film of lipids on the flask wall. The dry thin film was subsequently hydrated with 3 ml of 7.55 mM oxaliplatin solution, and was allowed to resume rotation in a rotary evaporator under normal pressure at 60 °C for 2 hours. Finally, membrane extrusion was performed using 100 nm polycarbonate membranes at 60 °C. The prepared liposomal formulations were stored at 4 °C. In the preparation of “void” liposomes or unloaded liposomes; ultrapure water was added instead of the drug solution during the hydration phase.

Since the composition of the lipid membrane tends to influence the characteristics of the prepared liposomes [90], part of the study has focused on the comparison of different lipid compositions and their influence on the liposome characterization. DSPG, a negatively charged lipid, was used in the liposome formulation to displace 5% of the total mole percent of each of the other lipid components used being DSPE, DSPC, and Cholesterol, as stated in Table 2-2.
Table 2- 2 Mole Ratio of lipids and oxaliplatin used to prepare DSPG containing liposomes

<table>
<thead>
<tr>
<th>Sample</th>
<th>DSPC</th>
<th>Cholesterol</th>
<th>DSPE</th>
<th>DSPG</th>
<th>DSPE-PEG2000</th>
<th>Total lipid number of moles (mmole)</th>
<th>Lipid Concentration (LC) (mM)</th>
<th>Oxaliplatin</th>
<th>Mole ratio Drug : Lipid</th>
<th>number of moles (mmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP</td>
<td>1.000</td>
<td>0.8000</td>
<td>0.100</td>
<td>-</td>
<td>1.100</td>
<td>0.0412</td>
<td>13.75</td>
<td></td>
<td>167 : 304</td>
<td>0.022653</td>
</tr>
<tr>
<td>LP-Void</td>
<td>1.000</td>
<td>0.8000</td>
<td>0.100</td>
<td>-</td>
<td>1.100</td>
<td>0.0412</td>
<td>13.75</td>
<td>0 : 1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LP-G1</td>
<td>1.000</td>
<td>0.8000</td>
<td>-</td>
<td>0.100</td>
<td>0.1000</td>
<td>0.0412</td>
<td>13.75</td>
<td>167 : 304</td>
<td>0.022653</td>
<td>0.0046</td>
</tr>
<tr>
<td>LP-G2</td>
<td>1.000</td>
<td>0.8889</td>
<td>0.111</td>
<td>0.111</td>
<td>0.1111</td>
<td>0.0412</td>
<td>13.75</td>
<td>167 : 304</td>
<td>0.022653</td>
<td>0.0046</td>
</tr>
<tr>
<td>LP-G3</td>
<td>1.000</td>
<td>0.7000</td>
<td>0.100</td>
<td>0.100</td>
<td>0.1000</td>
<td>0.0412</td>
<td>13.75</td>
<td>167 : 304</td>
<td>0.022653</td>
<td>0.0046</td>
</tr>
</tbody>
</table>

Table 2- 3 Mole Ratio of lipids and drugs used to prepare dual drug loaded liposomes

<table>
<thead>
<tr>
<th>Sample</th>
<th>DSPC</th>
<th>Cholesterol</th>
<th>DSPE</th>
<th>DSPE-PEG2000</th>
<th>Total lipid number of moles (mmole)</th>
<th>LC (mM)</th>
<th>Oxaliplatin</th>
<th>Hydrophilic/Hydrophobic drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP-Ox-AA</td>
<td>1.000</td>
<td>0.8000</td>
<td>0.100</td>
<td>1.100</td>
<td>0.0412</td>
<td>13.75</td>
<td>167 : 304</td>
<td>0.022653</td>
</tr>
<tr>
<td>LP-Ox-Stp</td>
<td>1.000</td>
<td>0.8000</td>
<td>0.100</td>
<td>1.100</td>
<td>0.0412</td>
<td>13.75</td>
<td>167 : 304</td>
<td>-</td>
</tr>
<tr>
<td>LP-Stp</td>
<td>1.000</td>
<td>0.8000</td>
<td>0.100</td>
<td>1.100</td>
<td>0.0412</td>
<td>13.75</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

Percent of total lipid: 10
Number of moles (mmole): 0.0046
For the preparation of dual-drug loaded liposomes, the hydrophilic/hydrophobic nature of the added drug influence the stage of its addition during liposome preparation. As stated in Table 2-4, hydrophobic drugs such as satraplatin are added to the lipid mixture prior to the formation of the thin film, whereas hydrophilic drugs such as ascorbic acid are added to the hydration solution. The ratio of the additional drug used is stated in Table 2-3. The dual drug loaded liposome containing oxaliplatin and ascorbic acid is encoded as LP-Ox-AA, and the liposomal formulation loaded with oxaliplatin and satraplatin is encoded as LP-Ox-Stp. In addition, a liposome formulation was prepared loaded only with satraplatin (LP-Stp) in order to evaluate the effect of loading a hydrophobic drug in the liposome lipid bilayer.

Table 2-4 Lipophilicity of drug used in liposome preparation

<table>
<thead>
<tr>
<th>Drug name</th>
<th>IUPAC name</th>
<th>Log P (Chemaxon)</th>
<th>Cell membrane permeability (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxaliplatin</td>
<td>Platinum(2+) ethanedioate (1R,2R)-1,2-cyclohexanediamine (1:1:1)</td>
<td>1.73</td>
<td>&lt;1×10⁻⁶ [91]</td>
</tr>
<tr>
<td>Ascorbic</td>
<td>acid (5R)-5-[(1S)-1,2-Dihydroxyethyl]-3,4-dihydroxy-2(5H)-furanone</td>
<td>-1.91</td>
<td>-</td>
</tr>
<tr>
<td>Satraplatin</td>
<td>Bis(acetato-κO)(ammine)dichloro(cyclohexanamine)platinum</td>
<td>1.17</td>
<td>-</td>
</tr>
</tbody>
</table>

3.3 Liposome characterization

3.3.1 Particle size, polydispersity index, and zetapotential

The particle size, polydispersity index (PDI), and Zeta potential (ζ potential) of liposomes were analyzed by Dynamic light scattering technique using a Zetasizer Nano Series (Malvern Instruments, UK). To ensure a convenient scattered intensity on the detector, formulations were diluted 1:50 (v/v) in ultrapure water prior to its measurement at 25°C.

3.3.2 Encapsulation efficiency

Two different techniques were used determine the encapsulation efficiencies for loaded drug within the liposomal delivery system.
A. Liposome separation procedure

i) Ultracentrifugation (UC)

Ultracentrifugation was used to separate liposomes from the bulk solution, and determine the amount of drug encapsulated in the recovered liposomes. Briefly, 0.5 ml of 1:2 (v/v) diluted liposome preparation is added to the Nanosep® centrifugal device, and was centrifuged at 7000 rpm at 4 °C for 1 h. This was followed by the analysis of the filtrate using HPLC.

The Encapsulation efficiency expressed in percentage (%) was calculated using the following equations.

\[
\text{Total amount of drug encapsulated in liposomes} = \text{Total amount of drug added} - \text{Un-entrapped drug fraction}
\]

\[
\text{Encapsulation efficiency\% (EE\%)} = \frac{\text{Total amount of drug encapsulated in liposomes}}{\text{Total amount of drug added}} \times 100
\]

ii) Pellet permeabilization (PP)

A total of 400 µl of 1:2 (v/v) diluted liposome preparation was centrifuged at 16500 rpm at 4 °C for 3 h. This was followed by collecting both the supernatant and the pellet, the pellet was diluted in 1:4 (v/v) 19.28 mM SDS in order to permeate the liposome entrapped drugs; then both the supernatant and the permeabilized pellets were analyzed using HPLC.

The Encapsulation efficiency expressed in percentage (%) was calculated using the following equations.

\[
\text{Total amount of drug} = \text{Total amount of drug encapsulated in pellet} + \text{Total amount of drug un-encapsulated in supernatant}
\]

\[
\text{Encapsulation efficiency\% (EE\%)} = \frac{\text{Total amount of drug encapsulated in pellet}}{\text{Total amount of drug}} \times 100
\]

B. Quantification method
The liposome un-entrapped fraction of drugs were quantified using UHPLC, with a photodiode array detector and a BDS Hypersil® C18 reverse-phase column (250 mm × 4.6 mm, 5 mm). Two methods were followed.

1) Oxaliplatin quantification

The mobile phase consisted of deionized water and acetonitrile (99:1) (v/v) at a flow rate of 1.2 ml/min, with the column temperature maintained at 40°C. The injection volume was 20 µL, and the effluent monitored at 210 nm. The sample oxaliplatin concentration was determined using the constructed calibration curve (refer to Appendix A).

2) Satraplatin and Ascorbic acid quantification

The mobile phase was composed of deionized water and acetonitrile (50:50) (v/v) at a flow rate of 1 ml/min, with the column temperature maintained at 40°C. The injection volume was 20 µL, and the effluent was simultaneously monitored at 210 nm for detection of satraplatin, and at 254 nm for detection of ascorbic acid. The sample satraplatin and ascorbic acid concentration was determined using their respective constructed calibration curves (refer to Appendix A).

3.3.3 Transmission electron microscopy (TEM)

The prepared stealth liposomes were analyzed by TEM. The measurements were carried out by means of a JEOL-JEM 2100 electron microscope operating at 160 kV. Fifty microliter of the sample was deposited over a carbon-coated copper grid with 200 mesh and dried. The sample was then negatively stained with 2% aqueous phosphotungstic acid and dried. The sample was then visualized and photographed.

3.4 In-vitro drug release analysis

The drug release testing was conducted according to a previously described method [92]. This was done for LP-Ox, LP-Ox-AA, LP-Ox-Stp, free oxaliplatin drug solution, Lipoxal™ (a commercial liposomal formulation of oxaliplatin), and finally LP-void as well as LP-Stp each spiked with an equivalent concentration of oxaliplatin. A volume of 0.5 ml of liposomal preparation was placed in a dialysis tubing (3.8 cm in length). Both ends were tied. The dialysis bag was suspended in 25 ml PBS at pH 7.4 and maintained at 37 ± 0.5 °C [92]. The dispersion
was rotated at 200 strokes/minute in a water bath shaker [92]. At predetermined time intervals of 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 48, and 72 h; 1 ml aliquots were sampled and replaced with 1 ml fresh pH 7.4 PBS, which was maintained at the same temperature as the samples being 37 ± 0.5 °C. Drug concentrations were determined using HPLC.

3.5 Release kinetics

Two methods were used to investigate the kinetics of drug release profile from the prepared liposomal formulations.

3.5.1 Model dependent methods

This method involved the fitting of the release data to one of the following seven release kinetic models.

i) Zero-order

\[ Q_t = Q_0 + K_0 t \]

Where, \( Q_t \) is the amount of drug released in time \( t \), \( Q_0 \) is the initial amount of drug in the solution and \( K_0 \) is the zero order release constant expressed in units of concentration/time. To study the release kinetics, data obtained from in-vitro drug release studies were plotted as cumulative amount of drug released versus time.

ii) First-order

\[ \log C_t = \log C_0 - K_1 t / 2.303 \]

Where, \( C_t \) is the concentration of drug released in time \( t \), \( C_0 \) is the initial concentration of drug, \( K_1 \) is the first order rate constant. The data obtained were plotted as log cumulative percentage of drug remaining versus time which would yield a straight line with a slope of \(-K/2.303\).

iii) Higuchi

\[ Q = K_H \times t^{1/2} \]

Where, \( Q \) is the amount of drug released in time \( t \), \( K_H \) is the Higuchi dissolution constant. The data obtained were plotted as cumulative percentage of drug released versus square root of time.
iv) **Hixson-Crowell**

\[ Q_0^{1/3} - Q_t^{1/3} = K_C t \]

Where, \( Q_t \) is the amount of drug remaining in time \( t \), \( Q_0 \) is the initial amount of the drug in liposome and \( K_C \) is the rate constant for Hixson-Crowell rate equation. The data obtained were plotted as Cube root of cumulative percentage of drug remaining versus time.

v) **Korsmeyer-Peppas**

\[ \frac{M_t}{M_\infty} = K_P t^n \]

Where \( M_t / M_\infty \) is a fraction of drug released at time \( t \), \( K_P \) is the release rate constant and \( n \) is the release exponent. The data obtained were plotted as log (cumulative percentage of drug released) versus log (time).

vi) **Baker Lonsdale**

\[ f_1 = \frac{3}{2} \left[ 1 - (1 - \frac{M_t}{M_\infty})^{2/3} \right] \frac{M_t}{M_\infty} = K_B(t) \]

Where \([M_t / M_\infty]\) is a fraction of drug released at time \( t \), \( K_B \) is the release rate constant. To study the release kinetics, data obtained from in-vitro drug release were plotted as \([d(M_t / M_\infty) / dt] \) against the root of time inverse on x-axis.

vii) **Michaelis-Menten (Hyperbola)**

\[ \frac{dC}{dt} = \frac{V_m C}{(K_m + C)} \]

Where \( V_m \) is the maximum release rate, \( K_m \) is the Michaelis-Menten constant, \( C \) is the amount of drug released, and \( t \) is time. The data obtained were plotted as drug release % versus time. Michaelis-menten was previously reported in describing the release kinetics of Ketorolac from silica nanoparticles [93].

### 3.5.2 Model independent method

This method utilizes the difference factor (\( f_1 \)), similarity factor (\( f_2 \)) to compare the release profiles of different formulations by measuring the percent difference and the percent similarity respectively.
Where, \( n \) is the number of release sample time intervals, \( R_t \) and \( T_t \) are the percent released at each time point, \( t \), for the reference and test drug release profiles, respectively.

### 3.6 Stability study

Stability of the prepared liposomal formulations was examined at a temperature of 4±1°C for 6 months, according to the guideline of the International Conference on Harmonisation [94]. The stored samples were tested for their physical changes, particle size distribution, zeta potential, and EE%.

### 3.7 In-vitro cytotoxicity study

The human mammary gland adenocarcinoma cell line, MCF-7; human liver hepatocellular carcinoma, HepG2; and human kidney normal cells, BHK-21 were exposed to variable concentrations of oxaliplatin. MTT assays were used to evaluate the cells viability as previously reported [95], [96], which utilize the conversion of the tetrazolium salt MTT to formazan by dehydrogenase enzymes in living cells. Briefly, cells were cultured in 96-well plate (10,000 cells per well) at 37 °C humidified with 5% CO\(_2\) in DMEM supplemented with 10% FBS and 5% Penicillin-Streptomycin mixture. Dilutions of oxaliplatin and liposomal formulations at 8, 12, 16, 20, and 28 µg/ml were made in the culture media. Samples were incubated with the cell line for 24 hrs, and wells containing cells treated only with media served as controls. After incubation, the media was discarded and the cells were further incubated in 20 µl MTT (5 mg/ml) and 100 µl fresh media for 3 hours, all media was then discarded, and the formazan crystalline precipitate formed were solubilized via the addition of 100 µl DMSO. The absorbance
of each well was measured at 450 nm using a microplate reader, and the reference absorbance measured at 620 nm. Cell viability was determined by calculating the absorbance of the test wells as a percentage of the control wells. GraphPad prism 6 software package was used for calculation of IC\(_{50}\).

Absorbance of well = \(A_{620} - A_{450}\)

\[
\text{Cell viability (\%) } = \frac{\text{Absorbance of experimental group}}{\text{Absorbance of control untreated group}} \times 100
\]

3.8 \(\gamma\)-H2AX assay

MCF-7 cell line for human mammary gland adenocarcinoma was exposed to 2 \(\mu\)M concentration of free oxaliplatin, LP-Ox, LP-Ox-Stp and Lipoxal for 1 hr. After incubation, the media was discarded and the cells were further incubated in fresh media for 24 hours. Cells were then fixed using formaldehyde and permeabilized with Triton X-100, and subsequently incubated with H2AX primary antibody for 1 hr. Then, cells were washed using PBS and were further incubated for 30 min. in FITC mouse secondary antibody and washed using PBS. Finally cells were stained using DAPI and observed under a fluorescence microscope.

3.9 Statistical analysis

All values are expressed as mean \(\pm\) S.D. Statistical analysis was performed using a two-tailed unpaired t-test, Tukey honest significant difference test, one way ANOVA, two way ANOVA and linear and non-linear regression.
4. Results

4.1 Characterization of liposomes

4.1.1 Original formulation

Table 4-1 The effect of single drug loading on size, Polymer dispersity index (PDI), and ζ potential, measures of stability

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (nm)</th>
<th>±SD</th>
<th>PDI ±SD</th>
<th>ζ Potential (mV) ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP-void</td>
<td>150.3</td>
<td>1.367</td>
<td>0.084</td>
<td>-40.72 ±0.638</td>
</tr>
<tr>
<td>LP-Ox</td>
<td>149.5***</td>
<td>2.811</td>
<td>0.040***</td>
<td>-35.8*** ±0.733</td>
</tr>
</tbody>
</table>

Data are means±SD from n=3. ***. P<0.001: difference from drug unloaded liposomal formulation, LP-void. ns: not significant versus LP-void.

With the aim of studying the effect of drug loading on the prepared liposomal system, three characteristic variables were studied for LP-void i.e. unloaded liposomes and oxaliplatin loaded liposomal formulation, LP-Ox. It was noted that the addition of oxaliplatin alter the PDI, and ζ potential of liposomes significantly (P<0.001), while the particle size did not change significantly; refer to Table 4-1.

4.1.2 Phosphatidyl glycerol addition

As reported in Table 4-2, the replacement of DSPE with DSPG (LP-G1-Ox) had a significant influence on reducing the size of liposomes (P<0.001), and on enhancing the encapsulation efficiency of oxaliplatin. The addition of DSPG at the expense of cholesterol (LP-G3-Ox) was found to result in a significantly larger liposomal size (P<0.001), the same effect was observed but to a less extent upon displacing 5 mole% of DSPC with DSPG (LP-G2-Ox) (P<0.05). As for the effect of DSPG on ζ potential, the incorporation of DSPG within the liposome had a significant reducing effect on ζ potential of all liposomal formulations (P<0.001). In addition, oxaliplatin loading was associated with a significant decrease in ζ potential in all DSPG containing liposomes (P<0.001).

4.1.3 Dual drug loading

Dual drug loaded liposomes with either ascorbic acid or satraplatin along with oxaliplatin had a direct influence on the final size, ζ potential, and EE% of oxaliplatin. The additional loading of ascorbic acid resulted in a significant increase in liposome size, and a subsequent increase in
oxaliplatin EE%, while reduced the liposome’s $\zeta$ potential. On the other hand, the additional loading of satraplatin was associated with reduction in liposome size, and an increase in liposomal $\zeta$ potential (see Table 4-3). LP-Ox-Stp had contradicting results in EE% calculated using ultracentrifugation (UC) and pellet permeabilization (PP) techniques. As UC-EE% determined a decrease in encapsulated oxaliplatin upon co-loading of satraplatin, while PP-EE% determined a direct relationship between oxaliplatin and satraplatin encapsulation. These results indicate that the dual loading of ascorbic acid results in enhancing the encapsulation of oxaliplatin within liposomes, while the dual loading of satraplatin influence on oxaliplatin encapsulation has contradictory results upon calculation of EE% using UC and PP protocols.

In addition, upon comparing the single drug loaded liposomes with satraplatin to dual drug loaded liposomes with satraplatin and oxaliplatin, a significant influence was observed in size reduction and increase in $\zeta$ potential for dual drug loaded liposomal system, with $P<0.001$, and $P<0.01$ respectively; and a minor influence of oxaliplatin on the EE% of satraplatin was observed.
Table 4-2 The effect of DSPG incorporation on liposomal formulation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (nm)</th>
<th>±SD</th>
<th>PDI ±SD</th>
<th>ζ Potential (mV) ±SD</th>
<th>Oxaliplatin (Oxpt) UC-EE% ±SD</th>
<th>Oxaliplatin (Oxpt) PP-EE% ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP-Ox</td>
<td>149.5</td>
<td>±2.811</td>
<td>0.040 ±0.025</td>
<td>-35.8 ±0.733</td>
<td>23.7 ±3.860</td>
<td>8.217 ±0.01738</td>
</tr>
<tr>
<td>LP-G1-Ox</td>
<td>136.7***</td>
<td>±1.183</td>
<td>0.056 ±0.034</td>
<td>-29.3*** ±2.18</td>
<td>26.04* ±0.3571</td>
<td>11.90*** ±0.1726</td>
</tr>
<tr>
<td>LP-G2-Ox</td>
<td>144.0#</td>
<td>±5.745</td>
<td>0.069 ±0.039</td>
<td>-29.4*** ±2.05</td>
<td>24.11 ±0.09466</td>
<td>9.007****±0.1577</td>
</tr>
<tr>
<td>LP-G3-Ox</td>
<td>155.6****ΔΔΔ</td>
<td>±7.098</td>
<td>0.055 ±0.026</td>
<td>-28.5*** ±1.43</td>
<td>25.11 ±0.1194</td>
<td>14.55****ΔΔΔ ±0.1591</td>
</tr>
<tr>
<td>LP-G1-void</td>
<td>146.2###</td>
<td>±3.780</td>
<td>0.077 ±0.026</td>
<td>-57.6### ±1.47</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LP-G2-void</td>
<td>149.6Δ</td>
<td>±4.555</td>
<td>0.123 ±0.0541</td>
<td>-50.2ΔΔΔ ±2.51</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LP-G3-void</td>
<td>146.5TT</td>
<td>±5.927</td>
<td>0.069 ±0.029</td>
<td>-53.8TT ±1.63</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are means ±SD from n=3. *: P<0.05 difference from liposomal formulation lacking DSPG, LP-Ox. ***: P<0.001 difference from liposomal formulation lacking DSPG, LP-Ox. #: P<0.05 difference from liposomal formulation lacking DSPE, LP-G1-Ox. ###: P<0.001 difference from liposomal formulation lacking DSPE. LP-G1-Ox. ΔΔΔ: P<0.001 difference from liposomal formulation with DSPG added at the expense of DSPC, LP-G2-Ox. Δ: P<0.05 difference from liposomal formulation with DSPG added at the expense of DSPC, LP-G2-Ox. ΔΔ: P<0.01 difference from liposomal formulation with DSPG added at the expense of Cholesterol, LP-G3-Ox. ΔΔ: P<0.01 difference from liposomal formulation with DSPG added at the expense of Cholesterol, LP-G3-Ox.

Table 4-3 Influence of dual drug loading on liposomal formulation and Lipoxal characterization results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (nm)</th>
<th>±SD</th>
<th>PDI ±SD</th>
<th>ζ Potential (mV)</th>
<th>Oxaliplatin (Oxpt) UC-EE%</th>
<th>Oxaliplatin (Oxpt) PP-EE%</th>
<th>AA / Stp UC-EE% ±SD</th>
<th>Oxaliplatin (Oxpt) PP-EE% ±SD</th>
<th>AA / Stp PP-EE% ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipoxal</td>
<td>118.5</td>
<td>±1.306</td>
<td>0.184 ±0.0178</td>
<td>-14.4±1.66</td>
<td>53.99 ±0.9310</td>
<td>-</td>
<td>-</td>
<td>7.390 ±0.1255</td>
<td>-</td>
</tr>
<tr>
<td>LP-Ox</td>
<td>149.5</td>
<td>±2.811</td>
<td>0.040 ±0.025</td>
<td>-35.8 ±0.733</td>
<td>23.70 ±3.860</td>
<td>-</td>
<td>-</td>
<td>8.217 ±0.01738</td>
<td>-</td>
</tr>
<tr>
<td>LP-Ox-AA</td>
<td>155.4***</td>
<td>±3.017</td>
<td>0.047 ±0.028</td>
<td>-22.3*** ±0.817</td>
<td>28.54*** ±3.593</td>
<td>97.81 ±2.142</td>
<td>9.108* ±0.3825</td>
<td>46.19 ±1.009</td>
<td></td>
</tr>
<tr>
<td>LP-Ox-Stop</td>
<td>130.2****ΔΔΔ</td>
<td>±1.421</td>
<td>0.057 ±0.026</td>
<td>-40.9****ΔΔΔ ±1.14</td>
<td>16.75**** Δ±4.177</td>
<td>49.34Δ ±2.550</td>
<td>19.10***ΔΔΔ ±0.05844</td>
<td>20.42ΔΔ ±0.1160</td>
<td></td>
</tr>
<tr>
<td>LP-Stop</td>
<td>155.4</td>
<td>±2.125</td>
<td>0.070 ±0.029</td>
<td>-36.1 ±1.94</td>
<td>-</td>
<td>-</td>
<td>48.32 ±2.601</td>
<td>-</td>
<td>19.12 ±0.07228</td>
</tr>
</tbody>
</table>

Data are means±SD from n=3. ***: P<0.001 difference from single drug loaded liposomal formulation, LP-Ox. ###: P<0.01 difference from dual drug loaded liposomal formulation, LP-Ox-AA. ΔΔΔ: P<0.001 difference from single drug loaded liposomal formulation, LP-Stop. ΔΔ: P<0.01 difference from single drug loaded liposomal formulation, LP-Stop. Δ: P<0.05 difference from single drug loaded liposomal formulation, LP-Stop.
4.2 Transmission electron microscopy (TEM)

The morphology of liposomes was evaluated using TEM, which in turn has indicated the spherical structure for most liposomes with uniform particle size and uniform dispersion, as in Figure 4-1. A white coated film was observed on the surface of the prepared liposomes that is attributed to the PEG coat over the surface of the liposomes, acting as a steric hindrant to mononuclear phagocytic cells of the RES.
Figure 4- 1 TEM images indicate the formation of small unilamellar liposomal vesicle with PEG coat on the surface. (A) LP-Ox (B) LP-Ox-AA (C) LP-Ox-Stp

4.3 FT-IR
Figure 4- 2 FT-IR spectra for the prepared liposomal formulations. (A) LP-Ox, (B) LP-Ox-AA, (C) LP-Ox-Stp

The FT-IR spectrum comparison for the drug loaded liposomal formulations versus their unloaded liposomes and free drug spectra has revealed that no change in chemical structure occurred during the preparation of satraplatin oxaliplatin dual drug loaded liposomes LP-Ox-Stp, while disappearance of the carbonyl group was noted for the LP-Ox and LP-Ox-AA liposomal formulations indicates the involvement of carbonyl groups in hydrogen bonding, confirmed by the broad hydroxyl peak observed at 3300-3500 cm\(^{-1}\).
4.4 Stability study

Relying on a two-way ANOVA analysis of the formulation stability data, it was found that the 6 month storage duration had no significant influence on the size of liposomes, but had a significant influence on $\zeta$ potential and PDI, $P<0.001$, $P<0.01$ respectively. In addition, there was a significant difference between all liposomal formulations in the variability of size, $\zeta$ potential and PDI during the 6 month storage duration, with $P<0.001$, $P<0.001$, and $P<0.01$ respectively. As illustrated in Table 4-4 and Figure 4-3, the type of drug loaded within the liposomal formulation had a significant influence on its stability during a six month storage duration which was significantly interpreted in size and $\zeta$ potential $P<0.001$. LP-Ox loaded only with oxaliplatin had a gradual yet non-significant decrease in size along with a significant decrease in $\zeta$ potential upon storage ($P<0.001$) associated with an increase in the UC-EE%, and a decrease in the PP-EE%. Whereas dual drug loaded liposomes LP-Ox-AA and LP-Ox-Stp had a more stable size with minimal non-significant variations. LP-Ox-Stp showed a significant gradual decrease in $\zeta$ potential, with $P<0.001$ upon storage for 6 month and an increase in UC-EE% for oxaliplatin associated with a concomitant decrease in UC-EE% of satraplatin, while the PP-EE% has shown a significant decrease in both oxaliplatin and satraplatin encapsulation. LP-Ox-AA had no significant difference in $\zeta$ potential after 6 month storage at 4°C, and a significant decrease in UC-EE% and PP-EE% of oxaliplatin and ascorbic acid after 6 month storage. In addition it was noted that for the stability evaluation for LP-Ox-Stp after an 8 month storage duration indicates high stability of the formulation.

![Figure 4-3 Cell means plot for (A) Size, and (B) $\zeta$ potential of samples stored for a 6 month duration at 4°C](image-url)
Table 4-4 Liposome characterization results over 8 month storage duration

<table>
<thead>
<tr>
<th>Sample</th>
<th>Storage duration (month)</th>
<th>Physical stability</th>
<th>Size (nm) ±SD</th>
<th>PDI ±SD</th>
<th>ζ Potential (mV) ±SD</th>
<th>Oxp UC-EE% ±SD</th>
<th>AA/Stencil UC-EE% ±SD</th>
<th>Oxp PP-EE% ±SD</th>
<th>AA/Stencil PP-EE% ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP-Ox</td>
<td>0</td>
<td>No Aggregates</td>
<td>149.5 ±2.81</td>
<td>0.040 ±0.025</td>
<td>-35.8 ±0.733</td>
<td>23.70 ±3.86</td>
<td>-</td>
<td>8.22 ±0.017</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>No Aggregates</td>
<td>150.4 ±5.45</td>
<td>0.032 ±0.029</td>
<td>-26.4*** ±0.576</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>No Aggregates</td>
<td>147.0 ±1.95</td>
<td>0.046 ±0.023</td>
<td>-23.2*** ±0.654</td>
<td>42.93*** ±1.06</td>
<td>-</td>
<td>7.55*** ±0.044</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>No Aggregates</td>
<td>145.9 ±1.38</td>
<td>0.048 ±0.030</td>
<td>-26.3***A ±0.931</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>No Aggregates</td>
<td>134.3 ±3.02</td>
<td>0.047 ±0.028</td>
<td>-22.3 ±0.817</td>
<td>28.54 ±3.59</td>
<td>97.81 ±2.1</td>
<td>9.11 ±0.383</td>
<td>46.19 ±1.01</td>
</tr>
<tr>
<td></td>
<td>@</td>
<td>No Aggregates</td>
<td>129.7 ±1.68</td>
<td>0.057 ±0.026</td>
<td>-40.9 ±1.14</td>
<td>16.75 ±4.18</td>
<td>49.34 ±2.55</td>
<td>19.1 ±0.058</td>
<td>20.42 ±0.116</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>129.1 ±1.90</td>
<td>0.057 ±0.023</td>
<td>-25.9 ±2.70</td>
<td>16.75 ±4.18</td>
<td>49.34 ±2.55</td>
<td>19.1 ±0.058</td>
<td>20.42 ±0.116</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>130.4 ±2.35</td>
<td>0.070 ±0.038</td>
<td>-29.1*** ±0.904</td>
<td>32.21*** ±3.40</td>
<td>46.32*** ±2.78</td>
<td>11.8*** ±0.072</td>
<td>11.36*** ±0.051</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>128.4 ±1.97</td>
<td>0.066 ±0.028</td>
<td>-27.6 ±0.568</td>
<td>37.95 ±3.18</td>
<td>54.59 ±2.33</td>
<td>7.19 ±0.161</td>
<td>8.208 ±0.004</td>
</tr>
</tbody>
</table>

Data are means±SD from n=3. ***: P<0.001 difference from 0 storage time. **: P<0.01 difference from 0 storage time. *: P<0.05 difference from 0 storage time. #: P<0.01 difference from 1 month storage duration, #: P<0.05 difference from 1 month storage duration. ΔΔ: P<0.01 difference from 3 month storage duration. Δ: P<0.05 difference from 3 month storage duration. @: The 8 month measurement was not evaluated for LP-Ox due to insufficient sample amount for 2 measurements at 8 and 12 month, thus the 12 month measurement was given a higher priority.
4.5 In-vitro drug release profile

The drug release profiles for the prepared liposomal formulations are illustrated in figure 4-4. In order to further understand the differences in release profiles and their underlying cause, the drug release profiles for the three prepared liposomal formulation LP-Ox, LP-Ox-AA, LP-Ox-Stp were evaluated relative to free oxaliplatin drug solution, Oxaliplatin spiked liposomal formulation LP-void and LP-Stp, and a commercial oxaliplatin liposomal formulation, Lipoxal.

![Graphs showing drug release profiles](image)

Figure 4-4 The drug release profiles for the prepare formulations. (A) Free Oxaliplatin release profile, (B) Lipoxal release profile of oxaliplatin, (C) LP-Ox release profile of oxaliplatin, (D) LP-Ox-AA release profile of oxaliplatin, (E)LP-OX-Stp release profile of oxaliplatin and satraplatin.

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Relative to the release profile of free oxaliplatin, LP-Ox-Stp was the only formulation having an oxaliplatin release profile significantly different from free oxaliplatin (P<0.01) showing the least cumulative % release of oxaliplatin, i.e. a more efficient system for controlled release, as illustrated in Figure 4-5(A). Upon comparison with Lipoxal drug release profile, it was found that Lipoxal has a significantly different release profile from all of the liposomal formulations prepared (P<0.001); however, it was noted that LP-Ox-Stp had the least significant difference from oxaliplatin release profile to that of Lipoxal (P<0.05), refer to Figure 4-5(B). In addition it was noted that the co-loading of ascorbic acid with oxaliplatin had no significant influence on the rate of oxaliplatin release from the liposomal system.

![Oxaliplatin comparative release](image1.png)

![Oxaliplatin comparative release](image2.png)

Figure 4-5 Comparative study for the oxaliplatin release profile for prepared liposomal formulation against (A) Free oxaliplatin, and (B) Lipoxal.

In an attempt to further understand the differences in the drug release profile obtained for the prepared liposomal formulations, an oxaliplatin spiked void liposomal formulation was used to examine its difference in terms of drug release from an oxaliplatin loaded liposomal formulation, LP-Ox, and free oxaliplatin solution (Figure 4-6). It was noted that there is no significant difference in the release profile between all three of them. A possible explanation for the similarity in oxaliplatin release profile between drug loaded liposomes (LP-Ox), and free oxaliplatin would be the rapid release of entrapped drug; as for the similarity of free drug release profile with that of spiked liposomes (LP-void+Oxpt.) outweigh the absence of any drug binding to the liposomal bilayer.
Figure 4-6 Comparative study for the oxaliplatin release profile for single drug loaded liposomal formulation LP-Ox against Free oxaliplatin, and oxaliplatin spiked void liposome LP-void+Oxpt.

Similarly, the dual drug loaded liposomal formulation LP-Ox-Stp was examined for its satraplatin and oxaliplatin release profiles relative to satraplatin loaded liposomes (LP- Stp), satraplatin loaded liposomes spiked with oxaliplatin (LP- Stp+Oxpt.), and oxaliplatin loaded liposomes (LP-Ox). In the case of satraplatin release profile, no significant difference was noted between dual drug loaded liposome, single drug loaded liposome, and single drug loaded liposome spiked with oxaliplatin as illustrated in Figure 4-7(A). On the contrary, oxaliplatin release profile was significantly different for the dual drug loaded liposome, LP-Ox- Stp, compared to single drug loaded LP-Ox, and oxaliplatin spiked satraplatin loaded liposomes, LP- Stp+Oxpt (P<0.05), refer to Figure 4-7(B). Thus, it can be concluded that satraplatin co-loading with oxaliplatin in a liposomal system has a significant retarding influence on the release of oxaliplatin (P<0.05). That could be due to one of the following reasons, either as a result of reduced permeability of the liposome lipid bilayer to oxaliplatin, or due to enhanced binding of oxaliplatin to the lipid bilayer. However, the lack of significant difference between the oxaliplatin spiked LP-Stp liposomes and oxaliplatin loaded liposomes negates the second reason, and outweigh the reduced liposome permeability to oxaliplatin as a result of satraplatin accommodation in the lipid bilayer.
Figure 4-7 Comparative study for dual drug loaded liposomal formulation LP-Ox-Stp (A) the satraplatin release profile against single drug loaded liposomal formulation LP-Stp, and oxaliplatin spiked liposomal formulation LP-Stp+Oxpt; (B) the oxaliplatin release profile against single drug loaded liposomal formulation LP-Ox, and oxaliplatin spiked liposomal formulation LP-Stp+Oxpt

This comparative analysis of the release data was further validated by the calculation of the similarity and difference factors, $f_2$ and $f_1$, for the release data in the same comparison pattern used, reported in Table 4-5. Taking into consideration that samples are considered different if $f_1 > 15$, and $f_2 < 50$, free oxaliplatin was found to have a different oxaliplatin release profile from that of Lipoxal, LP-Ox-Stp, and LP-Stp+Oxpt; while maintaining a similar oxaliplatin release profile to LP-Ox, and LP-Ox-AA. Lipoxal had a different oxaliplatin release profile from free drug and all liposomal formulations. LP-Ox had a similar oxaliplatin release profile to that of free drug, and LP-void+Oxpt. LP-Ox-Stp had a different oxaliplatin release profile from LP-Ox, and LP-Stp+Oxpt; and a similar satraplatin release profile to both LP-Stp, and LP-Stp+Oxpt.

### 4.6 Release Kinetics

Drug release data were fitted to seven dissolution-diffusion kinetic models (zero-order, first-order, Higuchi, Hixon-Crowell, Korsmeyer-Peppas, Baker-Lonsdale, and Michaelis-Menten), and their respective kinetic parameters and coefficient of determination ($R^2$) were calculated, refer to Table 4-6. In general, the zero-order, first-order, Higuchi, Hixon models were not suitable to explain the controlled drug release pattern obtained in this study. The plots had poor linear fit with low P-value and low coefficient of determination ($R^2 < 0.8$). However, the
Korsmeyer-Peppas, and Baker Lonsdale models had a perfect linear fit with the oxaliplatin release data for samples LP-Ox-Stp and LP-Stp+Oxpt, respectively ($R^2 >0.9$, $P<0.001$). However the value of $n$ the release exponent was found to be beyond the limits of korsmeyer-peppas model.

On the other hand, the two parameter, rectangular hyperbola model was found to fit the release data for all formulations perfectly with $R^2 >0.9$, $P<0.001$; except for the satraplatin release data from LP-Ox-Stp and LP-Stp, and oxaliplatin release from Lipoxal, with $R^2 <0.8$, $P<0.001$. The hyperbolic release pattern indicates that the rate of drug release is not dependent on the concentration.
Table 4-5 Difference and Similarity factors for comparative study

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Reference Free Oxpt</th>
<th>Reference LP-void+Oxpt</th>
<th>Reference Lipoxal</th>
<th>Reference LP-Ox</th>
<th>Reference LP-Stp</th>
<th>Reference LP-Stp+Oxpt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1</td>
<td>F2</td>
<td>F1</td>
<td>F2</td>
<td>F1</td>
<td>F2</td>
</tr>
<tr>
<td>Lipoxal</td>
<td>Oxaliplatin</td>
<td>71.65</td>
<td>8.940</td>
<td>65.27</td>
<td>15.34</td>
<td>-</td>
</tr>
<tr>
<td>Free Drug</td>
<td>Oxaliplatin</td>
<td>-</td>
<td>-</td>
<td>22.47</td>
<td>38.25</td>
<td>252.7</td>
</tr>
<tr>
<td>LP-void+Oxpt</td>
<td>Oxaliplatin</td>
<td>18.35</td>
<td>38.25</td>
<td>-</td>
<td>-</td>
<td>188.0</td>
</tr>
<tr>
<td>LP-Ox</td>
<td>Oxaliplatin</td>
<td>101.2</td>
<td>51.86</td>
<td>1.202</td>
<td>31.31</td>
<td>218.9</td>
</tr>
<tr>
<td>LP-Ox-AA</td>
<td>Oxaliplatin</td>
<td>5.482</td>
<td>55.25</td>
<td>15.76</td>
<td>44.62</td>
<td>233.3</td>
</tr>
<tr>
<td>LP-Stp</td>
<td>Satraplatin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LP-Stp+Oxpt</td>
<td>Oxaliplatin</td>
<td>18.99</td>
<td>37.47</td>
<td>0.787</td>
<td>91.76</td>
<td>185.7</td>
</tr>
<tr>
<td></td>
<td>Satraplatin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LP-Ox-Stp</td>
<td>Oxaliplatin</td>
<td>38.94</td>
<td>22.13</td>
<td>25.21</td>
<td>35.81</td>
<td>115.35</td>
</tr>
<tr>
<td></td>
<td>Satraplatin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4-6 Coefficient of determination, and drug release rates obtained from different mathematical model fitting of release data

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Zero-order</th>
<th>First-order</th>
<th>Higuchi</th>
<th>Hixson-Crowell</th>
<th>Korsmeyer-peppas</th>
<th>Baker-Lonsdale</th>
<th>Michaelis-Menten</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r²</td>
<td>K₀</td>
<td>r²</td>
<td>K₁</td>
<td>r²</td>
<td>K₈</td>
<td>r²</td>
</tr>
<tr>
<td>Lipoxal</td>
<td>Oxaliplatin</td>
<td>0.0378</td>
<td>0.0677</td>
<td>0.0377</td>
<td>-0.0003</td>
<td>0.1302</td>
<td>1.0755</td>
</tr>
<tr>
<td>Free Drug</td>
<td>Oxaliplatin</td>
<td>0.0787</td>
<td>0.3444</td>
<td>0.4773</td>
<td>-0.0172</td>
<td>0.1889</td>
<td>4.5644</td>
</tr>
<tr>
<td>LP-void+Oxpt</td>
<td>Oxaliplatin</td>
<td>0.0739</td>
<td>0.2749</td>
<td>0.1229</td>
<td>-0.0033</td>
<td>0.1891</td>
<td>3.7622</td>
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<tr>
<td>LP-Ox</td>
<td>Oxaliplatin</td>
<td>0.1358</td>
<td>0.4224</td>
<td>0.3988</td>
<td>-0.0127</td>
<td>0.2813</td>
<td>5.2102</td>
</tr>
<tr>
<td>LP-Ox-AA</td>
<td>Oxaliplatin</td>
<td>0.0213</td>
<td>0.1688</td>
<td>0.0027</td>
<td>0.0009</td>
<td>0.0801</td>
<td>2.7965</td>
</tr>
<tr>
<td>LP-Stp</td>
<td>Satraplatin</td>
<td>0.0987</td>
<td>-0.2321</td>
<td>0.1638</td>
<td>0.002</td>
<td>0.0231</td>
<td>-0.9608</td>
</tr>
<tr>
<td>LP-Stp+Oxpt</td>
<td>Oxaliplatin</td>
<td>0.1064</td>
<td>0.3297</td>
<td>0.2228</td>
<td>-0.0045</td>
<td>0.2344</td>
<td>4.1863</td>
</tr>
<tr>
<td></td>
<td>Satraplatin</td>
<td>0.1052</td>
<td>-0.2347</td>
<td>0.1688</td>
<td>0.002</td>
<td>0.0273</td>
<td>-1.0229</td>
</tr>
<tr>
<td>LP-Ox-Stp</td>
<td>Oxaliplatin</td>
<td>0.1766</td>
<td>0.326</td>
<td>0.2933</td>
<td>-0.003</td>
<td>0.3279</td>
<td>3.8003</td>
</tr>
<tr>
<td></td>
<td>Satraplatin</td>
<td>0.1308</td>
<td>-0.2302</td>
<td>0.1949</td>
<td>0.0017</td>
<td>0.0536</td>
<td>-1.2598</td>
</tr>
</tbody>
</table>

r² coefficient of determination, K₀ is the zero-order release rate constant, K₁ is the first-order release rate constant, K₈ is the Higuchi rate constant, K₈ is the cube root law release constant, n is the Korsmeyer peppas slope exponent, K₈ is the Baker-Lonsdale release rate constant, and K₈ is the Michaelis-Menten release rate constant.
4.7 In-vitro cytotoxic study

The cytotoxicity of the prepared liposomal formulations was examined on two cancer cell lines HepG2 and MCF-7, and one normal cell line BHK-21. In MCF-7, all tested liposomal formulations were found to cause a significantly higher cytotoxic effect than free oxaliplatin; with the following respective P-values LP-Ox, P<0.01; LP-Ox-AA, P<0.001; LP-Ox-Stp P<0.01; and Lipoxal, P<0.001 (Figure 4-8(A)). Similarly in HepG2 cell line, the cytotoxic effect of all liposomal formulations was significantly higher than free oxaliplatin with P<0.001, except for LP-Ox P<0.05 (Figure 4-8(B)). In addition, Lipoxal has shown a significantly higher cytotoxic effect in HepG2 cells over other liposomal formulations with P<0.001, except for LP-Ox-Stp P<0.01 (Figure 4-8(B)). On the contrary to cancer cells, there was no significant difference in cytotoxic effect between all tested formulations on normal cells, BHK-21; all formulations had an overall much weaker cytotoxic effect over normal cells relative to cancer cells (Figure 4-8(C)).
Figure 4-8 Effects of prepared liposomal formulations on cell viability of (A) MCF-7, (B) HepG2, and (C) BHK-21 cell lines relative to free oxaliplatin drug solution, the results are expressed as a percent of the control.

Upon comparing the IC\textsubscript{50} of the tested formulations in each cell line, it was found that the Lipoxal, LP-Ox-AA, and LP-Ox-Stp had a significantly lower IC\textsubscript{50} relative to free oxaliplatin, P<0.01, in HepG2 cell line; and in BHK-21 cell line LP-Ox-Stp and Lipoxal had a significantly lower IC\textsubscript{50} relative to LP-Ox with P<0.05, and P<0.01 respectively (Figure 4-9). However the IC\textsubscript{50} values obtained for LP-Ox, Free drug, and LP-Ox-AA in BHK-21 are extrapolated from cell viability data at lower concentrations since they are out of the oxaliplatin concentration range used in the experiment (0 - 28 µg/ml) (Table 4-7).

Table 4-7 In-vitro cytotoxicity of prepared liposomal formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>BHK-21</th>
<th>HepG2</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LP-Ox</td>
<td>90.19 ± 2.053</td>
<td>52.05 ± 1.758</td>
<td>34.11 ± 1.504</td>
</tr>
<tr>
<td>LP-Ox-AA</td>
<td>74.81 ± 1.779</td>
<td>40.78* ± 1.504</td>
<td>31.84 ± 1.477</td>
</tr>
<tr>
<td>LP-Ox-Stp</td>
<td>58.45## ± 1.650</td>
<td>36.77** ± 1.568</td>
<td>35.44 ± 1.507</td>
</tr>
<tr>
<td>Lipoxal</td>
<td>53.44## ± 1.677</td>
<td>27.56** ± 1.575</td>
<td>28.75 ± 1.545</td>
</tr>
<tr>
<td>Free Oxaliplatin</td>
<td>72.06 ± 1.932</td>
<td>69.82 ± 1.631</td>
<td>50.37 ± 1.555</td>
</tr>
</tbody>
</table>

Data are means±SD, n=2. *: P<0.05 difference from Free oxaliplatin. **: P<0.01 difference from Free oxaliplatin. #: P<0.05 difference from LP-Ox. ##: P<0.01 difference from LP-Ox.
Data are means±SD, n=2. *: P<0.05 difference from Free oxaliplatin. **: P<0.01 difference from Free oxaliplatin.

Figure 4-9 Comparative IC50 for the prepared formulations in different cell lines versus free oxaliplatin and Lipoxal

4.8 DNA damage preliminary data
Free oxaliplatin resulted in a relatively lower DNA damage as indicated from its immunofluorescence images showing few $\gamma$-H2AX foci and minimal pan-nuclear staining similar to Lipoxal and LP-Ox but with a slightly higher magnitude of DNA damage (Figures 4-10 and 4-11). Whereas LP-Ox-Stp demonstrated the highest DNA damage magnitude, exceeding 60% foci pan-nuclear staining.

![Immunofluorescence images for studying oxaliplatin induced DNA damage](image)

**Figure 4-10** Immunofluorescence images for studying oxaliplatin induced DNA damage

Data are means±SD, n=1.

**Figure 4-11** Magnitude of oxaliplatin and liposomal formulations induced DNA damage in MCF-7 cell line. (A) $\gamma$-H2AX foci analysis treated with 2μM oxaliplatin, (B) % cells with $\gamma$-H2AX foci pan-nuclear staining
5. Discussion

The combination of drugs in chemotherapy is often considered of great potential in yielding an enhanced cytotoxic efficacy that could be either due to an additive or synergistic effect [57]. The liposomal encapsulation of combinational chemotherapy would limit the associated toxicity by passively targeting cancer cells benefiting from the EPR effect in cancer tissues. These formulations also protect the chemotherapeutics from plasma protein and RBCs binding. Thus further enhance the intracellular accumulation of the combined chemotherapeutics within cancer tissues. It is therefore expected that the encapsulation of two cytotoxic agents in one liposomal system may synergistically or additively enhance their cytotoxic effects. In order to maximize the efficiency of liposomes as drug delivery systems certain key properties like size and surface charge need to be optimized. Generally, negatively charged liposomes containing anionic phospholipids such as phosphatidylglycerol (PG), and phosphatidylserine (PS) exhibit faster rates of endocytosis which enhance their cellular uptake, and are thus common in most FDA approved lipid-based formulations [97]. This is supported by the extensive use of PG in liposome preparation in several previously reported studies [40], [71], [72], [98]. One of the most interesting phospholipids in use in such liposomal preparations is phosphatidylethanolamine (PE) which is reported to facilitate membrane fusion mainly due to the poor hydration of its polar group [99]. However, the unique molecular shape of PE having small polar heads favor their arrangement in an inverted hexagonal phase (HII) rather than the formation of a lipid bilayer, where the lamellar to HII transition temperature is inversely proportional to the chain length and unsaturation of PE acyl chains [97], [99], [100]. It was reported that the incorporation of PE in a liposomal system containing 20-50 mol% of bilayer polymorphic lipids such as phosphatidylcholine (PC) can aid in PE stabilization within the bilayer structure of liposomes [99], [100].

In this study two liposomal systems were evaluated for use in dual drug delivery, these being DSPE-based liposome, LP, and DSPG-based liposome, LP-G. Despite the previous reports of neutral or slightly negative charge being exhibited by liposomes prepared using neutral lipids and 5 mol% DSPE-PEG2000, the LP liposomal system prepared and examined here was found to exhibit a significantly large negative charge [46], [97]. This unexpected negative charge exhibited by the LP liposomal system may be attributed to a reaction between dichloromethane
(DCM) the organic solvent used in the thin film formation phase of the liposome preparation, especially since this phase was conducted at a relatively high temperature of 60°C which might have induced a reaction between DCM and phospholipids, altering their structure. This conclusion is supported by a previous study that reported the possible decomposition or rearrangement of straight chain aliphatic amines into other compounds upon reaction with DCM [101].

Both liposomal systems investigated here are anionic in nature; however LP-G exhibits a higher negative charge. It is possible that having an anionic liposomal system would offer high encapsulation efficiency of oxaliplatin particularly upon aquation into cationic aquated species, mainly through electrostatic interactions. It has been reported that electrostatic interaction is a significant factor for binding between a cationic drug and a negatively charged liposome surface [102]. Consistent with this previous finding it was observed here that the net negative charge of the two liposomal systems decreased significantly upon oxaliplatin loading. This suggests possible neutralization of the liposomal negative charge by the cationic oxaliplatin aquated species. This observation was previously reported in studies suggesting that charged aquated species of oxaliplatin could form complexes with zwitterionic phosphatidyl groups [85]. However, it was noted that the significant difference in ζ potential between LP, and LP-G liposomal systems was not associated with an appreciable difference in oxaliplatin ultracentrifugation-encapsulation efficiency. That could be related to the DCM-induced possible structure modifications in lipid components thus altering the encapsulation behavior of liposomes. However, encapsulation efficiency calculated using the pellet permeabilization technique has determined that the incorporation of DSPG aid in enhancing the PP-EE% of oxaliplatin.

The dual drug loading experiments were conducted using the LP liposomal system. The study of the dual drug loading effect on the physical characterization of the liposomal system has revealed that the additional loading of ascorbic acid enhances the encapsulation of oxaliplatin in the liposomal system, associated with a decrease in the liposome ζ potential. On the other hand, the additional loading of satraplatin was associated with a significant decrease in oxaliplatin UC-EE%, with a concomitant increase in liposomal ζ potential. These results further support the involvement of electrostatic interaction in oxaliplatin loading. Previously reported studies are in
line with this conclusion indicating a direct correlation between the surface charge and the form of drug binding to the nanoparticles on the efficiency of drug loading and the rate of drug release from the nanoparticles [103]. The positive effect of ascorbic acid loading on the EE% of oxaliplatin could be explained by its anionic nature at neutral pH thus allowing for enhanced electrostatic attraction of oxaliplatin aqua species [104]. As for the negative effect of satraplatin additional loading on the UC-EE% of oxaliplatin, it might be a result of the accommodation of high densities of satrapatin within the dynamic structure of the saturated lipid membrane changing the behavior of the lipid bilayer in drug encapsulation, where satrapatin might disrupt the liposomal phase transition behavior [53], [97]. The TEM images (Figure 4-1) clearly indicate the formation of lipid bilayer in the form of large unilamellar vesicles (LUV) rather than HII phase. This suggests that the dual drug loading did not affect the structure of liposomes.

The prepared single and dual drug loaded LP liposomes were found to be highly stable for a 6 month duration upon storage at 4°C with results being in accordance with reported data for 5% DSPE containing liposomes even in the absence of DSPE-PEG [73]. In addition it was noted that LP-Ox-Stp had a high stability for 8 month at 4°C. However, it was noticed that the type of drugs loaded affect the stability of the liposomal system. LP-Ox-AA was the most stable liposomal formulation with no appreciable variation in size, ζ potential, or oxaliplatin EE%; but it encountered a significant decrease in ascorbic acid EE%, LP-Ox, and LP-Ox-Stp liposomal formulations on the other hand manifested a significant decrease in their ζ potential and a concomitant significant increase in their UC-EE% of oxaliplatin after 6 month storage at 4°C. This variation in EE% can be due to the formation of oxalate monodentate intermediates in aqueous solutions with an acid dissociation constant (pKa) of 7.23. This would result in limited dissociation in the presence of ascorbic acid (pKa = 4.17) and the consequent formation of ascorbate anion justifying the high stability and retained EE% upon storage of LP-Ox-AA. On the other hand, the oxaliplatin in LP-Ox and LP-Ox-Stp formulations are much more susceptible to this dissociation enhancing the interaction of oxaliplatin with the liposome lipid bilayer [105]. In addition, it was noticed that the liposomal formulation aid in enhancing the stability of ascorbic acid even upon long-term storage. This coincides with a previously reported study that has pointed out for the ability of liposomal systems in stabilizing ascorbic acid with more than 50% ascorbic acid retention after 1.5 month storage at 4°C [106]. This ascorbic acid stabilizing
effect of liposomes could be related to the high concentration of ascorbic acid accommodated within the liposomal system [106]. The relatively higher percent of ascorbic acid retained reported in this study after 6 month under the same storage conditions could be attributed to the use of a liposomal system characterized by the lower fluidity and permeability of its lipid bilayer relative to an egg phosphatidylcholine based liposomal formulation [106].

On the other hand, upon focusing on the PP-EE% for LP-Ox-Stp it can be noticed that the co-loading of satraplatin is associated with an enhancement of oxaliplatin PP-EE%. In addition, the interpretation of PP-EE% for the stability study was also found to differ from the UC-EE% particularly in LP-Ox, and LP-Ox-Stp. The oxaliplatin PP-EE% decreased significantly in LP-Ox and LP-Ox-Stp. This difference might be attributed to the absence of pellet washing step prior to the quantification of encapsulated drugs. Generally, it is believed that the PP-EE% protocol is capable of presenting more reliable data for the stability study about the encapsulation efficiency of the tested drugs over storage duration of 6 month at 4°C.

The in-vitro release profile for all liposomal formulations exhibited very similar patterns showing an initial bolus release of free drug followed by low rate release of encapsulated drug. The similarity of the in-vitro release profiles for LP-Ox and LP-Ox-AA liposomal formulations in comparison with free oxaliplatin and oxaliplatin spiked LP-void, suggest the presence of weak interactions between oxaliplatin and the liposome constituting phospholipids. These interactions are most likely hydrogen bonding, electrostatic and hydrophobic interactions that could be easily destroyed in a high ionic strength medium such as PBS. These results for LP-Ox and LP-Ox-AA are in contrast with recent report that have indicated the high encapsulation efficiency and higher retention of highly hydrophilic drug exhibited by long-chain saturated phospholipids in simulated physiologic conditions relative to short-chain ones [107]. Our results are also in contrast with previous reports indicating the poor interaction of zwitterionic phospholipids (dipalmitoyl phosphatidylcholine DPPC, and dipalmitoyl phosphatidylethanolamine DPPE) with cationic polynuclear platinum complexes relative to anionic phospholipids such as dipalmitoyl phosphatidylglycerol (DPPG) associated with both covalent and non-covalent interactions in the form of electrostatic and hydrogen bonding [108]. This might be attributed to the negative charge exhibited by the liposomal system LP and the use of long-chain saturated phospholipids characterized by their high hydrophobicity and dynamic membrane structure [53]. Thus capable
of efficiently encapsulating poorly membrane permeable drugs such as oxaliplatin, as well as promoting interaction of hydrophobic drug such as satraplatin with the lipid bilayer through hydrophobic interactions [53], [91], [99], [108]. Oxaliplatin was reported to be sensitive to 0.1M sodium chloride degrading into \( [(\text{oxalate})(\text{Cl}) \ Pt(\text{DACH})]^{1-} \) intermediate within 30 minutes which may either revert back to oxaliplatin or form \( [(\text{Cl})_{2}Pt(\text{DACH})] \). Here it is important to mention that the PBS used in our in-vitro release studies contained 0.154M sodium chloride thus there is an increased chance for oxaliplatin degradation maintaining a gradient for diffusion [109]. It was also reported that PE tends to invert into its HII phase when subjected to high ionic strength, however this hypothesis is eliminated at physiologic temperature as the lamellar – HII transition temperature for DSPE was reported to be 78.4 °C [99], [100], [110]. It is worth noting that in the in-vitro release profile for LP-Ox-AA it was not possible to observe the release profile for ascorbic acid due to its poor stability in aqueous media [111].

The percent of oxaliplatin release was significantly reduced in the LP-Ox-Stp formulation as a result of the formation of stronger interactions of oxaliplatin with the lipid bilayer saturated with satraplatin that could be associated with covalent bonds. Despite its low oxaliplatin encapsulation efficiency LP-Ox-Stp had the most efficient in-vitro release profile for controlled release. This is mainly attributed to the co-loading of satraplatin, releasing only 60% as an initial bolus release of the total oxaliplatin added. This indicates the presence of strong interaction between oxaliplatin and lipid bilayer where lipophilic satraplatin bind to membrane lipid molecules altering the fluidity of the liposomal membrane and consequently altering its behavior in encapsulation and release profiles of aqueous content [97], [108]. The release of satraplatin from LP-Ox-Stp was shown to be consistent with the un-encapsulated fraction based on the EE% results for satraplatin, see Table 4-3. However, it was noted that satraplatin showed slight degradation in the release medium which limit clear identification of whether LP-Ox-Stp liposomal system release the encapsulated fraction of satraplatin or not.

The fitting of the release profiles to hyperbolic Michaelis-Menten (hyperbola) kinetic model further indicates the presence of weak interactions between oxaliplatin and the liposomal delivery systems LP-Ox and LP-Ox-AA that tends to rapidly dissociate in simulated physiologic conditions independent on the concentration [112]. The LP-Ox-Stp formulation on the other hand had a slightly different release profile with an initial bolus release of the free fraction of the
drug followed by a slow rate of drug release of the bound and encapsulated oxaliplatin. The FT-IR spectra obtained are in line with these conclusions with the carbonyl group disappearance from spectra for LP-Ox and LP-Ox-AA indicating the involvement of the carbonyl group in hydrogen bonding. This is further corroborated by the intense broad OH peaks detected in the range of 3300 – 3500 cm⁻¹, as illustrated in Figure 4-2 (A) and (B) [113]. The inert nature of satraplatin on the other hand preserved the key functional groups in the LP-Ox-Stp FT-IR spectrum (see Figure 4-2 (C)) [10], [12], [13].

Relative to the liposomal formulations prepared here, Lipoxal had a much smaller particle size, and a relatively low ζ potential which was unexpectedly associated with high colloidal stability. Although several studies have related high surface charge of nanoparticles to colloidal stability, nanoparticles having a coat of polymers or large molecules attain high colloidal stability even at low surface ζ potential [103], [114]–[117]. The release profile for Lipoxal was as similar to that of LP-Ox-Stp, showing only 30% initial bolus release of the total added oxaliplatin indicating strong interactions between oxaliplatin and the lipid bilayer upon reflecting on the EE% results for Lipoxal, refer to Table 4-3. However on the whole, the Lipoxal release profile was significantly different from that of all prepared liposomal formulations. It is also interesting to note that Lipoxal was found to poorly fit all of the release kinetic models used in this study.

It was evident from the in-vitro cytotoxicity results that liposomal encapsulation of oxaliplatin enhances its cytotoxic efficiency on HepG2, and MCF-7 cancerous cell lines while showing minimal cytotoxic effect for BHK-21 non-cancer cells. Lipoxal also showed a significantly higher cytotoxic effect on HepG2 cells than other liposomal formulations. However, a closer examination of the calculated IC50 for the tested liposomal formulations, indicates that liposomal formulations with significantly higher cytotoxic efficacy relative to free oxaliplatin in HepG2 cell line are in the following order Lipoxal> LP-Ox-Stp >LP-Ox-AA. In MCF-7, the difference between all liposomal formulations and free oxaliplatin was relatively high where lipoxal had the highest cytotoxicity and the rest of the formulations followed in this order Lipoxal>LP-Ox-AA>LP-Ox>LP-Ox-Stp>free oxaliplatin. Generally, the liposome encapsulated combination of therapeutics LP-Ox-AA, and LP-Ox-Stp are more cytotoxic in HepG2 cells than the single drug loaded liposomes LP-Ox. The cytotoxicity reported for LP-Ox-AA is relatively high despite the low content of ascorbic acid within the liposomal formulation relative to
previously reported cytotoxic concentrations of ascorbic acid reaching 1mM needed to induce cell death by generating extracellular hydrogen peroxide [58], [118]. The co-loading of ascorbic acid in this work, even at non-cytotoxic concentration was found to enhance the encapsulation efficiency of oxaliplatin, the formulation stability and its cytotoxic efficiency. It can be noted from these results that only Lipoxal and LP-Ox-Stp offer a constant cytotoxic potency in the two tested cancer cell lines. The observed cytotoxicity of liposomes in BHK-21 non-cancer cell line could be due to the free un-encapsulated fraction of drug which does not allow us to conclude whether normal non-cancerous cells are targeted by the prepared liposomal formulations. The significant difference in IC50 values of each liposomal formulation between cancerous and non-cancerous cells could be considered as an indication for their lower cytotoxicity towards normal cells. However, the disability of MTT assay in significantly differentiating the prepared liposomal formulations based on their cytotoxic activity in MCF-7 cell line lead us to use a more sensitive γ-H2AX assay to observe the extent of DNA damage induced by the liposomal formulations relative to free oxaliplatin and Lipoxal.

Preliminary data on DNA damage have demonstrated significant DNA-damage induced by LP-Ox-Stp in MCF-7 cell line and relatively low DNA damage induced by Lipoxal. These results obtained for Lipoxal are comparable to previously reported studies where despite the high cellular uptake demonstrated by Lipoxal in HCT116 cells, only a small fraction was bound to DNA (≤ 5%) [119]. In addition, the DNA damage exhibited by free oxaliplatin had a lower magnitude than that exhibited by Lipoxal, this result is not supported by the results of a previous study that focused on DNA accumulation for free oxaliplatin and Lipoxal in HCT116 cells where free oxaliplatin was reported to have 4-fold more platinum to DNA compared with Lipoxal after 24 h incubation [119]. In the case of LP-Ox, and LP-Ox-Stp, the high magnitude of DNA damage may be due to satraplatin and oxaliplatin forming DNA adducts that are not recognizable by proteins involved in DNA MMR system [120], [121]. This may in turn result in stalling of DNA replication fork which is associated with the generation of single stranded DNA breaks (SSB) inducing the activation of ataxia telangiectasia mutated and Rad3-related (ATR) pathway [122], [123]. This would subsequently prevents the collapse of stalled replication forks into double stranded DNA breaks (DSB) [122], [123]. Conditions that result in replication fork collapse also induce H2AX phosphorylation, therefore a phosphorylated histone H2AX can act
as a marker for DSB [123], [124]. In this case, p38 mitogen-activated protein kinase (MAPK) is activated by two pathways, (i) ataxia telangiectasia mutated (ATM)-mediated indirect activation of p38 MAPK in response to DSB, and down regulation of ATR, (ii) increase p38 MAPK phosphorylation as a result of oxaliplatin treatment, as previously reported in HCT116, which in turn result in hyperphosphorylation of γ-H2AX [124], [125]. This will then result in excessive DNA damage throughout the chromatin as demonstrated by pan-nuclear staining rather than distinct foci in immunofluorescence imaging (see Figure 4-10)[126]. It is worth reporting that DNA damaged cells upon their attempt to pursue DNA replication can either move into cell cycle arrest or proceed with one or several apoptic pathways [122]–[126]. Despite the very significant DNA damage detected for LP-Ox-Stp in MCF-7 cell line and the low magnitude of Lipoxal induced DNA damage the cytotoxicity profile was more potent for Lipoxal than LP-Ox-Stp in the same cell line. The Pattern observed based on results of the in-vitro cytotoxicity in MCF-7 and DNA damage the higher the magnitude of DNA damage, the lower is the cytotoxic potency of the liposomal formulation. However, the γ-H2AX is a far more sensitive approach in quantifying the cytotoxic ability of drug molecules. In addition, it was previously reported that MTT assay is associated with some limitations as it depends on the metabolic activity of the tested cells, as well as possible interference of a some chemical compounds with the MTT assay [127]. Therefore, it would be recommended to confirm the γ-H2AX DNA damage results with a more sensitive approach for cell viability quantitation such as clonogenic survival assay.

It was reported that the reason for Lipoxal accumulation in cytoplasm is its proposed mechanism of fusion with the cell membrane of cancer cells [119]. However, LP liposomes are also expected to be subjected to cellular uptake by membrane fusion facilitated by its content of PE, and were found to be associated with massive DNA damage that could also be interpreted as DNA accumulation. In addition, PE was reported to be associated with induction of apoptosis in HepG2 cell line by stimulating a decrease in mitochondrial membrane potential and subsequent increase in Bax and caspase-3 expression [128].

Although in-vitro studies tend to aid in clarifying the biological activity of the liposomal systems they are not sufficient to give a comprehensive vies on the biological activity of prepared formulations, where in-vivo studies are necessary to take into account other factors that would
influence the cytotoxic activity of liposomal formulations such as tumor volume, vascular permeability, tumor interstitial pressure and possible metastasis [52].
6. Conclusion and Future work

This study was set out to explore the potential use of liposomal delivery systems as carriers for dual drug targeting combination therapy. The study worked on developing an anionic liposomal system offering high encapsulation efficiency for oxaliplatin with particle size less than 200 nm and has identified the effect of dual drug loading on particle size, zeta potential, encapsulation efficiency and long-term stability. The study has also sought to determine whether dual drug loading can result in effective anti-cancer efficacy with minimal toxicity in normal cells. Despite the wide use of oxaliplatin in combinational therapy, to the best of our knowledge previous studies did not cover the use of oxaliplatin in dual drug loaded delivery systems. The study sought to determine the ability of the developed liposomal system in being co-loaded by hydrophobic and hydrophilic drugs and two hydrophilic drugs while retaining its physical characteristics and stability. In addition, the study has evaluated the effect of dual drug loading on the cytotoxic efficacy and DNA damage induction.

Based on the characterization of single drug loaded liposomal formulation, it was found that the use of phosphatidylglycerol based phospholipids did not show a significant enhancement in oxaliplatin encapsulation relative to phosphatidylethanolamine phospholipids (23.7%). The dual drug loaded liposomes were prepared by the active extrusion method. Two dual drug loaded liposomal formulations were prepared being LP-Ox-AA, carrying oxaliplatin and ascorbic acid and LP-Ox-Stp, loaded with oxaliplatin and satraplatin. Characterization of the prepared liposomal formulations was done using zetasizer, TEM, and FT-IR. The encapsulation efficiency and the in-vitro release profile were also studied for each liposomal formulation. The study has determined that the use of liposomes as a drug delivery system for combinational chemotherapy aids in enhancing their intracellular accumulation and cancer cell targeting, thus maximize the cytotoxic efficiency and minimize the toxicity of drugs delivered. The combination of oxaliplatin with a chemo-sensitizing agent such as ascorbic acid at non-cytotoxic concentration in a liposomal delivery system was found to enhance the cytotoxic profile of oxaliplatin towards cancer cells. In addition, ascorbic acid aided in improving the encapsulation efficiency (28.5%) and the stability profile of oxaliplatin in the dual loaded liposomal formulation. The liposomal encapsulation of ascorbic acid had a stabilizing influence on ascorbic acid upon long-term storage at 4°C. As for the dual chemotherapeutic loaded liposomal system composed of
oxaliplatin and the hydrophobic drug satraplatin, it was shown that satraplatin loading had a negative influence on oxaliplatin encapsulation efficiency (16.8%), while enhanced oxaliplatin retention under simulated physiological conditions. In addition, the developed liposomal system was capable of encapsulating hydrophobic satraplatin at high levels (49.3%). The dual chemotherapeutic loaded liposome was found to utilize the potent cytotoxicity of both drugs in amplifying the cytotoxic potential of the whole liposomal formulation, even relative to the commercial Lipoxal formulation by inducing more potent DNA damage.

A general trend was observed in this study for the loading of oxaliplatin in a liposomal system, where the percent of oxaliplatin encapsulation was found to be directly proportional to the reductive change in magnitude of the negative surface potential of the liposome particles. This indicates the involvement of electrostatic interaction in the loading of oxaliplatin thus relating to the role of ascorbic acid in enhancing oxaliplatin encapsulation. Furthermore, the release profile depended to a great extent on the rapid dissociation of these electrostatic interactions in high ionic strength medium. However, the incorporation of hydrophobic satraplatin was found to enhance oxaliplatin retention even in high ionic strength medium by altering the fluidity of the lipid bilayer of the liposome delivery system.

Future investigations may include studies of possible reactions between DCM and phospholipids maintained at 60°C for 1 hour using mass spectrometry, to help determine the structural changes within amine containing phospholipids such as phosphatidylcholine. Since the MTT cytotoxicity assay results did not significantly differentiate the cytotoxic potency of the prepared liposomal formulations. Clonogenic survival assay may act as a more sensitive approach in quantifying the cytotoxicity potential of each liposomal formulation. In addition, animal studies will be required to determine the anti-cancer effect of the prepared dual drug loaded liposomal formulations LP-Ox-AA and LP-Ox-stp and their potential advantage in reducing the systemic toxicity of oxaliplatin and limit the drug in-vivo distribution to the tumor tissues.
7. References


transporter CTR1 to the cellular accumulation of cisplatin, carboplatin, and oxaliplatin.,”


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Appendix A: Calibration Curves

### Oxaliplatin Calibration Curve

- **Equation:** $y = 85.444x$
- **$R^2$:** 1

### Ascorbic Acid Calibration Curve

- **Equation:** $y = 124.67x$
- **$R^2$:** 0.998
Satraplatin Calibration Curve

Absorbance at 210 nm vs. Concentration of Satraplatin (mM)

- Equation: $y = 673.21x$
- $R^2 = 0.9992$