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Docetaxel in liposomes: the effect of lipid composition on the achieved drug entrapment

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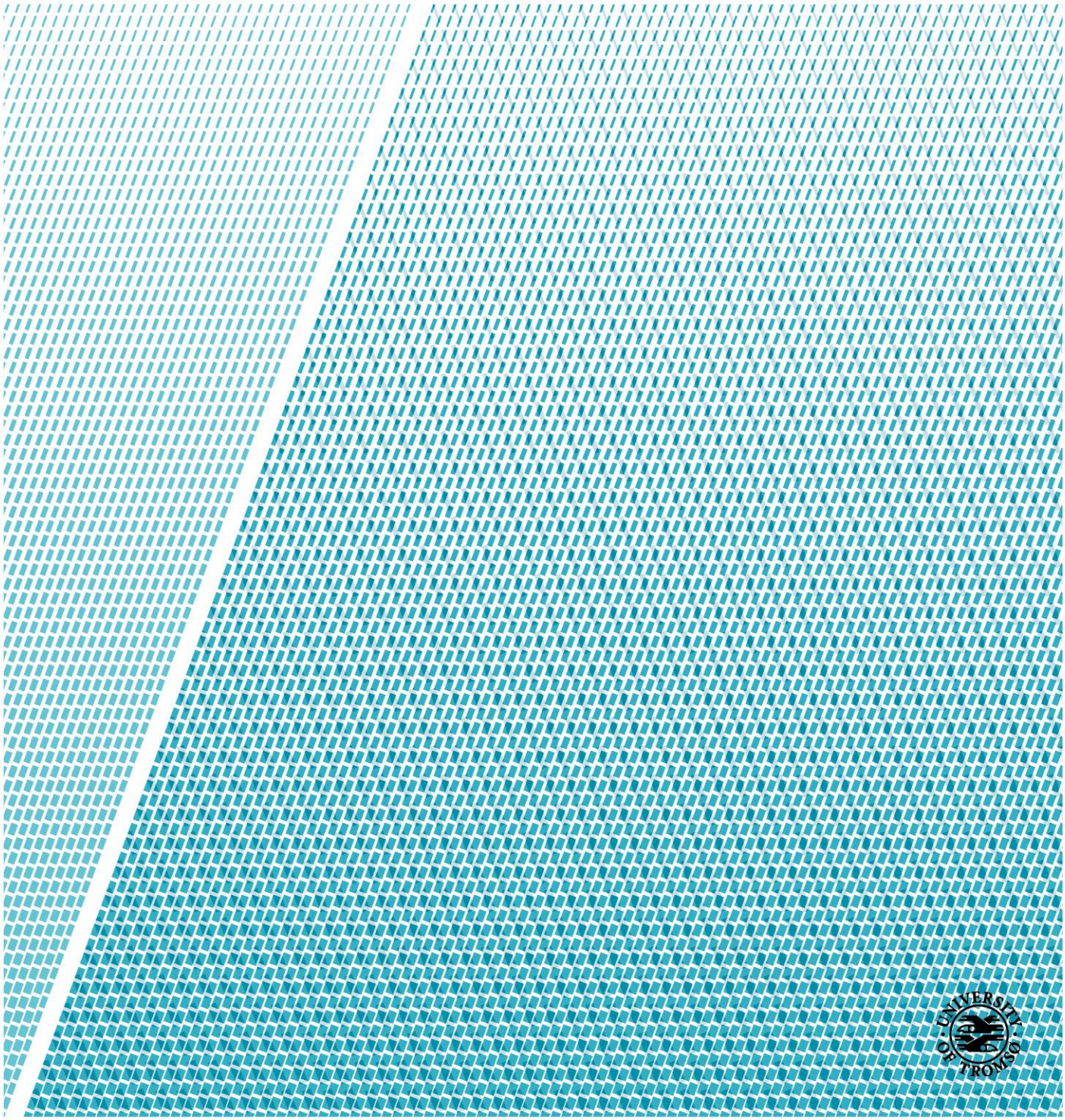

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Sammendrag

INTRODUKSJON: Docetaxel (DOC) er et potent kjemoterapeutisk legemiddel, men som har flere begrensninger inkludert dårlig løselighet. Dagens tilgjengelige formulering med DOC begrenses av rapporterte alvorlige bivirkninger som enten skyldes legemidlet i seg selv eller hjelpestoffene brukt i formuleringen. Nanomedisin har blitt brukt for å forbedre det terapeutiske utfallet av flere legemidler, men til nå er det ingen tilgjengelige nano-formuleringer med DOC. Det er derfor interessant å undersøke om en liposomal formulering med DOC kan løse legemidlet og forbedre det terapeutiske utfallet.

FORMÅL: Målet med denne oppgaven var å etablere en metode for tillaging og karakterisering av DOC-liposomer i liten skala, for videre å undersøke effekten av ulike lipidkomposisjoner for inkorporering av legemiddel.

METODE: DOC-liposomer ble fremstilt av ulike lipidkomposisjoner ved bruk av «thin-film hydration» metode og størrelsesredusert ved hjelp av sonikering. Sentrifugering ble brukt som metode for å fjerne fritt legemiddel fra den liposomale formuleringen. Effekten av ulike lipidkomposisjoner på inkorporering av legemiddel ble undersøkt med karakterisering av de ulike lipsomale formuleringene.

RESULTATER: De 14 ulike liposomale formuleringene med ulik lipidkomposisjon og legemiddel:lipid ratio på 10:1 (vekt/vekt) viste en inkorporering mellom 18 og 115 %. Tre av de liposomale formuleringene viste inkorporering av DOC nær 100 % og ble undersøkt videre ved å øke legemiddel:lipid ratio til 2:10 (vekt/vekt) for å se om mer DOC kunne inkorporeres i liposomene. Økt legemiddel:lipid ratio reduserte inkorporering av DOC i liposomene for alle tre formuleringene, men soya-fosfatidylkolin (SPC) og positive ladet 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) (8:2 vekt/vekt) formuleringen hadde den høyeste DOC-konsentrasjonen. Vi undersøkte derfor effekten av å variere konsentrasjonen av DOTAP i forhold til SPC. Resultatene viste at en høyere konsentrasjon av DOTAP ga et høyere utbytte av DOC.

KONKLUSJON: Vi lyktes med å etablere en småskala metode for å screene ulike liposomale formuleringer for deres evne til å ta opp DOC i liposommembranen. I screening studien fant vi at liposomale formuleringer som inneholdt det kationiske lipidet DOTAP viste bedre inkorporering av de ulike liposomkombinasjonene som ble undersøkt. En økende mengde DOTAP i SPC:DOTAP liposomer viste en økende inkorporering av DOC.

Nøkkelord: docetaxel, liposomer, lipider, lipidkomposisjon, legemiddelformulering, inkorporering av legemiddel.

Abstract

INTRODUCTION: Docetaxel (DOC) is a potent anticancer drug but has several limitations such as poor solubility. The currently commercially available formulation of DOC is limited by reported serious side effects which either is attributed to the drug itself or the solvent used. Nanomedicine has been used for improving the therapeutic outcome of several drugs, but so far there are none available with DOC. It would be interesting to apply a liposomal formulation for delivery of DOC in order to solubilize the drug and improve the therapeutic outcome.

OBJECTIVES: The aim with this master project was to establish a small-scale screening method for making and characterizing DOC liposomes and further investigate the effect of lipid composition on drug entrapment to find a suitable liposomal formulation of DOC.

METHODS: DOC liposomes were made of different lipid compositions by a thin-film hydration method and size reduced by probe sonication. Centrifugation was used to remove free DOC from the liposomal formulation. The effect on varying lipid compositions on drug entrapment were tested with characterization of the liposomal formulations.

RESULTS: The 14 different liposomal formulations with varying lipid compositions and a drug:lipid ratio of 1:10 (w/w) showed an entrapment efficiency between 18 and 115 %. Three of the liposomal formulations showed entrapment efficiency near 100 % and were further investigated by increasing the drug:lipid ratio to 2:10 (w/w) to see if more DOC could be entrapped in the liposomes. The DOC recovery were low for all three formulations, although the soy phosphatidylcholine (SPC):DOTAP liposomes showed a higher recovery compared to the other two. The SPC:DOTAP were brought further to investigate the effect of varying the concentration of DOTAP, and the results showed that a higher DOTAP concentration gave a higher recovery of DOC.

CONCLUSION: A small scale screening method for investigating the effect of different liposomal formulations on the archived DOC entrapment was established successfully. The liposomes with cationic lipid, DOTAP, showed best entrapment efficiency of the different liposomal combinations screened. Increasing amount of DOTAP within SPC:DOTAP liposomes showed a greater incorporation of DOC.

Key words: docetaxel, liposomes, lipids, lipid composition, drug carrier, formulation, entrapment efficiency.

List of Abbreviations

CC6	Ceramide C6
CC12	Ceramide C12
Chol	Cholesterol
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DMPE	1,2-dimyristoyl-*sn*-glycero-3-phosphoetanolamine
DMPG	1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)
DOC	Docetaxel
DOPC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DPPG	1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
DSPE	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine
EE %	Entrapment Efficiency %
EPR effect	Enhanced Permeability and Retention effect
HPLC	High Performance Liquid Chromatography
Lipoid S 100	Soybean lecithin, 100 % phosphatidylcholine
LUV	Large Unilamellar Vesicles
MLV	Multilamellar Vesicles
MPS	Mononuclear Phagocyte system
NP	Nanoparticle

PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PG	Phosphatidylglycerol
PI	Polydispersity index
PL	Phospholipid
POPC	1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine
POPE	2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphoetanolamine
SD	Standard deviation
SPC	Soy phosphatidylcholine
SUV	Small unilamellar vesicles
Tm	Phase transition temperature
ZP	Zeta potential

1 General introduction

Cancer causes millions of deaths each year and is the second leading cause of death globally (WHO, 2018). The first line treatment for most cancers today are surgery, radiation and chemotherapy (Kumari *et al*., 2016). Although docetaxel (DOC) is one of the most important cytotoxic agents in clinic today, it has several limitations such as poor solubility, nonselective distribution and fast elimination. In the currently commercially available formulation of DOC, it has been reported serious side effects such a neutropenia, musculoskeletal toxicity, peripheral neuropathy and hypersensitivity, which either can be attributed to the drug itself or to the solvent used (polysorbate 80) (Tan *et al*., 2012).

Nanomedicine is a technology in which special drug systems, drug delivery systems, uses nano sized particles, including liposomes for medical applications (Nehoff *et al*., 2014). In general, employment of drug delivery systems with a carrier and an entrapped drug can improve the pharmacological properties of the drug compared with the conventional "free drug" (Allen and Cullis, 2004).

Liposomes is an attractive drug carrier of several reasons. First, both hydrophilic and lipophilic drugs can be encapsulated or entrapped in liposomes (Tan *et al*., 2012). Second, the membrane composition of liposomes can be altered and composed of a variety of lipids and lipid combinations which in turn can change the organization of the membrane, charge and stability (Brandl, 2001). Third, size can be altered which is favourable in terms of longer half-life and altered pharmacokinetics (Fanciullino and Ciccolini, 2009; Bozzuto and Molinari, 2015). Fourth, liposomes are in general non-toxic, biocompatible and biodegradable (Akbarzadeh *et al*., 2013). Fifth, liposomal formulations are often applied to solubilize the drug which is beneficial with poorly soluble drugs (Laouini *et al*., 2012). Finally, liposomes could be modified with targeting moieties to achieve active targeting, or modified to achieve a triggered release mechanism, or altered with surface modifications to give long circulating liposomes.

In the last decades, extensive research has been done to incorporate drugs into liposomes to improve therapeutic outcome, and currently several formulations are commercially available and more formulations are under clinical trials (Bulbake *et al*., 2017).

It is desirable to overcome the limitations and side effects alongside with improving the anticancer effects of DOC compared to the currently commercially available DOC formulation. Hence there is comprehensive focus on studies on applying a nanocarrier with DOC entrapped to overcome these problems. As far as we are concerned, there is no commercially available nanocarrier with DOC to this date (Louage *et al*., 2017). Hence, it is desirable to develop a liposomal formulation which shows good tolerability, minimal side effects and a good therapeutic effect.

By applying a liposomal formulation for DOC entrapment to solubilize the highly lipophilic drug, one could avoid the use of Polysorbate 80. The partition coefficient and polarity of a drug determines where the drug will be located in the liposomal membrane, and further the entrapment efficiency of the drug (Bozzuto and Molinari, 2015). Lipophilic drugs, like DOC, can be entrapped in the lipid bilayers of the liposome (Tan *et al*., 2012) and because of their lipophilic nature they reside in the acyl chains of the liposome. The entrapment efficiency of drugs is dependent on the acyl chain properties, like length of acyl chain and packing density, in addition to changes in the drug:lipid ratio (Bozzuto and Molinari, 2015).

We aimed at development of a liposomal formulation with DOC incorporated in the lipid bilayer. To find a suitable formulation, we explored the effect of lipid composition in liposomes and what effect the composition would have on entrapment and drug load of DOC.

2 Introduction

2.1 Cancer-targeted drug delivery

According to the World Health Organisation (WHO), cancer is the second leading cause of death globally, responsible for 8.8 million deaths in 2015. Cancer is a collective term applied for a numerous of diseases which can affect any part of the body, and the most common causes of cancer deaths are lung cancer, liver cancer, colorectal cancer, stomach cancer and breast cancer. Cancer is caused by transformation of normal cells into tumour cells in a complex process which progresses from pre-cancerous lesion to malignant tumour (WHO, 2018).

Today the first line treatment for most cancers are surgery, chemotherapy and radiation. Chemotherapy is a highly non-specific strategy in targeting drugs to cancer tissues which leads to undesirable side effects to healthy tissues (Kumari *et al*., 2016). As a consequence, healthy proliferative cells in bone marrow, hair follicles and the gastrointestinal tract gets killed and thereby leading to common side effects like compromised immune defence because of decreased production of leukocytes, platelets and red blood cells, hair loss and inflammation and ulceration of mucus membranes in the gastrointestinal tract (Dawidczyk *et al*., 2014). Thus, conventional chemotherapy drugs suffers from several limitations, like severe toxicity to normal cells, non-specific biodistribution, inadequate drug concentrations at cancer cells, development of multiple drug resistance and poor aqueous solubility (Kumari *et al*., 2016).

Nanomedicine and application of nanoparticles (NPs) as drug delivery systems for treatment of cancer have received extensive attention in recent years (Kumari *et al*., 2016). Nanomedicine is a branch under nanotechnology which are focused on development of pharmaceuticals (Etheridge *et al*., 2013), where use of special drug delivery systems are used for medical applications like treatment of cancer, medical imaging and diagnostics (Nehoff *et al*., 2014).

The European Science Foundation (ESF) defined nanomedicine in 2004 as: *"The field of Nanomedicine is the science and technology of diagnosing, treating and preventing disease and traumatic injury, of relieving pain and of preserving and improving human health, using molecular tools and molecular knowledge of the human body…. The aim of "Nanomedicine" may be broadly defined as the comprehensive monitoring, control, construction, repair, defence and improvement of all human*

biological systems, working from the molecular level using engineered devices and nanostructures, ultimately to achieve medical benefit" (European Science Foundation, 2004).

A drug delivery system consists of a carrier and a therapeutic drug (Jain, 2008). The drug delivery system-carrier can be a NP generally composed of lipids or polymers which are designed to improve the therapeutic and pharmacological properties of the therapeutic drug. In general, employment of drug delivery systems with a carrier and an entrapped drug can improve the pharmacological properties of the drug compared with the conventional "free drug" (Allen and Cullis, 2004). The drug delivery process includes administration of the drug delivery system, release of the therapeutic drug by the carrier, transport of the therapeutic drug across the biological membranes to the desired site of action (Jain, 2008).There are several types of nanoparticles used for drug delivery carriers, such as liposomes, micelles, polymeric nanoparticles, polymeric micelles, dendrimers, carbon nanotubes, nano-shells, viral nanoparticles and inorganic (metal) nanoparticles (Cho *et al*., 2008; Kumari *et al*., 2016).

NPs offers several advantages in treatment of cancerous diseases which make them attractive compared to conventional chemotherapy. A major advantage with NPs is that they can improve solubility by solubilize poorly soluble, lipophilic drugs in hydrophobic compartments and therefore improve the pharmacokinetics of the drug compared to conventional medicine (Kumari *et al*., 2016).

Another important property of NPs is the size in nano-range that could alter the pharmacokinetics of the drug and favour nanomedicine over conventional medicine (Nehoff *et al*., 2014). Conventional drugs may have a widespread distribution in the body which may affect normal, non-target tissues. Employment of a drug entrapped in a NP will on a general basis affect the clearance of the drug in direction downwards, so the half-life increases, the distribution volume decreases and the area under the time versus concentration curve increases (Allen and Cullis, 2004; Nehoff *et al*., 2014). The lower distribution volume will reduce the impact and potential side effects in normal tissues (Allen and Cullis, 2004).

NP employed for cancer treatment can enhance safety, bioavailability and therapeutic efficacy compared to conventional therapy. NPs can exploit an inherent passive targeting phenomenon, or be altered and hence offer strategies of active targeting to cancerous tissue (Kumari *et al*., 2016).

Many solid tumours have a unique pathophysiologic characteristic that distinguish them from healthy tissue, which anticancer drugs can exploit (Natfji *et al*., 2017). Many cancer cells grow fast and therefore demands energy in form of nutrients and oxygen, which leads to development of new blood vessels, a process called neovascularization, or recruitment of nearby existing blood vessels to supply the tumour cells. This results with highly disorganized angiogenic blood vessels and dilated tumours with enlarged gaps between the endothelial cells, which enables macromolecules, including NPs, to permeate and accumulate in tumour tissues (Cho *et al*., 2008; Natfji *et al*., 2017).

In addition, tumours show lack of- or compromised lymphatic drainage, hence NP gets retained present in the interstitial fluid of tumours for a longer time compared to normal tissues which have functional lymphatics (Peer *et al*., 2007). The result of the enhanced permeability of the tumours vascularity together with compromised lymphatic drainage leads to passive and to a certain extent, selective accumulation of extravasated macromolecules inside tumour cells, which in turn reduces the clearance from the tumour tissue (Natfji *et al*., 2017). This is called the enhanced permeability and retention (EPR) effect, illustrated in Figure 1 (Kumari *et al*., 2016).

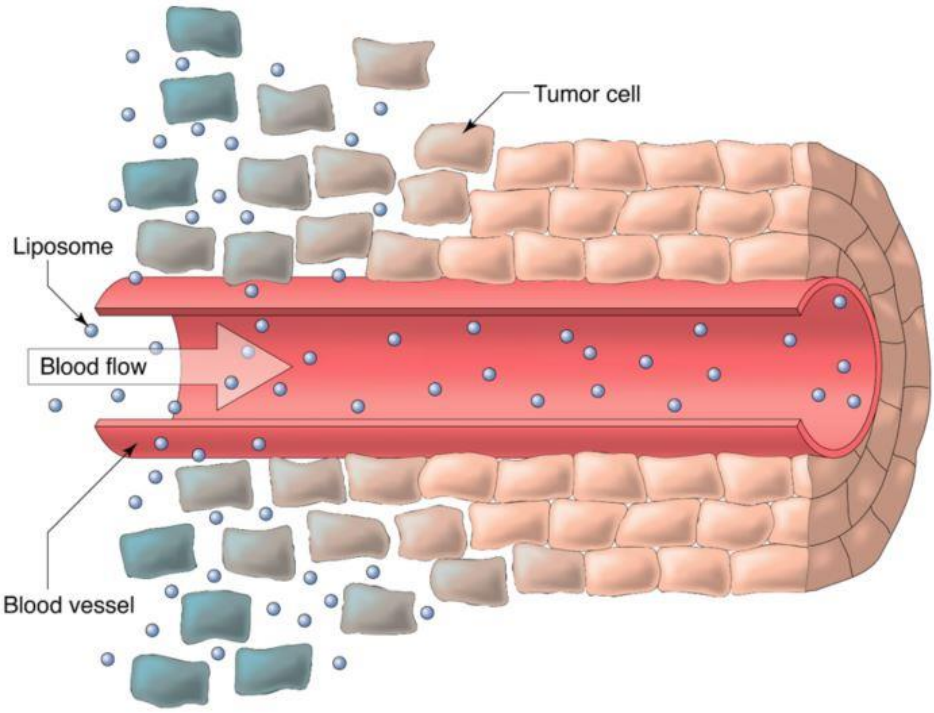


Figure 1*: A schematic illustration of the Enhanced Permeability and Retention effect is imagined to take place in leaky tumour vessels* (Sætern, 2004) *(with permission).*

NPs tends to exploit the enhanced permeability and retention (EPR) effect, especially to tumours and inflamed tissues. Because of their small size, NPs can extravasate through cellular barriers.

There are several factors that determine the efficacy of anticancer drugs. First, the ability to penetrate a variety of barriers and reach the tumour without losing activity or amount drug when traveling through the blood circulation is crucial. Second, the drug should have the ability and selectivity to avoid killing normal cells and tissue, but only affect tumour cells in a controlled manner (Cho *et al*., 2008).

It is crucial for the drug to stay in the blood circulation for sufficient enough time to effectively reach the tumour. The chance of being caught by the mononuclear phagocyte system (MPS) is high if the NPs have unmodified surface characteristics. The fate of injected NPs can, to a certain extent, be controlled by modifying the size of the NPs and modifying the surface characteristics like making the surface of a NP hydrophilic. The surface can be modified to be more hydrophilic by coating the surface with polyethylene glycol (PEG) which is a hydrophilic polymer, a process known as PEGylation. PEGylation will protect NPs from opsonization by macrophages by repelling them (Cho *et al*., 2008).

2.1.1 Docetaxel

N-debenzoyl-N-*tert*-(butoxycarbonyl)-10-deaxetyltaxol, or docetaxel (DOC) is a semisynthetic Taxol/paclitaxel analogue (Guéritte-Voegelein *et al*., 1991) which belongs to the taxane family, a class of anticancer drugs (Immordino *et al*., 2003). DOC is prepared by semi synthesis from 10-deacetylbaccatin-III, which are an inactive precursor that are isolated from needles of the European yew tree, *Taxus baccata* (Zhang and Zhang, 2013). DOC is used for treatment of breast cancer, non-small cell lung cancer, prostate cancer, gastric adenocarcinoma and head and neck cancer (Louage *et al*., 2017).

Both DOC and paclitaxel are poorly soluble drugs. The chemical structure of DOC in Figure 2 shows a complex taxane ring that is linked to an ester at the C-13 position. The hydrophobic domains of the fused ring system and side chain of DOC contributes to poor aqueous solubility (Straubinger and Balasubramanian, 2005). DOC is slightly more water-soluble than paclitaxel

because of the chemical structure, specifically a tertbutyl carbamate ester in the phenylpropionate side chain in addition to a hydroxyl group on C-10 (Tan *et al*., 2012).

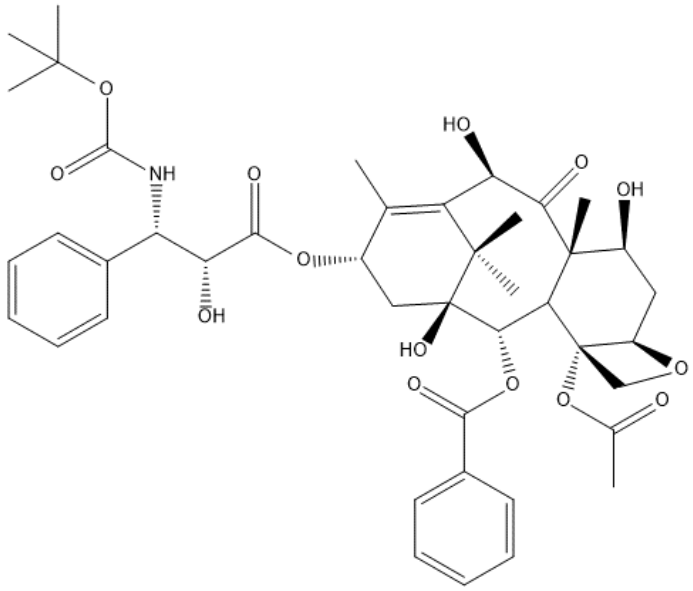


Figure 2: *Chemical structure of docetaxel.*

DOC lipophilic nature causes a limited solubility in aqueous medium (Tan *et al*., 2012). Since DOC is practically insoluble (4.03 µg/mL) in water, the only currently available formulation is parenterally administrated (Yin *et al*., 2009), formulated in a 50:50 (v/v) ethanol:polysorbate 80 formulation, commercially branded Taxotere® (Pereira *et al*., 2016; Louage *et al*., 2017). Polysorbate 80 is a surfactant which can cause serious hypersensitivity reactions and induce fluid retention and therefore have to be pre-treated with antihistamines and/or corticosteroids to avoid severe or fatal allergic reactions, and diuretics if swelling due to fluid retention (Louage *et al*., 2017). The currently commercially available formulation of DOC have reported to cause serious side effects like neutropenia, musculoskeletal toxicity, peripheral neuropathy and hypersensitivity reactions which either is attributed to polysorbate 80 or to DOC itself (Tan *et al*., 2012).

In order to solubilize DOC, one could employ a liposomal formulation. There have been developed alternative dosage forms such as liposomes (Deeken *et al*., 2013; Mahalingam *et al*., 2014), micelles, polymeric nanoparticles and cyclodextrin complexes in order to eliminate Polysorbate 80 based formulation of DOC and hopefully and ideally to eliminate toxicity and

adverse reactions (Manjappa *et al*., 2013; Naik *et al*., 2010). However, none of these formulations are commercially available but under clinical investigations for now.

Two of the formulations under clinical trials are liposomal formulations; ATI-1123 and LE-DT (Louage *et al*., 2017). The ATI-1123 liposomal formulation of DOC was composed of phospholipids (PLs), cholesterol (Chol), human serum albumin and sucrose (Mahalingam *et al*., 2014). The LE-DT liposomal formulation of DOC was composed of negatively charged synthetic PLs and Chol (Deeken *et al*., 2013). Both ATI-1123 and LE-DT is currently under phase I and II clinical trials, respectively, and subject to a patent situation (Louage *et al*., 2017) and therefore there is little information about which PLs that were used. The results from the clinical trials of ATI-1123 and LE-DT have reported good tolerability, predictable and manageable toxicity and promising antitumor effect (Mahalingam *et al*., 2014; Deeken *et al*., 2013).

2.1.1.1 Mechanism of action

DOC is an antineoplastic agent in which the antitumor mechanism of action is hyper-stabilization of microtubules. By binding to the β-subunit protein of tubulin on the microtubules, DOC promotes assembly of tubulin into stable microtubules and simultaneously inhibition of microtubule depolymerization. The normal dynamic equilibrium between polymerization and depolymerization within the microtubule system is disrupted because of the formation of stable microtubule bundles and hence lead to cell cycle arrest at the G2/M phase and cell death (Zhang and Zhang, 2013; Tan *et al*., 2012). Cell death is a result of a significant reduction in free tubulin, inhibition of mitotic cell division and prevention of cancer cell proliferation (Xie *et al*., 2016). To achieve therapeutic efficacy, DOC is dependent on being released and delivered to the cytoplasm of the cell to access microtubules (Dawidczyk *et al*., 2014).

2.2 Liposomes

2.2.1 Characteristics of liposomes

Liposomes are lipid based nanoparticles with a spherical shape in where an aqueous core lies between a lipid bilayer (Kim, 2016). The size of liposomes can range from a few nanometres to several micrometres. Liposomes seem to have ideal properties as a drug carrier system because of their ability to entrap different substances together with their morphology which is similar to cellular membranes (Bozzuto and Molinari, 2015). Because of this, a lot of interest and research have been carried out with this nanocarrier system since the discovery of liposomes in the 1960s by Alec D. Bangham (Bozzuto and Molinari, 2015; Bangham *et al*., 1965).

The liposomes are primarily composed of phospholipids (PL) that are either originated from plants or egg. In addition, liposomes can include Chol, sphingolipids, glycerolipids, long-chain fatty acids, membrane proteins and nontoxic surfactants (Kaur *et al*., 2014).

The liposomes are arranged in bilayers, as shown in Figure 3, where the lipids arrange themselves so that hydrophilic head groups of the PLs points toward the aqueous phases, that is both outside and inside the vesicle, making the core hydrophilic, while the hydrophobic chains of the PLs is forming the inner core of the lipid bilayers (Kumari *et al*., 2016). Liposomes can have one or more lipid bilayers, named unilamellar (ULVs) and multilamellar vesicles (MLVs), respectively (Kraft *et al*., 2014). Poorly soluble, lipophilic drugs or compounds can be entrapped in the lipid bilayers as shown in Figure 3, while hydrophilic, water-soluble drugs or compounds can be encapsulated in the hydrophilic core of the liposome (Tan *et al*., 2012).

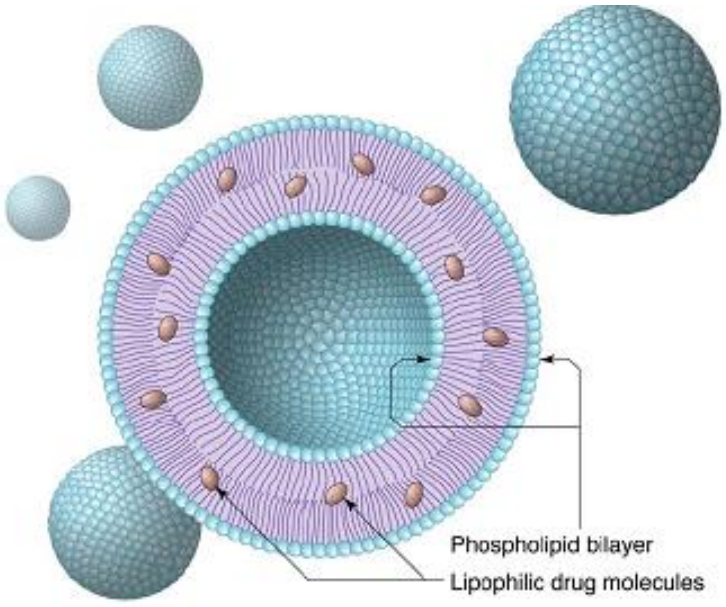


Figure 3: *Cross section of a liposome with a phospholipid bilayer. Lipophilic drugs are entrapped in the phospholipid bilayer* (Sætern, 2004) *(with permission).*

The partition coefficient and polarity of a drug determines where the drug will be located in the liposomal membrane, and further the entrapment efficiency of the drug (Bozzuto and Molinari, 2015). Because of DOCs high lipophilicity, it is conceivable that the drug will reside in the fatty acyl chains of the liposome. The entrapment efficiency of DOC is dependent on the acyl chain properties, like length of acyl chain and packing density, in addition to changes in the drug:lipid ratio (Bozzuto and Molinari, 2015).

The liposomal properties vary substantially with composition of lipids, preparation methods, surface charge and size of the vesicles.

2.2.2 Membrane components in liposomes

The membrane composition of the liposomal membrane might be varied by selecting different lipids and lipid combinations, that will ultimately change liposomal features like phase transition temperature (T_m), stability and charge. Which lipid(s) that are chosen will affect the stability of the liposomes both *in vitro* and *in vivo* and the stability of the drug. Choice of lipid affects the organisation and properties of the PL membrane like elasticity, permeability and binding of a drug (Brandl, 2001). Several studies have showed that composition of the lipid bilayer affects the entrapment of DOC in liposomes (Pereira *et al*., 2016; Naik *et al*., 2010; Manjappa *et al*., 2013; Immordino *et al*., 2003).

The lipids used to form the bilayer will determine the rigidness or "fluidity" of the membrane and the charge of the bilayer (Akbarzadeh *et al*., 2013). A key parameter for liposomal systems is the gel to liquid crystalline T_m in which the structure of the bilayer loses the ordered packing because the hydrocarbon chains melts. The longer the length of the hydrocarbon chain is, the higher the T_m is (Taylor *et al*., 2005), due to van der Walls interactions which is stronger and thus require more energy to disrupt the ordered packing (Bozzuto and Molinari, 2015). In addition, strong head group interactions and increasing saturation of the fatty acid will increase the T_m (Taylor *et al*., 2005). If saturated PLs with long acyl chains is chosen as components, it will form a rigid and impermeable bilayer, whereas unsaturated PLs from natural sources gives a less stable bilayer that is more permeable (Akbarzadeh *et al*., 2013).

2.2.2.1 Phospholipids

Phospholipids (PLs) or glycerophospholipids are a subclass of lipids which are a key component of all cell membranes (Singh *et al*., 2017). PLs contain phosphorus, an polar part and a non-polar part (Li *et al*., 2015). They are amphiphilic molecules which means that they are composed of a hydrophilic head group and hydrophobic acyl chain-tails (Bozzuto and Molinari, 2015), that are linked to alcohol (Figure 4). The polar head groups will be oriented interiorly or exteriorly to the aqueous phases (Akbarzadeh *et al*., 2013).

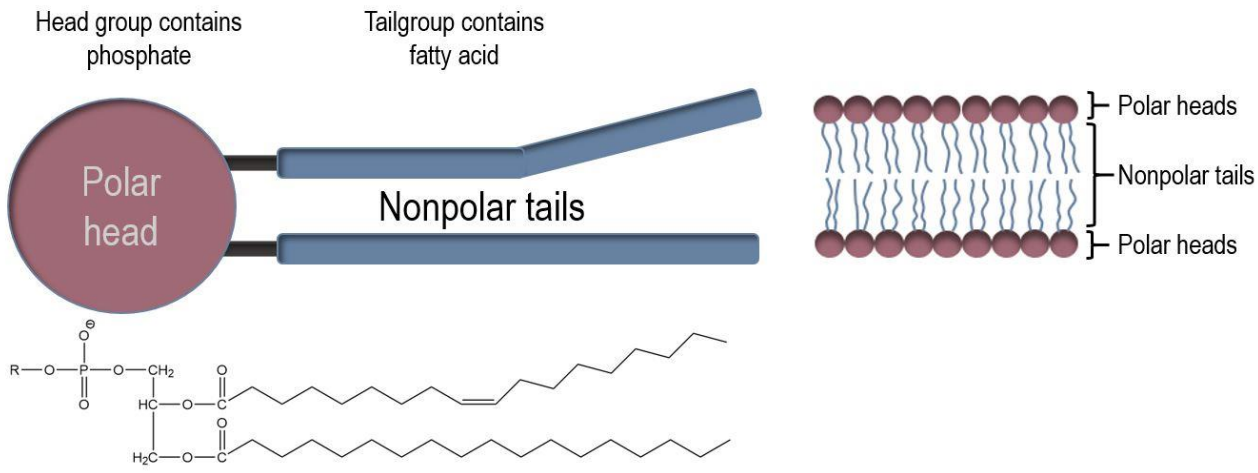


Figure 4: *Illustration of the general structure of phospholipids and how they arrange themselves in bilayers.*

PLs might differ in their composition by containing different alcohols, head group, acyl chains, or also by source of PL. PLs that vary in the alcohols can be divided into glycerophospholipids and sphingomyelins. PLs that vary in the structure of the head group gives rise to different PLs like phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) (Li *et al*., 2015) like shown in Figure 5.

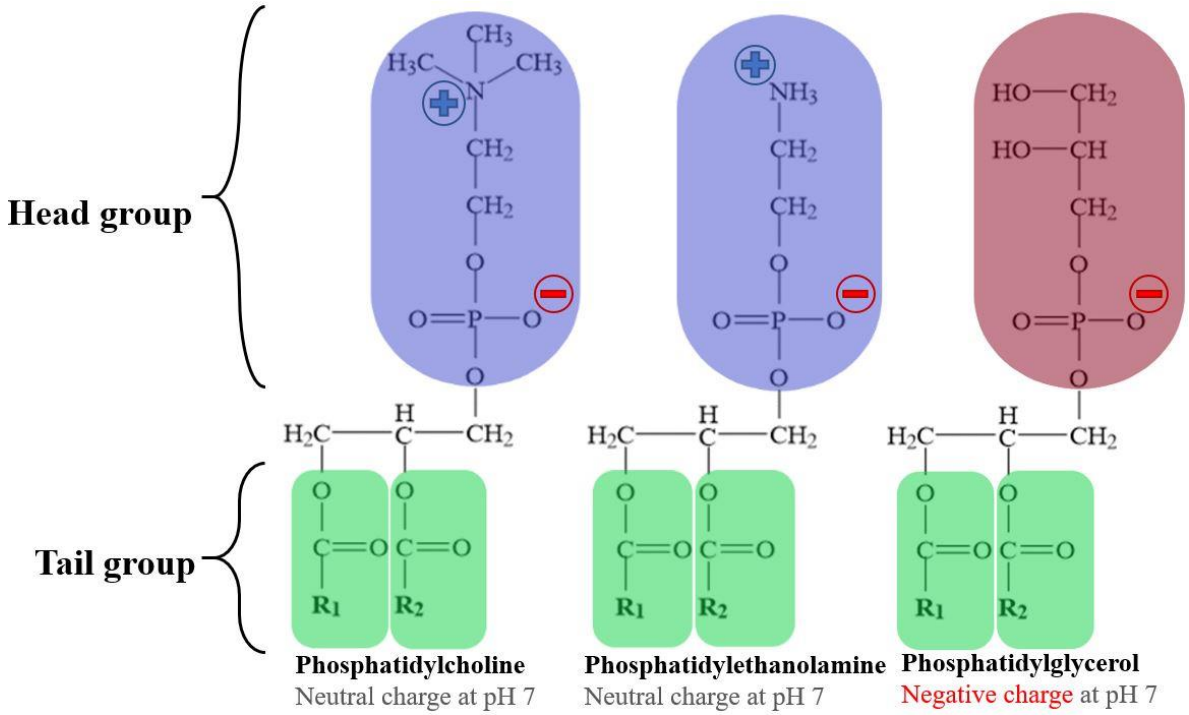
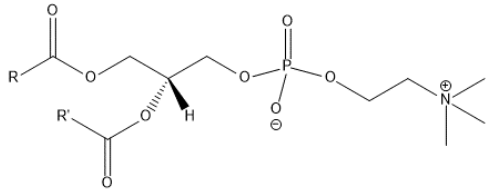
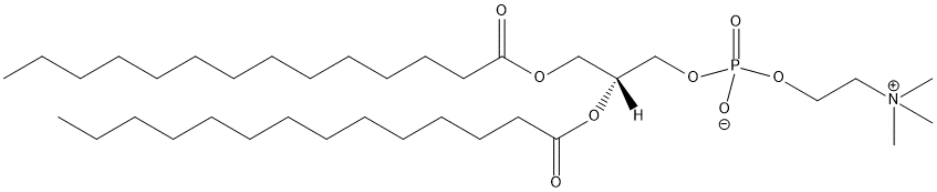
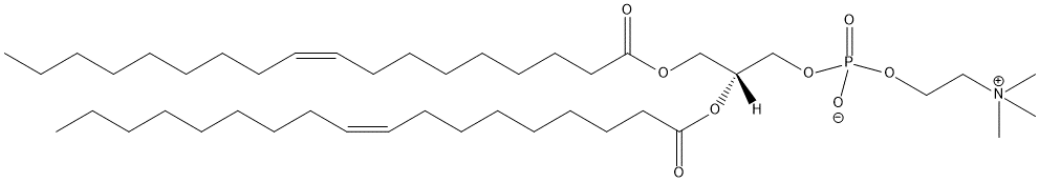
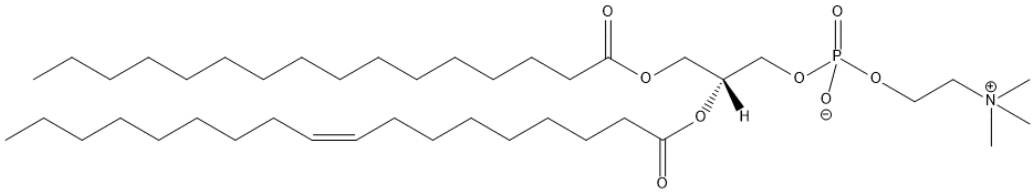
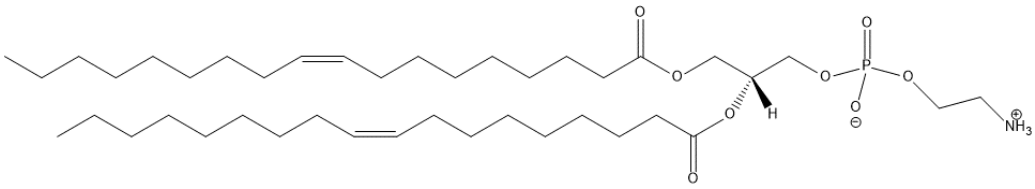
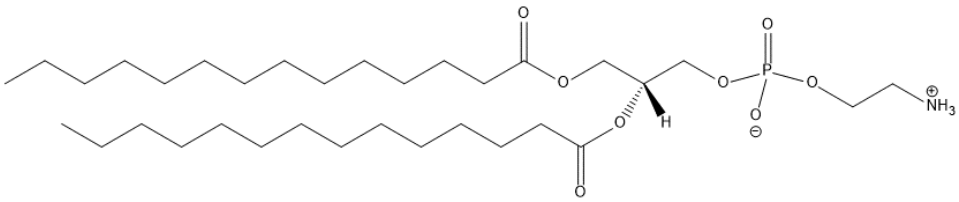
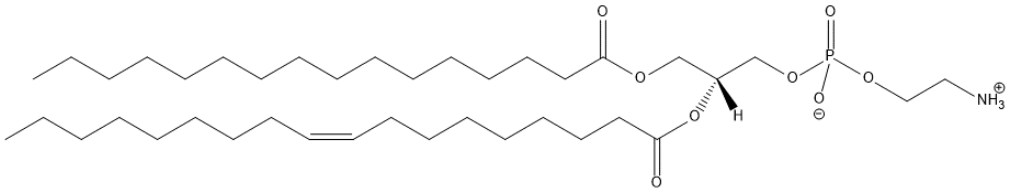


Figure 5: *Illustration of commonly used phospholipids for liposomal formulations. The R_1 and R_2 groups represents the fatty acyl tail groups (Inspired by* (Kraft *et al*., 2014)*).*

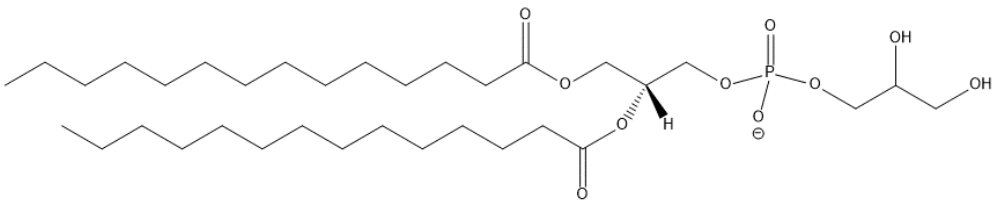
The fatty acyl chains (tail group presented in Figure 5 by R_1 and R_2) of the PL are typically 14-18 carbons in length and have varying saturation (Kraft *et al*., 2014). Dipalmitoyl with 16 carbons and distearoyl with 18 carbons, both saturated fatty acid chains, are popular choices for lipid bilayers. Dioleoyl is an unsaturated fatty acid chain with 18 carbons which also are commonly used (Alipour *et al*., 2017).

Table 1 comprises examples of different lipids with different head groups and fatty acyl chains. These lipids are used in this master project. The head groups is either PC, also referred to as lecithin which has a neutral charge and are a major building block in membranes, PE which carry a neutral charge at physiologic pH 7.4 and PG which carry a negative net charge at physiological pH 7.4 (Kraft *et al*., 2014) as shown in Figure 5.

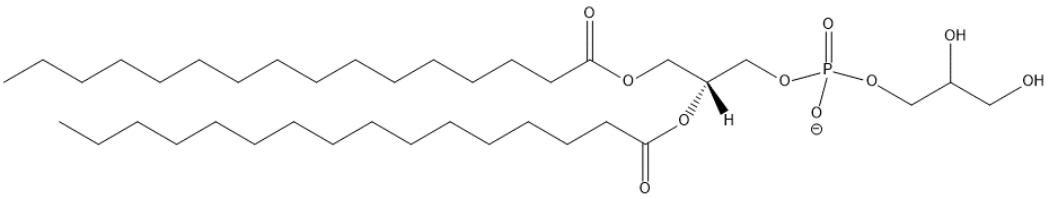
Table 1: *Examples of different lipids with different head groups: PC, PE and PG. The fatty acyl groups vary in length and saturation.*

Lipid	Structure
SPC	 R, R' = fatty acid residues
DMPC	
DOPC	
POPC	
DOPE	
DMPE	
POPE	

DMPG



DPPG



The source of the PLs can be categorized accordingly (Samad *et al*., 2007):

- Natural source.
- Modified from a natural source.
- Semisynthetic.
- Synthetic.

PLs have excellent biocompatibility which makes them attractive as pharmaceutical excipients and applications in drug delivery systems. When PLs is hydrated in aqueous medium, they will form in different assemblies like liposomes or micelles (Li *et al*., 2015). In the case of liposomes, PLs and eventually other adjacent lipid molecules interact and align to form a contiguous bilayers sheet, which will form enclosed vesicles in solution (Kraft *et al*., 2014).

2.2.2.2 DOTAP

1,2.Dioleoyl-3-trimethylammonium-propane (DOTAP) is a cationic lipid which consists of two unsaturated fatty acids, oleoyl chains which is bound by an ester bond to a glycerol backbone. The cationic head group is a quaternary ammonium salt (Zhi *et al*., 2018). The chemical structure of DOTAP is shown in Figure 6, and is one of the lipids used in this master project.

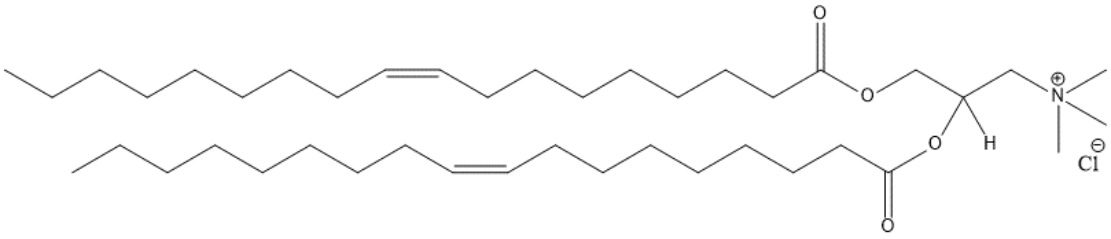


Figure 6: *The chemical structure of* 1,2-Dioleoyl-3-trimethylammonium-propane *(DOTAP).*

2.2.2.3 Ceramides (Sphingolipids)

Ceramides is a class of sphingolipids which are composed of two distinct functional components, the sphingosine structure and the esterified fatty acyl chain (Stillwell, 2016a). The acyl chains vary in length, where endogenous ceramide species commonly have 16-24 carbon atoms. In this master project we used ceramide C6 shown in Figure 7 and ceramide C12 shown in Figure 8.

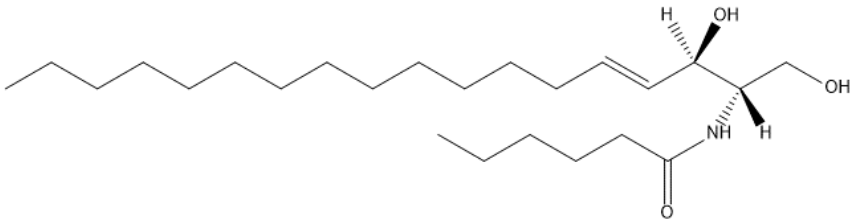


Figure 7: *The chemical structure of Ceramide C6*

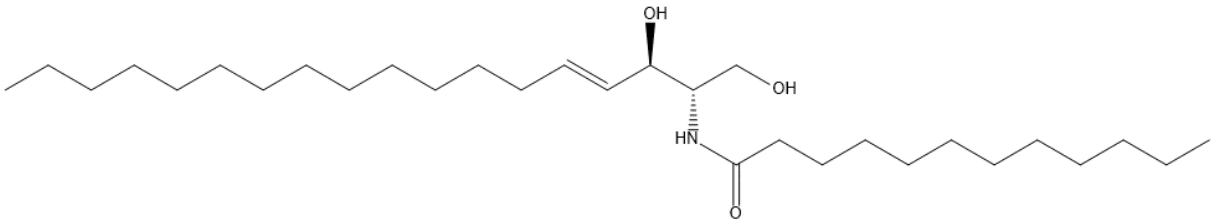


Figure 8: *The chemical structure of Ceramide C12*

2.2.2.4 Cholesterol (sterols)

Cholesterol (Chol) is a hydrophobic molecule, shown in Figure 9, with the exception of a polar -OH group which is anchored to the aqueous interface (Stillwell, 2016b). Chol can increase the packing of PL molecules and decrease the mobility of hydrocarbon chains, and therefore reduce the permeability of the liposomal bilayer (Demel and De Kruyff, 1976), hence preventing loss

of drug (Brandl, 2001; Dawidczyk *et al*., 2014). Chol also change the fluidity of the intravesical interactions between the head groups and hydrocarbon chains which make the lipid bilayer more rigid (Briuglia *et al*., 2015) and hence decrease the T_m (Taylor *et al*., 2005). Studies have showed that inclusion of a small amount of Chol does not have a negative effect on entrapment of drugs, however, increasing amount of Chol in a liposomal formulation had a negatively effect on drug loading (Mohammed *et al*., 2004; Immordino *et al*., 2003; Chen *et al*., 2017).

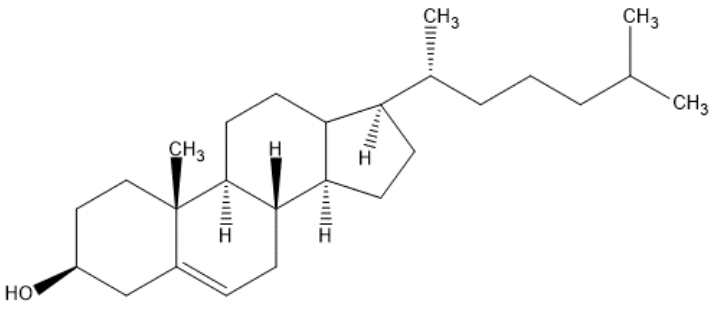


Figure 9: *The chemical structure of cholesterol.*

2.2.2.5 PEGylation

Conventional liposomal formulations have been hampered by short circulation time in the bloodstream because of uptake by the mononuclear phagocyte system (MPS) (Kim, 2016). Polyethylene glycol (PEG) is a polymer which often is attached to the surface of nanoparticles to make the detection by the MPS more difficult. This process is called PEGylation (Bulbake *et al*., 2017; Kumari *et al*., 2016) and have been shown to improve the stability and circulation time of liposomes after intravenous administration (Bozzuto and Molinari, 2015).

PEGylated liposomes are also referred to as Stealth™ liposomes or long circulating liposomes. Among various surface modifying molecules, PEG is popular because of its properties including conformationally flexibility, high mobility and hydrophilicity which contribute to decreased interactions with various plasma proteins and uptake by the MPS (Kim, 2016). DSPE-PEG2000, shown in Figure 10, is a PEGylated PL which is applied in different preparations, including Doxil® and used in this master project. The PEG layer is grafted onto PE and usually serves as a steric barrier to stabilize the molecule assemblies (Li *et al*., 2015).

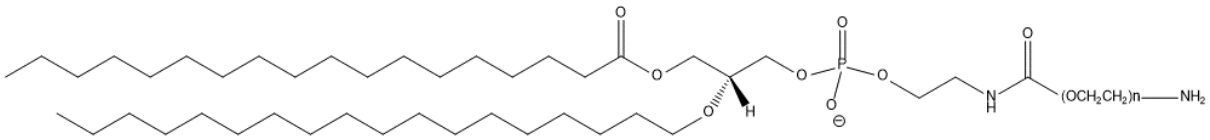


Figure 10: *The chemical structure of* 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) with polyethylene glycol (PEG)-2000.

2.2.3 Methods for preparation of liposomes

2.2.3.1 Film-hydration method

There are several methods for preparation of liposomes, and one of the most common used is the thin-film hydration method, also called Bangham-method (Bozzuto and Molinari, 2015; Bangham *et al*., 1965), which is the method applied in this project. Other methods for preparation of liposomes include reverse-phase evaporation, freeze-drying and ethanol injection (Laouini *et al*., 2012; Kim, 2016). In the thin-film hydration method the lipids and drug are dissolved in an organic solvent, then the solvent is evaporated by rotary evaporation to get a lipid film. The lipid film is rehydrated with an aqueous solvent (Bulbake *et al*., 2017).

2.2.3.2 Sonication

There are several ways to control and reduce the size of liposomes such as extrusion, homogenization and/or freeze-thawing, and sonication (Bulbake *et al*., 2017). Sonication is the method applied in this project. Sonication is an extensively used method for reduction of size, and there are two different techniques to sonicate: probe sonication and bath sonication (Akbarzadeh *et al*., 2013). Probe sonication is the technique applied in this project and involves a probe, the tip of the sonicator, that is directly applied into a liposomal dispersion. The probe transmits energy in form of ultrasonic irradiation to the lipid dispersion and thereby reduces the size of the vesicles (Bozzuto and Molinari, 2015). Under the ongoing process the probe is heated, and this heat is also transferred to the liposomal dispersion. To avoid extensive heating it is therefore important to keep the container in an ice bath under the process (Akbarzadeh *et al*., 2013).

2.2.4 Size and surface charge of liposomes

The ideal size of a liposome depends on several biological conditions. The size should be small enough to avoid being captured by macrophages in the MPS and to exploit the gaps between the endothelial cells of the tumour with leaky vasculature ranging from 100-500 nm to enter the interstitial space (Bozzuto and Molinari, 2015) and at the same time be big enough to prevent being leaked into the capillaries from the tumour site (Cho *et al*., 2008).

The size of conventional liposomes determines the fraction cleared by the MPS and it have been shown that small liposomes (e.g. size below 100 nm) are opsonized less rapidly and to a lower extent compared bigger liposomes (e.g. bigger than 100 nm) (Fanciullino and Ciccolini, 2009). On the other hand, liposomes with a diameter < 10 nm rapidly cleared by the kidney (Kumari *et al*., 2016).

Studies indicate that particles should have a diameter below 200 nm to effectively get extravasated into tumours (Peer *et al*., 2007), and that a reduction on size of liposomes has been correlated with increased accumulation in tumours. It has been displayed that small liposomes shows a longer half-life compared to larger liposomes (Fanciullino and Ciccolini, 2009). Therefore, the ideal size of liposomes for reaching the tumours should be 50-100 nm in diameter (Bozzuto and Molinari, 2015; Kumari *et al*., 2016).

The charge of liposomes depends on the lipid composition and head group of lipids, which is typically expressed as surface charge or zeta potential, ζ (Kraft *et al*., 2014). The zeta potential is one of the characteristics measured of liposomes and give an index of the magnitude of the repulsive interaction between colloidal particles (González-Paredes *et al*., 2010). Liposomes may carry a negative, neutral or positive net charge (Kraft *et al*., 2014). The surface charge may influence kinetics, stability, interaction with and uptake of liposomes by target cells and extent of biodistribution (Lian and Ho, 2001). Increasing zeta potential shows a tendency that charged particles will repel one another and hence not aggregate (Alipour *et al*., 2017).

Cationic lipids, lipids with a positive charge, have been shown to be rapidly eliminated by the MPS (Allen and Cullis, 2013; Kraft *et al*., 2014). Also, negative surface charge is recognized by receptors on different cells, like macrophages, and when entering the circulation, the negatively charged liposomes are subject to opsonization. On the other hand, a negative surface

charge show enhanced cellular uptake through endocytosis compared to natural counterparts (Kraft *et al*., 2014).

2.2.4.1 Classification of liposomes according to structure and size

Based on structure classification, the liposomes are classified with respect to type of vesicle together with size and number of lipid bilayers that enclose the aqueous phase (Samad *et al*., 2007; Maheswaran *et al*., 2013):

- Unilammelar vesicles (UVs): all diameter size range, with one lipid bilayer.
- Small unilamellar vesicles (SUVs): 20-100 nm in diameter, with one lipid bilayer.
- Medium unilamellar vesicles (MUVs): >100 nm in diameter, with one lipid bilayer.
- Large unilamellar vesicles (LUVs): >100 nm in diameter, with one lipid bilayer.
- Giant unilammelar vesicles (GUVs): >1 µm in diameter with one lipid bilayer.
- Oligolamellar vesicles (OLVs): 0.1-1 µm in diameter with approximately 5 lipid bilayers.
- Multilamellar vesicles (MLVs): > 0.5 µm in diameter with 5-25 lipid bilayers.
- Multi vesicular vesicles (MVs): >1µm in diameter where the lipid bilayers have multi compartmental structure (Samad *et al*., 2007; Maheswaran *et al*., 2013).

Lamellarity is a feature of the membrane structure which indicate number of bilayers the membrane it is composed of. If the membrane has a single bilayer, it is called unilamellar, but if the membrane has many bilayers it is called multilamellar, as shown in Figure 11 (van Swaay and deMello, 2013). Often, liposomes could be in between the categories in the above mentioned classification, and without any characterization with small angle X-ray scattering evaluation or electron microscopy it is difficult to know how many lamella there is within the liposomes (Škalko *et al*., 1998).

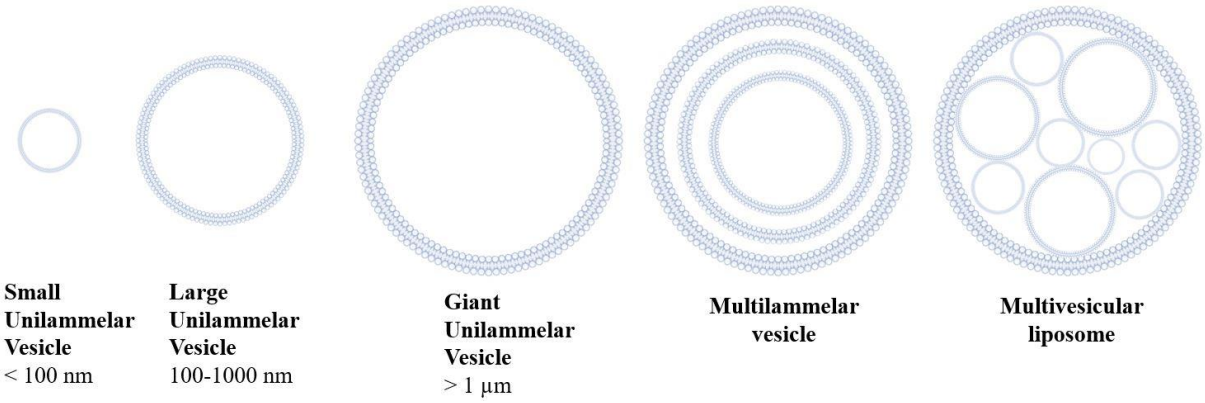


Figure 11: *The size and lamellar structure of different classes of liposomes (Inspired by* (van Swaay and deMello, 2013).

2.2.5 Liposomes used as drug delivery systems

The first drug delivery system which have made success translating into clinical applications are liposomes. In 1995 Doxil®, a liposomal formulation of doxorubicin, entered the U.S. marked. Doxil® was approved for treatment of ovarian cancer and AIDS-related Kaposi´s sarcoma. Most of the liposomal formulations that have been developed are used for cancer treatment, but there are also liposomes used for treatment of fungal- or virus infections or pain management (Bulbake *et al*., 2017). To this day there is approximately 15 liposomal formulations on the marked (Bulbake *et al*., 2017; Kim, 2016).

The research conducted on liposomes has progressed from conventional liposomes, also referred to as "first-generation liposomes" to long circulating liposomes in which surface modifications, lipid composition and size of the vesicle is modified (Immordino *et al*., 2006). Liposomes can also be modified with targeting moieties such as monoclonal antibodies, peptides or receptor ligands to achieve active targeting, or liposomal formulations can be modified to achieve triggered release, achieved by pH- or temperature sensitivity (Allen and Cullis, 2013).

Liposomes offers both several advantages together with limitations, which are summarized in Table 2.

Table 2: *Advantages and disadvantages of liposomes used in drug delivery systems.*

Advantages	**Disadvantages**
Liposomes are in general non-toxic, flexible, biocompatible, completely biodegradable and non-immunogenic both for systemic and non-systemic administrations (Akbarzadeh *et al*., 2013).	Production cost is high (Akbarzadeh *et al*., 2013) and mass production is challenging (Kim, 2016).
Entrapment of both hydrophilic and lipophilic drugs is feasible (Kim, 2016).	A short half-life after i.v. administration due to rapid clearance from the bloodstream by the mononuclear phagocytic system (MPS) (Naik *et al*., 2010; Immordino *et al*., 2006).
Changed biodistribution of the drug might lead to reduced exposure of toxic drugs to sensitive or normal tissues (Akbarzadeh *et al*., 2013).	
Can increase drug stability via incorporation, by protecting the drug by entrapment and thereby isolating the drug from the surrounding environment (Akbarzadeh *et al*., 2013; Peer *et al*., 2007).	Stability issues might lead to a burst drug release (Peer *et al*., 2007). PLs are prone to chemical degradation reactions, like oxidation and hydrolysis (Akbarzadeh *et al*., 2013).
Flexible in formulation: size, charge and surface functionality can be modified either through addition of agents to the lipid membrane or by alteration of the surface (Peer *et al*., 2007)**.**	
Active targeting is possible when coupled with site-specific ligands (Kumari *et al*., 2016)**.**	

3 Aim of the study

The aim of this master project was to establish a suitable small-scale screening method for making DOC-liposomes, and for separating the unentrapped DOC from the DOC-liposomes for further determination of the drug entrapment efficiency. This methodology was applied to examine different PL compositions aiming to decide which factors are affecting how well DOC is taken up and becomes a part of the liposomal membrane.

Specific aims:

- Pilot project: validate the method for making and testing the different DOC liposomal formulations.
- Screening study: screening of 14 different lipid combinations to investigate how the different liposomal formulations affect the DOC entrapment efficiency.
- Further, to challenge the liposomal formulations showing best entrapment of DOC in the Screening study, by increasing the DOC concentration to examine if increasing the DOC concentration also would give an increase in DOC entrapment.
- Finally, varying the concentration of positively charged lipids, DOTAP, in the liposomal membrane to investigate if DOTAP concentration affect the DOC entrapment.

4 Materials, instruments and experimental section

4.1 Materials

4.1.1 Chemicals

Table 3: *Specifications of the chemicals used in this study.*

Chemical	Quality	Batch/Lot nr.	Manufacturer
Acetic acid, CH_3COOH	ACS reagent ≥ 99.8 %	Lot: #SZBD1760V	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Acetonitrile, C_2H_3N	For HPLC, gradient scale	17B101326	VWR chemicals, BDH Prolabo, France
Ammonium molybdate tetrahydrate, $(NH_4)Mo_7O_{24} \cdot 4H_2O$		Lot: #SLBN5957V	Sigma Aldrich Chemie GmbH, USA
Chloroform	99.0-99.4 (GC)	Lot: #STBF8245V	Sigma Aldrich Chemie GmbH, Steinheim, USA
Distilled water			Distillation unit Distinction D4000, Bibby Sterlin LDT, Staffordshire, UK
Docetaxel		20140701	Euroasian chemicals pvt Ltd., Mumbai, India
Ethanol, C_2H_5OH	96 % (v/v)	Lot: 17E224011	VWR Chemicals, BDH Prolabo, France
Fiske Subbarow reducer		Lot: #SLBQ3794V	Sigma Aldrich Chemie, GmbH, USA.
Formic acid, CH_2O_2	Eluent additive for LC-MS	Lot: #BCBJ6551V	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Hydrochloric acid, HCl	ACS reagent	Lot: SZBE1360V	Sigma Aldrich, Austria
Hydrogen peroxide, H_2O_2	30 % H_2O_2 GR for analysis	Lot: K25310009 830	Merck: KGaA, Darmstadt, Germany
Methanol, CH_3OH	For HPLC ≥ 99.9 %	Lot: #SZBC272MV	Sigma Aldrich Chemie GmbH, Steinheim, Germany
MilliQ water			MilliQ gradient, Millipore Water purification system with Millipak® M 0.22µm filter (LOT NO C5MA58154), Damstadt, Germany
Phosphorus standard solution, KH_2PO_4	0.65 mM (phosphorous)	Lot: #SLBR5870V	Sigma Aldrich Chemie GmbH, Steinheim, Germany

Sulfuric acid, H_2SO_4	Analytical reagent	May & Baker Ltd., Dagenham, England.

4.1.2 Lipids

Table 4: *Specifications of the lipids used in the liposomal formulations in this study.*

Lipid	Full name	Purity	Batch/Lot nr.	Manufacturer
Ceramide C6	N-Hexanoyl-D-*erythro*-sphingosine	> 99 %	Lot: #060CER-15	Avanti Polar Lipids Inc.
Ceramide C12	N-Lauroyl-D-erythro-sphingosine		Lot: #120CER-10	Avanti Polar Lipids Inc.
Cholesterol		> 99 % from lanolin	Lot: # BCBK3087V	Sigma Aldrich Co, St. Louis, USA
DMPE	1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine	> 99 %		Lipoid GMBH, Ludwigshafen, Germany
DMPC	1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine	> 99 %	LP-04-058-H1228	Genzyme pharmaceuticals, Sygena Facility, Liestal, Switzerland.
DMPG	1,2-Dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)	> 99 %	LP-04-015-J1007	Lipoid GMBH, Ludwigshafen, Germany
DOPC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine	> 99 %	Lot: #SLBN3634V, product number: P6354-1G	Sigma Aldrich Co, St. Louis, USA.
DOPE	1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine	> 99 %	699146-1/18	Lipoid GmbH, Ludwigshafen, Germany
DOTAP chloride	1,2-Dioleoyl-3-trimethylammonium-propane	> 99 %	Lot: #SLBC6246V, product number: D6182-250MG	Sigma Aldrich Co, St. Louis, USA
DPPG sodium salt	1,2-Dipalmitoyl-*sn*-glycero-3-phosho-(1'-*rac*-glycerol)	> 99 %	LP-04-016-597 1724	Genzyme pharmaceuticals, Sygena Facility, Liestal, Switzerland.
DSPE-PEG2000	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]	> 99 %	Lot # 180PEG2PE-121	Avanti Polar Lipids Inc.
Lipoid S 100 (SPC)	Soybean phosphatidylcholine		Batch: 579000-1170718-03/902	Lipoid GmbH, Ludwigshafen, Germany

POPC	1-Palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine	≥ 99 %	Lot # BCBT9936	Sigma Aldrich, Japan
POPE	1-Palmitoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine	≥ 95 %	Lot # BCBV6370	Sigma Aldrich, Japan

4.1.3 Solutions

4.1.3.1 Mobile phases for HPLC-analysis:

Mobile Phase A:

I.	MilliQ Water	1998.0	mL
II.	Formic acid	2.0	mL

Mobile Phase B:

I.	Acetonitrile	1998.0	mL
II.	Formic acid	2.0	mL

4.1.3.2 Solutions prepared for PC-assay:

Fiske SubbaRow reducer:

3.0 g Fiske SubbaRow reducer

18.9 mL distilled water.

Ammonium Molybdate 0.22 % (v/v):

0.88 g ammonium molybdate powder

Ad 400 mL distilled water

H_2SO_4 10 N:

10.88 mL H_2SO_4

29.12 mL distilled water

HCl 0.5M:

0.411 mL HCl

Ad 100 mL water

4.2 Equipment and instruments

Balances: Sartorius BP211D, Sartorius LP4200S, Sartorius CP225D, Sartorius AG, Göttingen, Germany.

Benchtop centrifuge: Biofuge Stratos with Heraeus rotor #3048 and #8172, Heraeus Instruments, UK.

Drying oven: Termaks TS8056 Bench Top Drying Oven, Termaks a/s, Bergen, Norway

HPLC: Waters e2795 separations module connected to a Waters 2489 UV/Visible detector and a C-18 column: XSELECT CSH XP (2.5 µm 3.0x75 mm). (Waters, Dublin, Ireland).

LAF-bench: Holten LaminAir, Maxi Safe 2000, Allerød, Denmark.

Particle Size Analysis and Zeta Potential Measurement: Zetasizer Nanoseries ZS, Malvern Instruments Limited, Worcestershire, UK.

Rotary evaporator: Büchi Waterbath B480, Büchi Vac V-500, Büchi vacuum controller B-721, Büchi rotavapor R-124, Büchi labortechnik, Flawil, Schwitzerland.

Sonicator: Sonics Vibra Cell Probe sonicator, autotune series high intensity ultrasonic processor VC 754 750 watt Ultrasonic processor, CVR 234 Converter, Probe 19 mm, Sonics and Materials, USA.

UV-Spectrophotometer: SpectraMax 190 Microplate Reader UV Spectrophotometer, Molecular Devices Corporation, California, USA.

Vortex mixer: Vortex Genie 2™, Bender & Hobein AG, Zurich, Switzerland.

4.3 Computer programs

HPLC: Empower™ 3 Software, Build 3471, Waters, 2010.

Particle Size Analysis and Zeta Potential Measurement: Malvern Zetasizer Software for the nano, APS and UV. Version 7.11. Malvern Instrument Limited, Malvern, UK.

UV microplate reader: SoftMax Pro software version 5, Molecular Devices Corporation, California, USA.

4.4 Experimental section

When working with the cytotoxic drug DOC, precautions were made not to be exposed to the drug. Thus, the dry powder was always handled in a dedicated LAF-bench, and DOC-containing containers were kept sealed when handled outside the LAF-bench.

4.4.1 DOC stock-solution

A DOC stock-solution containing 20 mg/mL DOC was prepared prior to making the HPLC standard curve (Section 4.5.3) and for accurate transfer of the aimed amount of DOC to the liposomal formulations (Section 4.4.2). DOC was weighed in a small sample tube on an analytical balance (Sartorius CP225D) placed in the LAF-bench, and the locked vial control weight in a more precise balance (Sartorius BP211D) before transferred to a volumetric flask and dissolved in 10 mL methanol. The DOC stock-solution was kept in the refrigerator at 4 °C when not in use.

4.4.2 Preparation of liposomes with DOC

The DOC-liposomes were prepared through three distinct steps: 1) lipid film formation using the thin-film hydration method, 2) lipid film hydration, as shown in Figure 12, and finally, 3) size reduction. The first step assured an even distribution of DOC in the lipid membrane, hydration the lipid film with water was the liposome forming step, whereas the sonication reduced the liposome size into a suitable size for intravenous administration.

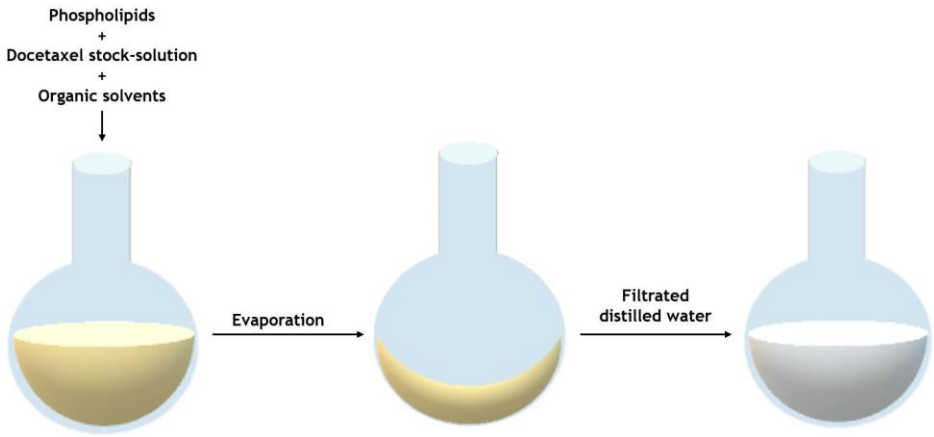


Figure 12: *A schematic presentation of the two first steps applied when preparing liposomes; the lipid film formation step evaporating the organic solvent and the lipid hydration step leading to the formation of a liposomal dispersion.*

4.4.2.1 Lipid film formation

The thin-film hydration method (Bangham *et al*., 1965) was applied for making the lipid films. First, 200 mg of the selected lipid was transferred to a 100 mL round bottom flask, and 1 mL of the DOC stock-solution (corresponding to 20 mg DOC) was transferred by pipetting and mixed with the lipids. To dissolve the lipids, chloroform and/or methanol were added in different ratios depending on the lipids applied to dissolve the lipid(s) and the DOC.

The round bottom flask containing the lipid-drug solution was placed in a Büchi Rotavapor R-124 on Büchi Water Bath B-480 (Büchi laborteknik, Switzerland) with a Büchi Vac V-500 vacuum pump system and Büchi Vacuum Controller B-721 to control the pressure under the rotavapor process. To keep the lipids fluid and homogeneous, the water bath was preheated and kept at a temperature higher than the lipids T_m (usually 44 °C). The pressure and temperature were adjusted to assure that the solutions did not boil but evaporate. The pressure was decreased gradually from 1005 mBar to 55 mBar, at a rotation speed of 80 rpm, that was gradually increased to 150 rpm to make sure that all solvent was removed from the lipid film.

4.4.2.2 Lipid film hydration

The lipid film was hydrated with 10 mL preheated filtrated (0.2 µm) distilled water to form a liposomal dispersion containing 20 mg/mL lipid and 2 mg/mL DOC. The flask was vortexed to properly disperse and dislodge the lipid film, making sure that all lipid and drug was detached from the walls of the flask. The water added kept the same temperature as the lipid film. The liposomal dispersions were stored at 4 °C overnight before sonication.

4.4.2.3 Size reduction of liposomes

A probe sonicator (Sonics Vibra Cell high intensity ultrasonic processor VS 754 750 Watt with Ultrasonic processor, CVR 234 Converter with a Probe 19 mm, Sonic and Materials, USA) was used to reduce the size of the liposomal dispersion. Prior to sonication, the liposomal dispersion was brought to room temperature and transferred to a 45 mL falcon tube. The sonication probe was positioned in the centre of the tube making sure that it did not touch the walls. The tube was placed in an ice bath to prevent the sample from getting warm. Amplitude was set to 40 %. The duration of the sonication varied with the lipid composition and judged from the turbidity of the preparation and finally size measurements (Section 4.5.1). In general, sonication runs were between 0.5 and 4 minutes with a one-minute cooling break between each run, to avoid overheating. A liposome size around 100 nm was targeted.

4.4.3 Removal of unentrapped drug from the liposomes by centrifugation

The liposomal dispersions were allowed to equilibrate in the refrigerator overnight, before separating the unentrapped drug from the liposomes through centrifugation. The liposoma dispersion was separated in 15 mL falcon tubes, and centrifugated at 3000 rpm (min^{-1}) (corresponding to 1800 g) for 20 minutes at 25 °C in a Biofuge stratos (Heraeus Instruments, Oslo) with Heraeus rotor #3048 and #3047. The centrifuge needed one minute to increase the velocity from 0-3000 rpm, and one minute in the end to decrease the velocity from 3000-0 rpm. The supernatant was transferred to new 15 mL falcon tubes and further examined, as described in next section.

4.5 Liposomal characterization

A Zetasizer Nanoseries ZS (Malvern, UK) was used to determine both the size distributions and zeta potential of the prepared liposomal dispersions.

4.5.1 Liposome size determination

The liposomal dispersion was homogenized by vortexing and diluted 1:25 (v/v) with freshly filtrated (0.2 µm) distilled water before size measurement. The test cuvette (12 mm square polystyrene cuvettes, Malvern Zetasizer Nano Series) was rinsed thoroughly with 1 mL ethanol and 2 mL filtrated (0.2 µm) distilled water before and after measurement. Each sample was analysed in triplicate. The measurements gave information of the average intensity weighted size distribution of the liposomes and polydispersity index (PI).

4.5.2 Determination of zeta potential

The liposomal dispersions were homogenized with a vortex machine and diluted 1:20 (v/v) with filtrated (0.2µm) tapped water before zeta potential measurement. The disposable folded capillary cells (Malvern Zetasizer Nano Series) were rinsed thoroughly with ethanol, filtrated tapped water and flushed with 1 mL of the sample before filling the cuvette with the sample to be analysed. A 1 mL syringe was used to clean and fill the cells with the sample. The measurement was set to 3 cycles and 100 runs. Each sample was measured twice.

4.5.3 HPLC: determination of DOC recovery

The DOC concentration in the liposomal dispersions both before and after centrifugation (supernatant) was quantified by high performance liquid chromatography (HPLC) using a Waters e2795 Separations Module connected to a Waters 2489 UV/Visible detector and a C-18 column: XSELECT CSH column XP, 2.5 µm 3.0x75 mm (Waters, Dublin, Ireland).

The method applied had a 12 minutes run time. The sample volume injected varied were 10 µL. The flowrate was set to 0.5 mL/min. A gradient elution was applied, where Mobile phase A,

contained Milli-Q-water with 0.1 % formic acid and Mobile phase B, contained acetonitrile with 0.1 % formic acid. The gradient flow condition applied is given in Table 5. Temperature of both the column and sample were set to 25 ± 1°C. The retention time of the DOC peak was at 7.74 minutes and the detection wavelength applied using the Waters 2489 UV/Visible detector was at λ = 232 nm.

Table 5: *Gradient flow conditions for mobile phase A and B applied in the DOC-HPLC method.*

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.00	80.0	20.0
8.00	10.0	90.0
10.00	10.0	90.0
10.10	80.0	20.0
12.00	80.0	20.0

The standard curve was created from DOC standards with known concentrations in the range of 0.5-1200 µg/mL. The calibration standard curve showed linearity in the range of 0.50-1200 µg/mL, with a R^2=0.9986 (Figure 13). The amount of DOC in the liposomal formulations was subsequently quantified based on the standard curve.

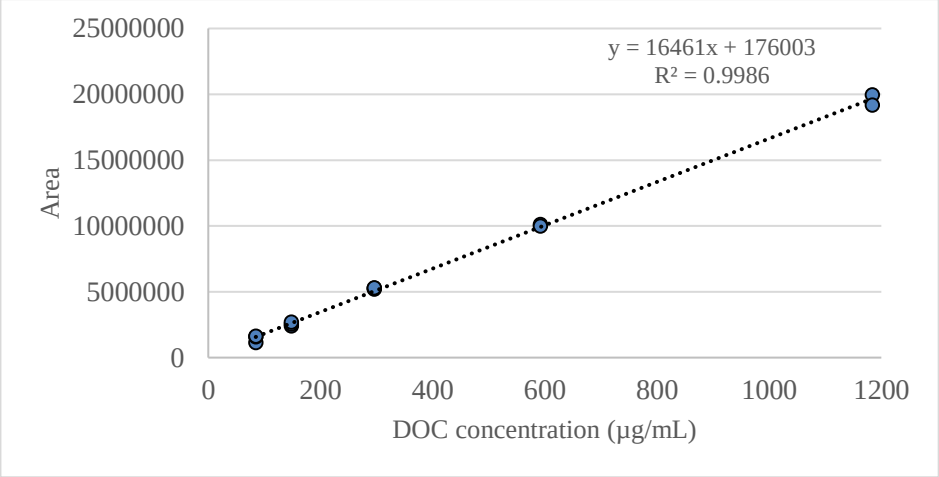


Figure 13*: The docetaxel standard curve obtained using HPLC.*

The eluted DOC peak had a retention time of approximately 7.7 minutes, as illustrated in Figure 14.

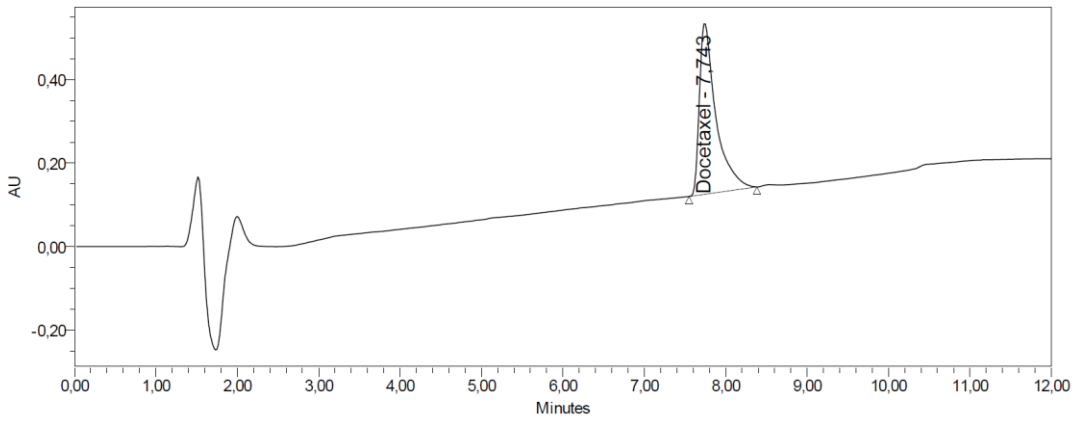


***Figure 14**: A typical chromatogram obtained from the HPLC when quantifying the DOC content.*

Before DOC quantification, the DOC-liposomal dispersion was diluted with methanol to reach concentrations within the span of the calibration curve. Methanol disrupt the liposomal structure, and forms a solution containing DOC and PLs that were separated when injected onto the HPLC for accurate quantification of DOC. The samples were prepared in quintuplicate (five preparations of each sample) in HPLC vials and every sample was injected twice.

The amount of DOC recovery in the supernatant was compared with the amount of DOC present in the total sample before centrifugation to calculate the recovery of DOC in supernatant. The recovery of DOC was calculated by Equation 1.

Equation 1:

$$\text{Recovery of DOC (\%)} = \frac{\text{DOC concentration in supernatant (µg/mL)}}{\text{DOC concentration in total sample (µg/mL)}} \times 100\ \%$$

4.5.4 Phospholipid assay – determination of the PL recovery

In order to determine the recovery of phospholipids (PL) in the liposome-containing supernatant after centrifugation (Section 4.4.3), the amount of PL was determined both in the liposomal dispersions before centrifugation and the supernatant after centrifugation. For this, the phosphatidylcholine assay (PC-assay) was applied using a method obtained from Bartlett (Bartlett, 1959) with some modifications (Naderkhani *et al*., 2015). PLs such as PC, PE and PG contains phosphorous and through the PC-assay it is possible to quantify the amount of

phosphorus in the liposomal dispersions and hence give an estimation of amount lipid in the liposomal formulations. This was further used to adjust for loss of lipid when calculating the entrapment efficiency in Section 4.5.5.

The reagent solutions used in the PC-assay (Bartlett, 1959; Naderkhani *et al*., 2015) are described in Section 4.1.3.2. In addition to these solutions, the samples (liposomal dispersions), the phosphorous standards for the phosphorous standard curve and the phospholipid (PL) reference samples were prepared:

The liposomal formulations were diluted with distilled water, transferring 50 µL liposomal sample to a volumetric flask and adding distilled water to a total volume of 10 mL, giving a final lipid concentration of approximately 0.1 mg/mL.

Phosphorus standard solutions were prepared to make a standard curve. A phosphorus standard solution with a concentration of 19.5-20.4 µgPhos/mL (Sigma Aldrich Chemie GmbH, Steinheim, Germany) was added to glass tubes and diluted with distilled water to reach a total volume of 1 mL and the following phosphorus concentrations: 1, 2, 3, 4, 5 and 8 µg/mL.

Reference PL-samples were made by transferring 7.5 mg SPC into a 50 mL volumetric flask and filling it up with 50 mL 0.5M HCl. The PLs were vortexed until completely dispersed and left to stir overnight, where after the samples appeared opaque.

All solutions, including the phosphorus standard solutions, the blank medium (distilled water), reference PL-samples and the liposomal dispersions were prepared in triplicates and 3 x 1 mL were transferred to glass tubes before adding 0.5 mL H_2SO_4 10N, to a total volume of 1.5 mL. The glass tubes were covered by marbles, and then heated in the oven at 160 °C for three hours. After cooled down to room temperature, 2 drops of 30 % (v/v) hydrogen peroxide (H_2O_2) were added to each sample, before heated at 160 °C for 1.5 hours. In the final step, and after cooled down to room temperature, 4.6 mL ammonium molybdate 0.22 % (v/v) and 0.2 mL Fiske SubbaRow reducer solution were added to the samples. Samples were mixed thoroughly by vortexing before heating the samples at 100°C for 7 minutes.

Each sample was measured in triplicate, transferring 0.2 µL sample into three wells of a 96 well plate. Absorbance were measured with a UV spectrophotometer (SpectraMax 190, Molecular Devices Corporation, California, USA) at an optical density at $\lambda = 830$ nm.

A phosphorous standard curve was prepared for every experiment, after withdrawing absorbance values measured for the blanks (distilled water). The 96 well plates were measured both on the same day as the assay was performed and the day after. The best results were obtained the day after the PC-assay, judged from the R^2-value of the standard curve, and therefor applied for further calculation of the PL recovery in the liposomal formulations.

The calibration standard curve showed linearity in the range of 1-8 µg/mL with R^2= 0.9972, R^2= 0.997, R^2= 0.9989, R^2= 0.9976 and R^2= 0.999 in the different experiments when absorbance was measured the day after sample preparation. A representative standard curve is given in Figure 15.

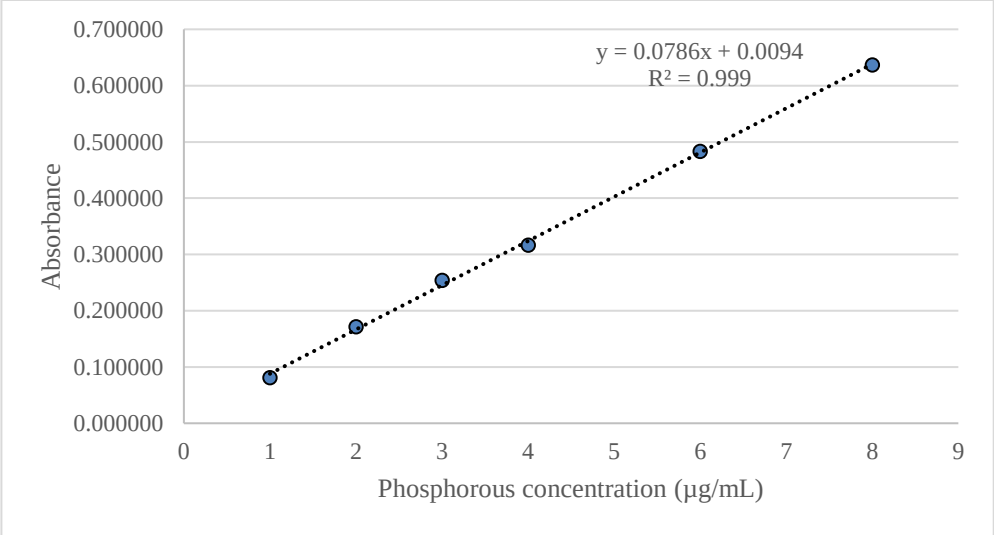


Figure 15: *One of the phosphorous standard curves obtained from the PC-assay.*

From the phosphorous standard curve (Figure 15), the phosphorous content of the reference PL-samples was quantified, and the ratio between the known lipid concentration (7.5 mg/50mL =0.148 mg/mL) and the measures mean phosphorous concentration calculated from the phosphorous standard curve. From these information, the PL content of the liposomal formulations were calculated, as given in Equation 2.

Equation 2:

$$\text{PL conc. in liposome} = \text{Phosphorous conc. in liposome} \times \left(\frac{\text{PL conc. reference PL-sample}}{\text{Phosphorous conc. reference PL-sample}}\right)$$

The ratio between the quantified PL-content in liposomal dispersion before centrifugation (total sample) and after centrifugation (in the supernatant), expressed as PL recovery, was then calculated using Equation 3.

Equation 3:

$$\text{PL recovery (\%)} = \left(\frac{\text{PL in supernatant (µg/mL)}}{\text{PL in total sample (µg/mL)}}\right) \times 100\ \%$$

4.5.5 Liposomal DOC entrapment determination

Finally, the drug entrapment efficiency (EE %) was calculated, adjusting for the liposomes lost during centrifugation, that is correlated with the PL recovery found using the PC-assay (Section 4.5.4). Bigger liposomes or liposome-aggregated will, due to the centrifugal forces, follow the drug precipitate into the pellet, and thus the lipid lost should be accounted for, as a low lipid recovery means that liposomal entrapped DOC is lost in the pellet during centrifugation (see section 4.5.4). The calculation of drug entrapment, adjusting for the liposome lost during centrifugation is shown by Equation 4.

Equation 4:

$$\text{EE (\%)} = \frac{\text{Recovery of DOC (\%)}}{\text{Recovery of phospholipid (\%)}} \text{ x } 100\ \%$$

Drug load capacity gives the drug:lipid weight ratio, calculated from the measured amount of DOC (Section 4.5.3) and lipids (Section 4.5.4) present in the liposomal formulation after removal of the unentrapped drug by centrifugation, as measured in the supernatant. The calculation is shown by Equation 5.

Equation 5:

$$\text{Drug load capacity} = \frac{\text{DOC concentration in supernatant (µg/mL)}}{\text{PL concentration in supernatant (µg/mL)}} *$$

*If lipids without phosphorous were used, this was adjusted for.

4.6 Preparation of DOC-lipid solutions – DOTAP study

In the final part of the study, the DOTAP study, not only DOC was added as stock-solutions (Section 4.4.1), but also DOTAP and SPC were prepared in known concentrations using the same solvents as in the previous studies. The exact volume was pipetted out from these solutions to assure that the actual weight ratio between the lipids and DOC was exactly as intended (Table 6).

Table 6: *Preparation of solutions with DOC, DOTAP and SPC for the DOTAP study.*

Liposomal formulation (DOTAP % (w/w) of total lipid)	**Volume DOC stock-solution (20 mg/mL in methanol)**	**Volume DOTAP stock-solution (80 mg/mL in chloroform/methanol (2:1 v/v)**	**Volume SPC stock-solution (200 mg/mL in methanol)**
1 (0 %)	1500 µL	-	1 mL
2 (10 %)		250 µL	900 µL
3 (20 %)		500 µL	800 µL
4 (30 %)		750 µL	700 µL
5 (50 %)		1250 µL	500 µL

The solutions were mixed in injection vials and sealed with a lid and frozen to -80°C until further processing following the normal procedure described in Section 4.4.2 and 4.4.3. The liposomal formulations were further examined and characterized as previously described (Section 4.5.1-4.5.3).

5 Results and Discussion

The focus of this project has been to investigate how liposomal compositions affect the DOC entrapment. The project was separated into four sub-parts that were interconnected. The first part was the "Pilot project", where we established the methods used in this thesis, validating the reproducibility of the methods applied by conducting repeating experiments with two different liposomal formulations.

The second part was the "Screening study", where 14 different lipid combinations in liposomal formulations were screened with the purpose to identify what lipids should be preferred or avoided to obtain the optimal DOC entrapment efficiency and drug load. The formulations that showed best properties with regard to entrapment efficiency were brought to the third part, the "Optimization study", in which we investigated if an increased DOC:lipid ratio could increase the drug entrapment of these formulations. In the fourth and final part, the "DOTAP study", the best liposomal formulation, containing SPC and DOTAP, was further investigated using different DOTAP concentrations.

5.1 Pilot project

To validate the liposome preparation method, and the suitability of the methods applied for quantifying DOC and PL, a Pilot project with two different liposomal formulations was carried out. These liposomes were made from SPC and SPC and Chol (8:2 w/w), respectively. Both formulations contained DOC in a 1:10 (w/w) ratio with the lipids and were prepared as described in Section 4.4. Both formulations were prepared in triplicates.

Since the method applied during liposome preparation should be suitable for comparing different liposomal formulations and their potential of entrapping DOC in the liposomal membrane, we first had to look at the reproducibility of the method and also whether or not the liposomes were of a suitable size and size distribution, enabling good separation of the unentrapped drug and liposomes, assuring the liposomes remaining in the supernatant during the centrifugation procedure, and trapping the precipitated drug in the pellet. For this to happen, the liposomes should be sufficiently small, and as the rigidity of the liposomal bilayer will differ for different lipid compositions, the sonication time needed to reach the targeted liposome size

of < 100 nm was not predefined but judged by the appearance of the liposomal dispersions that becomes less opaque as the liposome size was reduced. In Table 7, the final sonication time applied for the three batches of the two "Pilot liposomal formulations" are given together with their size and PI values and measures of the zeta potential.

Table 7: *Liposomal characteristics of the two "Pilot liposomal formulations".*

Liposomal formulation	**Sonication time**	**Average diameter (nm ± SD)**	**PI (AU ± SD)**	**Zeta potential ± SD**
SPC	A (3 min)	79.27 ± 0.54	0.24 ± 0.01	-0.11 ± 0.36
	B (2 min)	85.77 ± 0.36	0.23 ± 0.01	-0.12 ± 0.06
	C (2 min)	80.43 ± 0.19	0.25 ± 0.01	-0.35 ± 0.05
SPC:Chol (80:20 w/w)	A (3 min)	63.54 ± 0.38	0.22 ± 0.00	-1.88 ± 0.52
	B (3 min)	58.28 ± 0.40	0.21 ± 0.01	-1.98 ±0.22
	C (3 min)	57.58 ± 0.26	0.20 ± 0.01	-2.56 ± 0.50

The three parallels of the SPC liposomes showed a mean average diameter of 81.82 ± 3.46 (Figure 16), and mean zeta potential was -0.19 ± 0.13. The three parallels of the SPC:Chol liposomes showed a mean average diameter of 59.80 ± 3.26 (Figure 16) and mean zeta potential was -2.14 ± 0.37. Although some intra variability in between the liposomal formulations, the results were satisfying with respect to reproducibility.

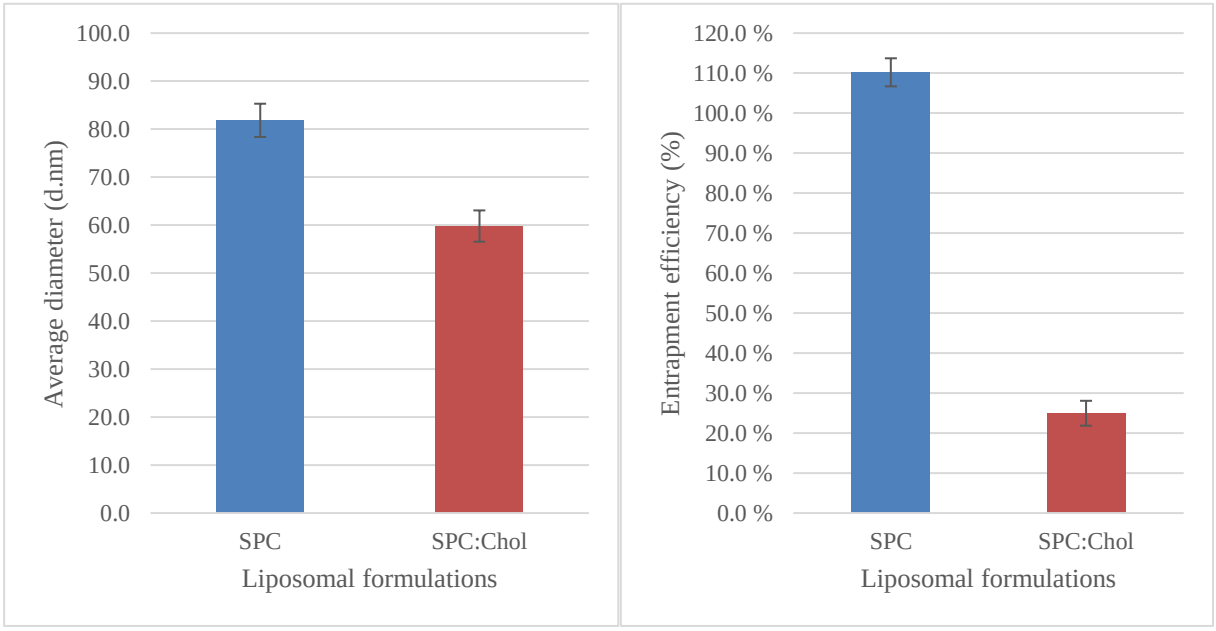


Figure 16: *Size distribution and mean entrapment efficiency of SPC and SPC:Chol liposomes. The value denotes the mean of 3 separate experiments ± SD.*

The success of separating liposomes and free drug was judged by visually inspecting the vial after centrifugation, and finally from the results obtained from the PC-assay, quantifying the PLs present in the liposomal dispersion before (total sample) and after (supernatant). These results are given in Table 8. The recovery of PL in the supernatant was higher for the SPC:Chol liposomes than for the SPC liposomes, something that might be explained by smaller size of these liposomes. However, this variation in PL recovery was found acceptable, and the method kept unchanged for further studies.

Table 8: *Liposomal characteristics and PL-content of the two "Pilot liposomal formulations".*

Liposomal formulation	**Average diameter (nm ± SD)**	**PI (AU ± SD)**	**PL concentration (mg/mL)**		**PL recovery (% ± SD)**
			Total sample	Supernatant	
SPC	79.27 ± 0.54	0.24 ± 0.01	22.21	18.48	85.9 ± 0.05
	85.77 ± 0.36	0.23 ± 0.01	21.83	18.17	
	80.43 ± 0.19	0.25 ± 0.01	19.75	18.00	
SPC:Chol (80:20 w/w)	63.54 ± 0.38	0.22 ± 0.00	17.44	16.95	97.3 ± 0.04
	58.28 ± 0.40	0.21 ± 0.01	17.16	16.09	
	57.58 ± 0.26	0.20 ± 0.01	16.49	16.67	

The liposomes lost in the pellet were considered when calculating the drug entrapment efficiency (Equation 4), as shown in Table 9. Both the higher entrapment efficiency than 100 % and a higher PL-concentration in the total sample before centrifugation, than expected from the amount of lipids added (20 mg/mL and a 16 mg/mL PL concentration in the total sample for the SPC liposomes and the SPC:Chol liposomes, respectively), indicates that the real PL recovery was higher than calculated from the measured results from the PC-assay. Thus, the real DOC entrapment might be corresponding to a value somewhere between the measured DOC recovery in the supernatant and the calculated DOC entrapment values, that takes into account the results from the measured PL recovery.

Table 9: *Drug content and entrapment of DOC in the "Pilot liposomal formulations".*

Liposomal formulation	**DOC concentration (µg/mL)**		**DOC recovery (%)**	**EE (%)***
	Total sample	Supernatant		
SPC	195.59 ± 2.35	184.64 ± 3.00	94.4	113.5
	190.29 ± 4.72	175.19 ± 2.23	92.1	110.6
	213.07 ± 3.61	206.85 ± 2.86	97.1	106.5
SPC:Chol (80:20 w/w)	226.91 ± 5.99	47.12 ± 1.31	20.8	21.4
	211.56 ± 8.52	53.04 ± 2.43	25.1	26.7
	218.72 ± 8.29	59.19 ± 1.19	27.1	26.8

** DOC entrapment (%) calculated when taking into account the PL lost during centrifugation.*

The mean EE (%) of the SPC liposomal formulations was 110 % as shown in Table 9. The reason why the entrapment exceeds 100 % could be explained by the correction of lipid content. The mean entrapment efficacy of DOC was much lower for the SPC:Chol liposomes and around 25 %, as shown in Figure 16. Thus, it seems that Chol is expelling the DOC from the liposomal membrane, and is not good for the entrapment of DOC.

5.2 Screening study: Liposomal lipid composition

After validating the method in the Pilot project, the second step in the project was to screen different lipids in a liposomal DOC formulation to optimize entrapment of DOC. The 14 liposomal formulations included in the study had different lipid composition, but the same drug:lipid ratio of 10:1 (w/w). Information on the liposomal formulations, the ratio of lipid(s) investigated, and the organic solvent applied for the lipid film preparation are summarized in Table 10.

Table 10: *Screening of liposomal formulations with different lipid compositions. No. = Formulation number. Tm = transition temperature.*

No.	Lipid composition	Lipid ratio (w/w)	Tm °C	Solvent
1	SPC	100	-20 to 30	Methanol *
2	SPC:Cholesterol	80:20		Chloroform:methanol (2:1 v/v) *
3	SPC:DOPE	80:20	-16	Chloroform:methanol (2:1 v/v) *
4	SPC:DOPC	80:20	-20	Methanol *
5	SPC:DSPE-PEG2000	80:20	65	Chloroform
6	SPC:DOTAP	80:20		Chloroform:methanol (2:1 v/v) *
7	SPC:Ceramide C6	80:20		Chloroform
8	SPC:Ceramide C12	80:20		Chloroform
9	SPC:DPPG	80:20	41	Chloroform:methanol (2:1 v/v)*
10	SPC:DMPG	80:20	23	Chloroform:methanol (2:1 v/v) *
11	SPC:DMPC	80:20	24	Methanol*
12	SPC:DMPE	80:20	50	Chloroform
13	SPC:POPC	80:20	-9	Chloroform:methanol (4:1 v/v)
14	SPC:POPE	80:20	25	Chloroform:methanol (6:1 v/v)

**(Flaten, 2003)*

The amount of organic solvent varied and was depended on the solubility of the different lipids used. Another factor varied was the temperature of the water bath, which were increased to exceed the T_m of the different lipids (Li *et al*., 2015; Laouini *et al*., 2012).

During sonication, the span of sonication was judged empirically from the appearance of the liposomal dispersions. The liposomal dispersions were visually inspected every 30 seconds to look at the transparency of the dispersions and evaluate the size. The dispersions went from being opaque and white to more transparent as they became smaller. Since this formulation is intended to be administrated by injection, the liposomes ought to be smaller than 200 nm

(Harashima *et al*., 1994; Allen and Cullis, 2004) and preferably around 50-100 nm (Bozzuto and Molinari, 2015; Kumari *et al*., 2016). The size was measured before centrifugation to assure an average diameter around 100 nm, but the polydispersity was very high. Hence, appearance was a decisive factor for sonication time. After purification, the average diameter and PI showed an acceptable range of the liposomal formulations. The different liposomal formulations required different sonication times for getting smaller, and therefore it was difficult to standardize the sonication time.

The liposomal characteristics of liposomal formulation 1-14 are presented in Table 11. All formulations contained 200 mg of lipid and 20 mg of DOC.

Table 11: *Liposomal characteristics.*

No.	Lipid composition	Sonication time	Average diameter (nm ± SD)	PI (AU ± SD)	Zeta potential ± SD
1*	SPC	3, 2, 2 min	81.82 ± 3.46	0.24 ± 0.01	-0.19 ± 0.13
2*	SPC:Chol	3, 3, 3 min	59.80 ± 3.26	0.21 ± 0.01	-2.14 ± 0.37
3	SPC:DOPE	1.5 min	91.31± 0.55	0.24 ± 0.00	-5.46 ± 0.48
4	SPC:DOPC	3 min	78.11 ± 0.50	0.28 ± 0.01	-2.59 ± 0.16
5	SPC:DSPE-PEG2000	0.5 min	97.55 ± 0.24	0.36 ± 0.01	-3.31 ± 0.34
6	SPC:DOTAP	3 min	77.97 ± 0.25	0.29 ± 0.00	76.28 ± 0.90
7	SPC:CC6	2 min	77.35 ± 0.52	0.24 ± 0.00	-1.87 ± 0.04
8	SPC:CC12	2 min	96.93 ± 0.83	0.42 ± 0.06	-1.33 ± 0.07
9	SPC:DPPG	0.5 min	103.67 ±0.71	0.24 ± 0.01	-31.52 ±0.62
10	SPC:DMPG	0.5 min	98.69 ± 0.82	0.25 ± 0.00	-31.90 ± 0.40
11	SPC:DMPC	0.5 min	109.37 ± 0.68	0.21 ± 0.00	-2.29 ± 0.05
12	SPC:DMPE	4 min	182.57 ± 8.98	0.86 ± 0.03**	-6.63 ± 0.24
13	SPC:POPC	4 min	81.48 ± 0.26	0.21 ± 0.00	-0.34 ± 0.10
14	SPC:POPE	2 min	86.03 ± 0.93	0.32 ± 0.02	-5.60 ± 0.48

**n=3 (average of the results in the Pilot project) ** The estimated diameter of the liposome vesicles is too polydisperse, that means the PI values that exceeds 0.7 are not valid.*

The average size of the different liposomal formulations in the Screening study was around 100 nm ranging from 77-109 nm, with the exception of SPC:Chol liposomes which were 60 nm and SPC:DMPE which were 183 nm as shown in Figure 17.

The liposomal formulations were observed to exhibit a smaller size and lower PI after centrifuged to remove unentrapped DOC. This might be explained by removal of unentrapped

DOC in the supernatant (Pereira *et al*., 2016). Thus, the size and zeta potential measurements conducted for total lipid dispersion during sonication were only applied as a guidance for whether to stop the sonication and not included in the final results, as the results obtained after centrifugation (Table 11 and Figure 17) was considered more accurate and reliable.

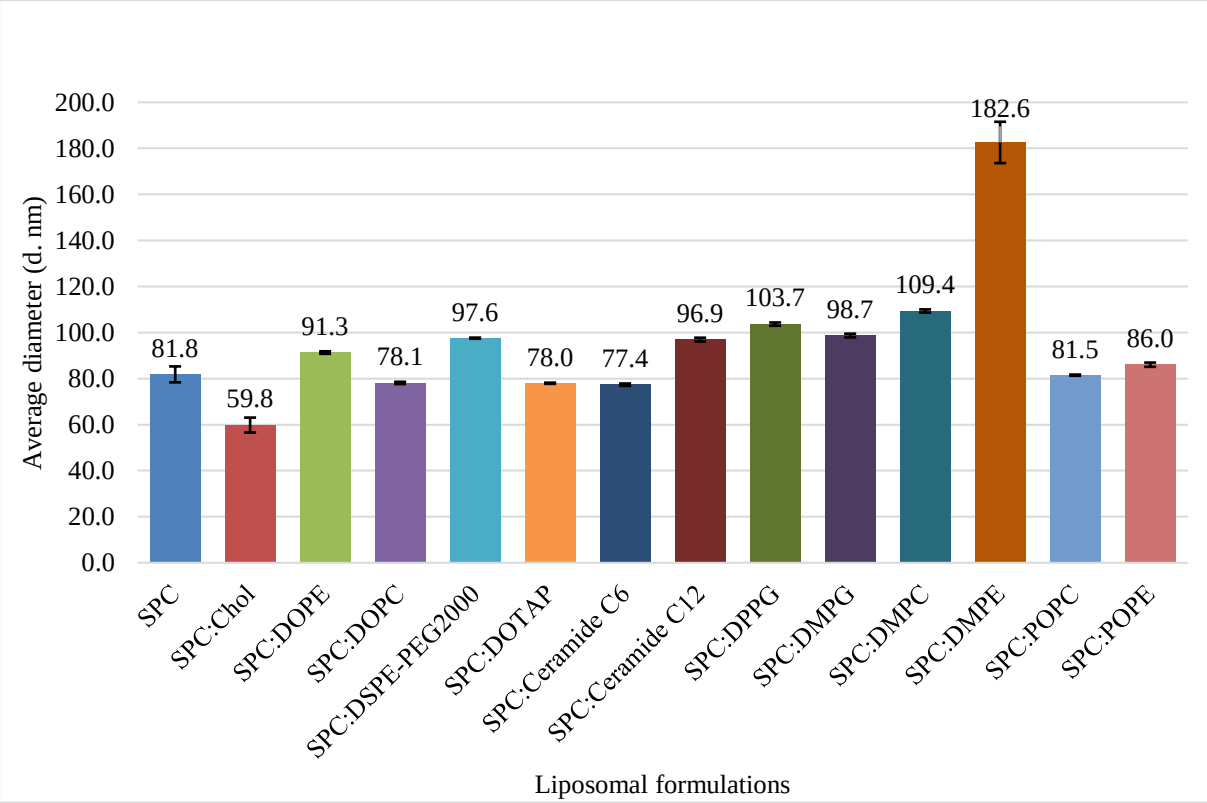


Figure 17: *Liposomal size distributions in the Screening study.*

PI is a measure of heterogeneity, or the width of the distribution of particles (Woodbury *et al*., 2006). Small values of PI (< 0.2) indicate homogenous distribution while larger PI values (> 0.2) indicates high heterogeneity and broad distribution of particles. The PI value should not exceed 0.7 because in that case the liposomal formulations is too polydisperse and cannot be trusted (Malvern, 2013).

The majority of the liposomal formulations displayed a PI < 0.3, which indicate that the measurement with the Malvern Zetasizer is a suitable method for these samples. SPC:Ceramide C12 (No. 8) and SPC:POPE (No.14) showed a PI over 0.3, respectively 0.42 and 0.32, which

indicate that they show a broader polydispersity, but were still below 0.7. However, the SPC:DMPE (No. 12) displayed a PI of 0.86 and hence it was found too polydisperse.

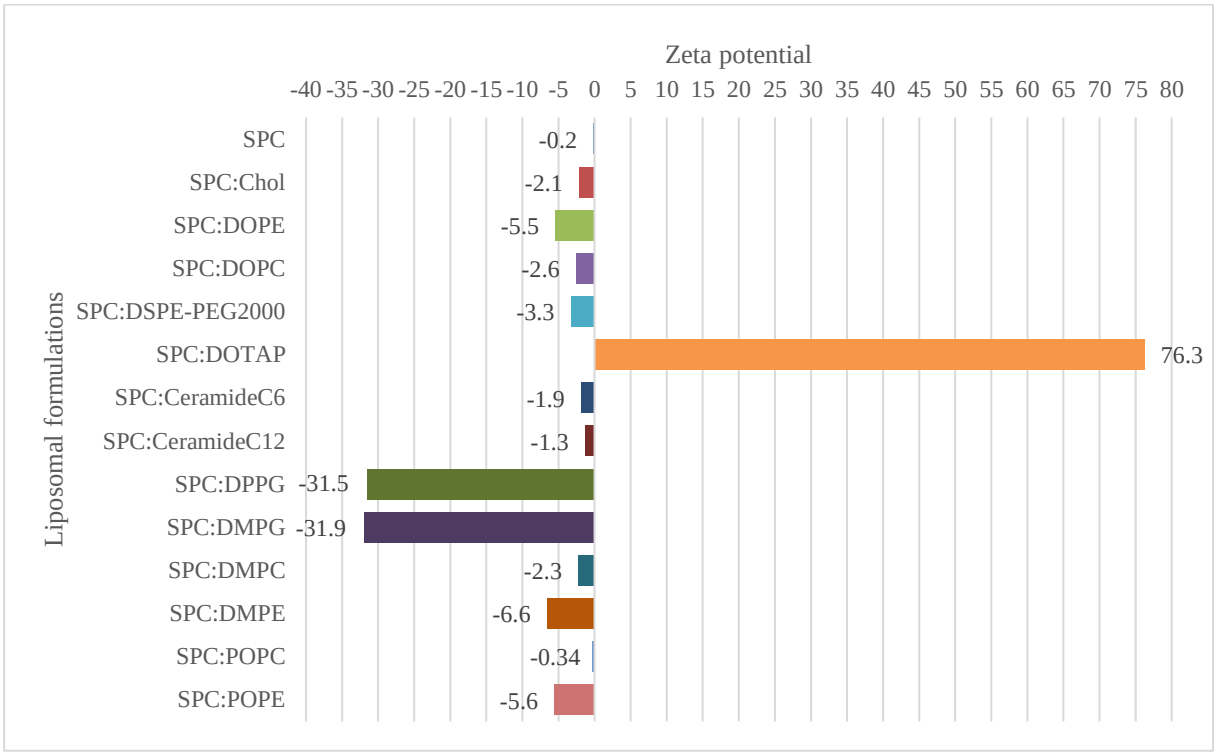


Figure 18: *Liposomal charge in the Screening study.*

The majority of the liposomal formulations carried a slightly negative charge, as shown in Figure 18. The head group of the lipids decides the charge (Kraft *et al*., 2014). The liposomal formulations composed of PE or PC were almost neutral, as expected as these head groups are neutral, as seen in Table 1. The SPC:DPPG (No. 9) and SPC:DMPG (No. 10) carried a high negative charge around -30 mV, as expected since the PG head group carry a net negative charge as seen in Table 1 and hence contributes to the highly negative zeta potential. The SPC:DOTAP (No. 6) was the only liposomal formulation in this study found to have a positive charge, as expected because DOTAP is a cationic lipid (Zhi *et al*., 2018).

Both SPC and SPC:Chol liposomes were slightly negative charged, and close to neutral, as expected since either SPC or Chol bear a charge. Yang *et al*. (2007) prepared liposomes with SPC:Chol (90:10 molar ratio) with paclitaxel and reported a zeta potential which were

coinciding with our results, even though the lipid ratio and drug were not the same (Yang *et al*., 2007).

Neutral liposomes have a lower tendency to be cleared by the MPS but have a higher tendency to aggregate. Both a negative and a positive charge contributes to reduce tendency to aggregate and hence increase the stability (Kraft *et al*., 2014), but on the contrary, a negatively and a positively charge increases the chance of being opsonized and cleared by the MPS and hence a shorter period of the in the circulation after being administrated (Lian and Ho, 2001; Allen and Cullis, 2013).

The results of entrapment efficiencies and drug:lipid ratio for the liposomal formulations are presented in Table 12. Drug recovery is the ratio between DOC concentrations in the supernatant after centrifugation divided by the total DOC concentration before centrifugation, determined by HPLC. The DOC concentration was adjusted by the PL recovery to compensate for variable PL recovery presented as entrapment efficiency (EE %). The drug load capacity represents how much DOC that is associated with the liposomes (the drug:lipid ratio), and gives a more comparable measure of the drug load in the liposomal membrane, as it is not associated with the initial drug:lipid ratio applied in the formulation (Pereira *et al*., 2016).

***Table 12**: Liposomal characteristics of 14 liposomal formulations investigated in the Screening study.*

No.	Lipid composition	DOC recovery in supernatant (%)	PL recovery in supernatant (%)	EE (%)	DOC:lipid ratio (w/w)
1	SPC	94.5 ± 0.03	85.9	110.2 ± 0.04	0.104 ± 0.98
2	SPC:Chol	24.3 ± 0.03	97.3	25.0 ± 0.03	0.040 ± 0.49
3	SPC:DOPE	69.9	84.4	82.5	0.079
4	SPC:DOPC	48.0	89.3	53.8	0.051
5	SPC:DSPE-PEG2000	64.4	95.1	67.7	0.058
6	SPC:DOTAP	101.7	88.8	114.6	0.122
7	SPC:CC6	15.7	89.8	17.5	0.027
8	SPC:CC12	20.8	89.3	23.3	0.039
9	SPC:DPPG	65.2	90.7	71.8	0.064
10	SPC:DMPG	92.3	95.9	96.2	0.089
11	SPC:DMPC	48.7	84.6	57.6	0.057
12	SPC:DMPE	39.3	99.2	39.6	0.041
13	SPC:POPC	53.8	95.7	56.3	0.062
14	SPC:POPE	45.9	97.4	47.1	0.052

** Liposomes that included cholesterol (Chol), DOTAP, Ceramide C6 (CC6) and Ceramide C12 (CC12) had their lipid content adjusted for in the calculations; lipid amount = phospholipid content * 1.25. This since these lipids, accounting for 20 % (w/w) of the lipids in the formulation, do not contain phosphorous.*

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The recovery of DOC after purification ranged between 16-102 % for the 14 different liposomal formulations. The drug recovery and entrapment efficiency of formulation no. 2, 4, 7, 8, 11, 12 and 14 was rather low, all of them with recovery of DOC below 50 % and <60 % EE. All formulations showed lipid recoveries ranging between 84 and 100 %, hence the lipid loss during purification of liposome cannot be responsible for the amount of DOC that was lost during purification. A possible explanation for this could be that DOC was not fully incorporated in the liposomal bilayer, but rather associated with the outer surface and hence lost during centrifugation leading to a reduction in entrapment efficiency. This was also suggested by Pereira *et al*. (2016) which made liposomal formulations of DOC, but with non-comparable compositions of the liposomes. In their study, a liposomal formulation composed of DOPC:Chol prepared with a molar ratio of 100:50 with a lipid:drug ratio of 40:1, 20:1 and 10:1, respectively, showed a maximum entrapment efficiency around 90 % for all three lipid:drug ratios (Pereira *et al*., 2016).

The 14 different liposomal formulations showed highly varying results with respect to DOC:lipid ratio, as shown in Table 12. The SPC (No. 1) and SPC:DOTAP (No. 6) liposomal formulations showed that the amount of DOC that was associated with the liposomes was approximately 0.1 DOC/1 lipid (w/w), which was consistent with the initial drug:lipid ratio of 1:10 (w/w). The SPC:DMPG (No. 10) showed a DOC:lipid ratio of 0.089 DOC/1 lipid (w/w) which was slightly lower than the SPC and the SPC:DOTAP liposomal formulation.

The SPC:Chol (No. 2), SPC:Ceramide C6 (No. 7) and SPC:Ceramide C12 (No. 8) liposomal formulations showed the lowest DOC:lipid ratio of all 14 liposomal formulations, all with a DOC:lipid ratio of ≤ 0.040 DOC/1 lipid (w/w). These results were consistent with the low entrapment efficiency of the same liposomal formulations.

The entrapment of DOC of the 14 different liposomal formulations ranged between 18 % and 115 % as shown in Figure 19.

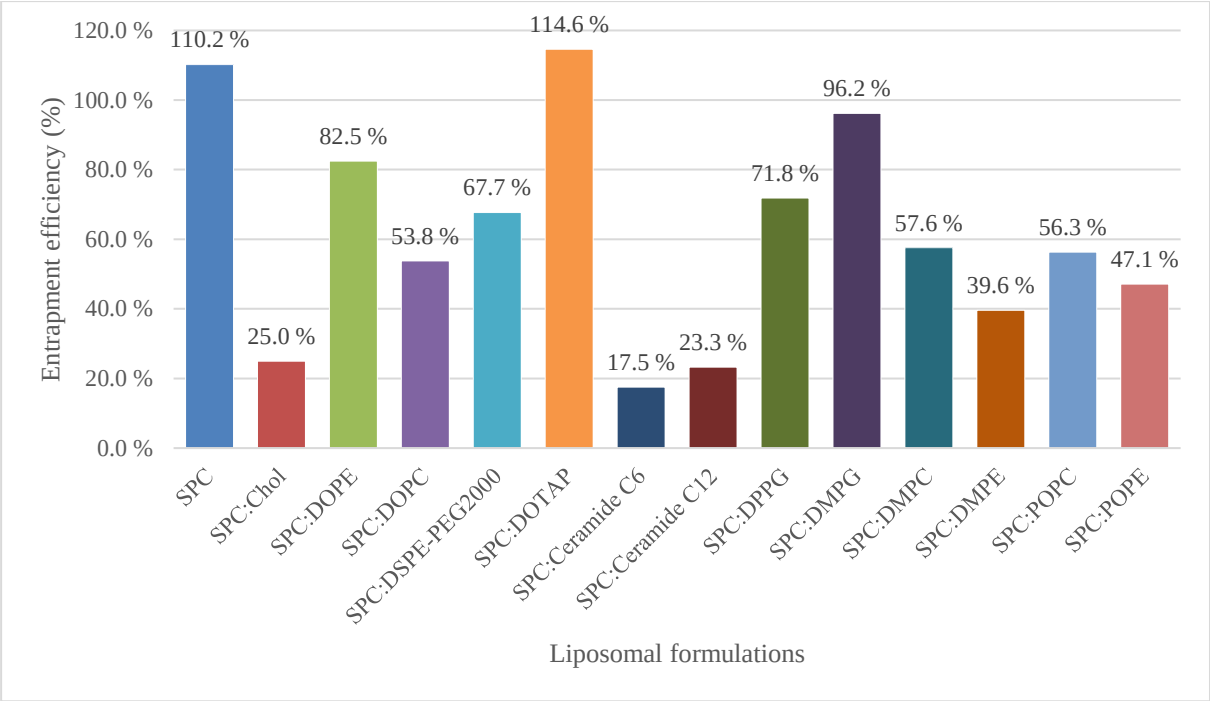

Figure 19: *Entrapment efficiency of liposomal formulation 1-14 in the Screening study.*

As shown in Figure 19 the entrapment of DOC varied a lot. The entrapment of DOC is dependent on the properties of the acyl chains of the PLs in the liposome and DOCs partition coefficient and polarity. Because of DOCs lipophilic nature it will reside in the fatty acyl chains of the liposome, and hence properties of the PLs used is a decisive factor for entrapment (Bozzuto and Molinari, 2015).

This study showed no correlation between saturation of the lipids and entrapment efficiency. SPC:DOTAP (No. 6) is unsaturated, as shown in Figure 6 and has the highest entrapment efficiency, but SPC:DMPG (No. 10) is unsaturated (Table 1) and has entrapment around 100 %. Formulation no. 3, 4, 6, 7, 8, 9, 13 and 14 were all unsaturated and displayed varying entrapment efficiency in the range of 18-115 %, and formulation no. 10, 11 and 12 is composed of saturated lipids and show an entrapment ranging from 40-96 %.

Immordino *et al*. (2003) investigated different liposomal formulations of DOC, but with non-comparable compositions of lipids. In their study it was observed that changing an unsaturated lipid (egg PC) with saturated lipids decreased the encapsulation efficiency. This was not found in our project and hence we cannot confirm or disapprove if the saturation will affect the

entrapment efficiency. They also observed that a higher amount of Chol or an increased DOC:lipid molar ratio affected the encapsulation efficiency negatively (Immordino *et al*., 2003).

Pereira *et al*. (2016) reported that liposomes composed of unsaturated lipids showed the highest DOC loading compared to saturated lipids. They used other lipid combinations in which are non-comparable with this study (Pereira *et al*., 2016). In this study we screened 14 different lipid combinations, in which we included 1-2 lipids in the different liposomal formulations, while both Immordinio *et al*. (2003) and Pereira *et al.* (2016) included more than 2 lipids in their liposomes. Hence it is several factors that affect how well DOC is incorporated and becomes a part of the liposomal membrane, and it is difficult from our findings to conclude it saturation affects the entrapment efficiency. It could appear like that DOC have a higher affinity for cationic lipid blends as compared to the non-charged and negative charged. This has also been described for other anticancer agents with lipophilic properties, like Camptothecin (Sætern *et al*., 2004).

We evaluated if different lengths of the fatty acids and variety head group of the PLs used in the different liposomal formulations could affect the entrapment of DOC. The head group of PLs determine the charge. Both PC and PE are non-charged (Kraft *et al*., 2014) as shown in Figure 5. In this study we used four different PLs with PC: SPC (No 1), DOPC (No. 4), DMPC (No. 11) and POPC (No. 13) which showed entrapment of 110, 54, 58 and 56 %, respectively. Interestingly, the liposomal formulations composed of DOPC, DMPC and POPC displayed a rather similar entrapment of DOC, even though they had different length and saturation of the fatty acids, where DMPC have saturated C14 chains, DOPC have unsaturated C18 chains, and POPC have one C18 saturated and one C18 unsaturated chain, as shown in Table 1.

We used four different PLs with PE as head group: DOPE (No. 3), DSPE (No.5), DMPE (No. 12) and POPE (No. 14) which showed varying entrapment of DOC with 83, 68, 40 and 47 %, respectively. Hence, for these liposomal formulations, it seems like properties of the fatty acids was determinant of entrapment.

PG is a negatively charged head group (Kraft *et al*., 2014), as seen in Figure 5. We used two lipids with PG: DPPG and DMPG, both which displayed a relatively high entrapment, 72 and

96 %, respectively. In this case it seems like a shorter fatty acyl chain gives higher entrapment, as saturation is the same for both dipalmitoyl in DPPG and dimyristoyl in DMPG (Table 1).

When comparing lipids with the same fatty acyl acids, but with different head groups in between the different liposomal formulations, such as DMPG (No. 10), DMPC (No. 11) and DMPE (No. 12), the entrapment decreases from 96 % with the PG group, to 58 % with the PC group, to 40 % with the PE group. These lipids have the same fatty acyl group: dimyristoyl which are saturated with 14 carbons, as seen in Table 1. The same trend could be seen with POPC (No. 13) and POPE (No. 14) where entrapment decreases from 56 % with PC to 46 % with PE. These two lipids have the same fatty acyl group: palmitoyl-oleyl which have one saturated 16 carbon chain and one unsaturated 18 carbon chain. From these observations, it could seem like the head group of PLs affects entrapment in the following order: PG > PC > PE. However, this is not the case with the fatty acid dioleoyl in DOPE (No.3) and DOPC (No. 4) where DOPE have a higher entrapment of DOC with 83 % compared to DOPC with 54 %.

The liposomal formulations which contained ceramides showed poor entrapment of DOC. Ceramide C6 (No. 7) with a C6 fatty acyl chain, shown in Figure 7, showed an entrapment of 18 %, while ceramide C12 with a C12 fatty acyl chain, shown in Figure 8, showed a slightly higher entrapment with 23 %.

The liposomal formulation containing Chol (No. 2) also showed a poor entrapment of DOC with 25 %. Previous studies have found that Chol in liposomal formulations with hydrophobic drugs (paclitaxel and DOC) exhibits a negative effect on encapsulation efficiency (Crosasso *et al*., 2000; Immordino *et al*., 2003) and can be explained by Chol occupying the hydrophobic space in the membrane (Chen *et al*., 2017). However, Immordino *et al*. (2003) showed that inclusion of a small amount of Chol did not have a negative effect on encapsulation efficiency of DOC, but in concentrations over 30 mol% the encapsulation efficiency and stability decreased (Immordino *et al*., 2003). In our case, the Chol concentration was ~33 mol%, and therefore our results correspond well with the work of Immordino and colleagues.

Chen *et al*. (2017) made liposomes with egg PC:Chol:DSPE-PEG2000:DOC at 56:40:4:4 molar ratio. They observed that the optimal Chol content was 40 mol% for their formulation. These results are not supported by the findings done in our study. The formulation and preparation method of Chen *et al*. (2017) are not comparable to the method applied in this project, as they

hydrated their lipid film with PBS and both probe sonicated and extruded their liposomes (Chen *et al*., 2017).

Yang *et al*. (2007) made paclitaxel liposomes with SPC:Chol (90:10 lipid molar ratio) and SPC:Chol:DSPE-mPEG2000 (90:10:5 lipid molar ratio), and reported an entrapment efficiency of 61 and 57 %, respectively. Compared to this study, the SPC:Chol (No. 2) liposomes with 2:1 molar ratio, has a much higher amount of Chol and a lower EE %. Interestingly, the SPC:DSPE-PEG2000 (No. 5) liposomal formulation in our study showed an entrapment efficiency of 68 %, with approximately 14:1 molar ratio of SPC:DSPE-PEG2000. The results might not be comparable as Yang *et al*. (2007) used paclitaxel as investigated drug and a different method of preparation, a modified thin-film hydration method where they evaporated solvents and flushed the lipid film with nitrogen and under vacuum overnight before hydrating the lipid film whit a phosphate buffer saline (PBS), thereafter extruding the dispersion to reduce the size of the liposomes (Yang *et al*., 2007). However, paclitaxel and DOC are both taxanes and are quite similar in structure, both highly lipophilic (Louage *et al*., 2017). It would be interesting to investigate the DSPE-PEG2000 further in a lipid combination, as PEGylation have shown decreased uptake by the MPS (Kim, 2016; Bozzuto and Molinari, 2015).

Formulation number 1 (SPC), 6 (SPC:DOTAP) and 10 (SPC:DMPG) shown in Table 12 were the most promising liposomal formulations after screening of the different lipids combinations in liposomal formulations, as they show an entrapment efficiency near 100 %.

The SPC:DOTAP formulation (No. 6) showed superior entrapment efficiency. This was the only cationic lipid used in the Screening study, and the formulation in this study showed a cationic charge at 76.3 mV. The fatty acyl chains of DOTAP, dioleyl is unsaturated with C18 chains. In comparison with the two other PLs with dioleoyl fatty acids, DOPC and DOPE used in this study, the DOTAP liposomal formulation displayed a higher entrapment with 115 % relative to DOPE with 83 % and DOPC with 54 %. The structure which separate DOTAP (Figure 6) from DOPE and DOPC (Table 1) is the head group which, among other things, does not have a phosphorous group. Both DOTAP and DOPC have a trimethylamine in their head group, and both showed a higher entrapment of DOC compared to DOPE which have a NH_3 (ammonia group).

A study on liposomes with paclitaxel in 3 and 4 mol% with respect to lipid, and DOTAP and different phosphatidylcholine in different lipid ratios investigated incorporation effect (Campbell *et al*., 2001). The results indicated a high incorporation of paclitaxel near 100 % with increased mol% of DOTAP. The DSPC:DOTAP showed a 100 % incorporation with 3 mol% paclitaxel and 60 % incorporation with 4 mol% paclitaxel with a 20 mol% of DOTAP. Our results with SPC:DOTAP, with approximately 20 mol% DOTAP and 8.5 mol% DOC showed an entrapment efficiency near 100 %. They also found that increasing amount of DOTAP increases the incorporation of paclitaxel up to a certain point and with some exceptions. It must be pointed out that these results are not comparable with our study, as they used different lipid compositions, a different drug and other preparation and characterization methods (Campbell *et al*., 2001).

Yang *et al*. (2009) reported an entrapment efficiency of 98 % of SPC liposomes containing DOC with a 25:1 (w/w) lipid:drug ratio. Although they chose a different preparation method, the ethanol injection method, followed by extrusion to obtain liposomes with the targeted size range, 100 nm, the lipid composition is similar (Yang *et al*., 2009). In this study we showed that a lipid:drug ratio of 10:1 (w/w) of SPC liposomes was achievable with close to 100 % DOC entrapment efficiency (Table 12). Even though we managed to solubilize DOC with a lipid:drug ratio of 10:1 (w/w) and achieved a concentration of approximately 2 mg/mL, the commercially available DOC formulation in the Norwegian market have a concentration of 20 mg/mL (Felleskatalogen, n.d.). Thus, if this were to be the final formulation to be administrated, it would most likely require a higher volume of injection to reach therapeutic range.

The three liposomal formulations with EE near 100 % and a DOC:lipid ratio of approximately 1:10 (w/w), SPC (No. 1), SPC:DOTAP (No. 6) and SPC:DMPG (No. 10), respectively, were brought further to the Optimization study to see if these liposomal formulations could be further optimized.

5.2.1 Quantification of lipid loss

The PC-assay examined the amount of phosphorus in the different liposomal dispersions in order to give an estimation of lipid concentration (Bartlett, 1959; Naderkhani *et al*., 2015). The results are presented in Table 13. The theoretically concentration of lipid was 20 mg/mL. Since Chol, DOTAP, Ceramide C6 and Ceramide C12 does not contain a phosphorous group like the other lipids, the amount of these lipids was not quantified, and hence, this was adjusted for in calculation. Thus, the expected quantified PL-concentration in these liposomal formulations were 16 mg/mL.

Table 13: *PL-content and loss of lipid during centrifugation.*

No.	**Lipid composition**	**Average diameter (nm ± SD)**	**PL concentration (mg/mL) ± SD**		**PL recovery (%)**
			Total sample	Supernatant	
1	SPC	81.82 ± 3.46	21.26 ± 1.33	18.22 ± 0.24	85.9 ± 0.05
2	SPC:Chol	59.80 ± 3.26	17.03 ± 0.49	16.57 ± 0.44	97.2 ± 0.04
3	SPC:DOPE	91.31± 0.55	20.89 ± 0.12	17.62 ± 0.12	84.4
4	SPC:DOPC	78.11 ± 0.50	22.63 ± 0.29	20.22 ± 0.32	89.3
5	SPC:DSPE-PEG2000	97.55 ± 0.24	18.89 ± 0.06	17.96 ± 0.04	95.1
6	SPC:DOTAP	77.97 ± 0.25	27.01 ± 0.17	23.98 ± 0.06	88.8
7	SPC:CC6	77.35 ± 0.52	17.68 ± 0.05	15.87 ± 0.06	89.8
8	SPC:CC12	96.93 ± 0.83	16.38 ± 0.04	14.64 ± 0.04	89.3
9	SPC:DPPG	103.67 ±0.71	23.00 ± 0.04	20.86 ± 0.05	90.7
10	SPC:DMPG	98.69 ± 0.82	22.39 ± 0.07	21.48 ± 0.07	95.9
11	SPC:DMPC	109.37 ± 0.68	23.04 ± 0.08	19.49 ± 0.08	84.6
12	SPC:DMPE	182.57 ± 8.98	22.36 ± 0.04	22.18 ± 0.05	99.2
13	SPC:POPC	81.48 ± 0.26	22.77 ± 0.06	21.84 ± 0.09	95.7
14	SPC:POPE	86.03 ± 0.93	20.78 ± 0.02	20.24 ± 0.05	97.4

The PL recovery was around 84-99 % which indicate that the loss of lipid during purification process with centrifugation was relatively low. The amount of lipid added to the liposomal formulations was 20 mg/mL with exception of SPC:Chol, SPC:DOTAP, SPC:Ceramide C6 and SPC:Ceramide C12 liposomes in which the amount was 16 mg/mL, and after centrifugation one could expect that the liposomal formulations with smaller size would have a higher PL

recovery. This could be seen with SPC:Chol with small liposome size and high PL recovery, compared with several of the other liposomal formulations with bigger size and a slightly lower PL recovery, as shown in Table 13.

Interestingly, the liposomal formulation with the highest PL recovery was the SPC:DMPE, which also displayed the biggest liposome size with 182 nm. The SPC:DMPE liposomal formulation was made two times, in which the first displayed an average size of 210 nm and a PI > 0.9 (data not shown) after two minutes sonication. Therefore, we de decided to make a new formulation (No. 12) to target a smaller size which was sonicated for 4 minutes. The targeted size was not achieved, and it seemed like this liposomal formulation needed longer sonication time to become smaller. Because of low entrapment and limited time for optimization, we decided not to make a new sample of this liposomal formulation. The high PL recovery of this liposomal formulation needs further investigation, but it could be that the size was small enough to be under the cut off line for what was become a part of the pellet, even though the size was bigger than the targeted size.

5.3 Optimization study

This part of the study involved three of the liposomal formulations from the previous Screening study, SPC (formulation no 1), SPC:DOTAP (formulation no 6) and SPC:DMPG (formulation no 10). In order to see if these formulations could be further optimized, the lipid:drug ratio was increased from 10:1 (w/w) used in the Screening study to 10:2 (w/w). The purpose was to see if increased amount of DOC could be further solubilized in the liposomes with the same amount of lipid.

The concentration of DOC was doubled compared to the Screening study, from 2 mg/mL to 4 mg/mL. All formulations contained 200 mg of lipid and 40 mg DOC. The liposomal characteristics of the three different formulations are presented in Table 14 and the drug content is presented in Table 15.

Table 14: *Liposomal characteristics.*

Liposomal formulation	**Lipid ratio (w/w)**	**Sonication time**	**Average diameter (nm ± SD)**	**PI (AU ± SD)**	**Zeta potential ± SD**
SPC	100	2 min	80.06 ± 0.61	0.28 ± 0.01	-0.45 ± 0.03
SPC:DOTAP	80:20	1 min	104.47 ± 0.06	0.25 ± 0.01	57.70 ± 0.93
SPC:DMPG	80:20	0.5 min	106.50 ± 0.10	0.23 ± 0.00	-28.77 ± 0.75

The average diameter of the liposomal formulations shown in Table 14 was around 100 nm, which is similar to the average diameter of the same liposomal formulations in the Screening study, with exception of SPC:DOTAP which could be explained by a shorter sonication time in this Optimization study. The PI value of all three liposomal formulations were < 0.3 which indicates that they have a homogenous relatively small width distribution. The zeta potential of the SPC was slightly negative as expected and observed in the Screening study. The charge of the SPC:DOTAP liposomes were highly positive, and the SPC:DMPG liposomes were negatively charged, both as observed in the Screening study. Thus, as expected, increased DOC content in preparation did not affect the zeta potential of the liposomes.

Table 15: *Drug content in the liposomal formulations.*

Liposomal formulation	**Sonication time**	**DOC concentration (µg/mL)**		**DOC recovery in supernatant (%)**
		Total sample	Supernatant	
SPC	2 min	406.24 ± 6.75	83.82 ± 1.37	21 %
SPC:DOTAP	1 min	348.70 ± 6.97	151.65 ± 2.93	43 %
SPC:DMPG	0.5 min	375.70 ± 7.72	74.50 ± 1.61	20 %

The recovery of DOC in the supernatant after centrifugation was 21 %, 43 % and 20 % for SPC, SPC:DOTAP and SPC:DMPG, respectively. Compared with formulation 1, 6 and 10 in the Screening study, which are the same formulations but with a lipid:drug ratio of 10:1, we can see that the drug recovery has decreased dramatically as shown in Figure 20. It might be that the increased concentration of precipitated drug in the liposomal dispersion capture more liposomes in the pellet. Unfortunately, we did not perform any recovery assessment of PL in this part of the study, and thus this theory cannot be confirmed. However, both studies indicate the positive effect and superiority of the DOTAP formulation.

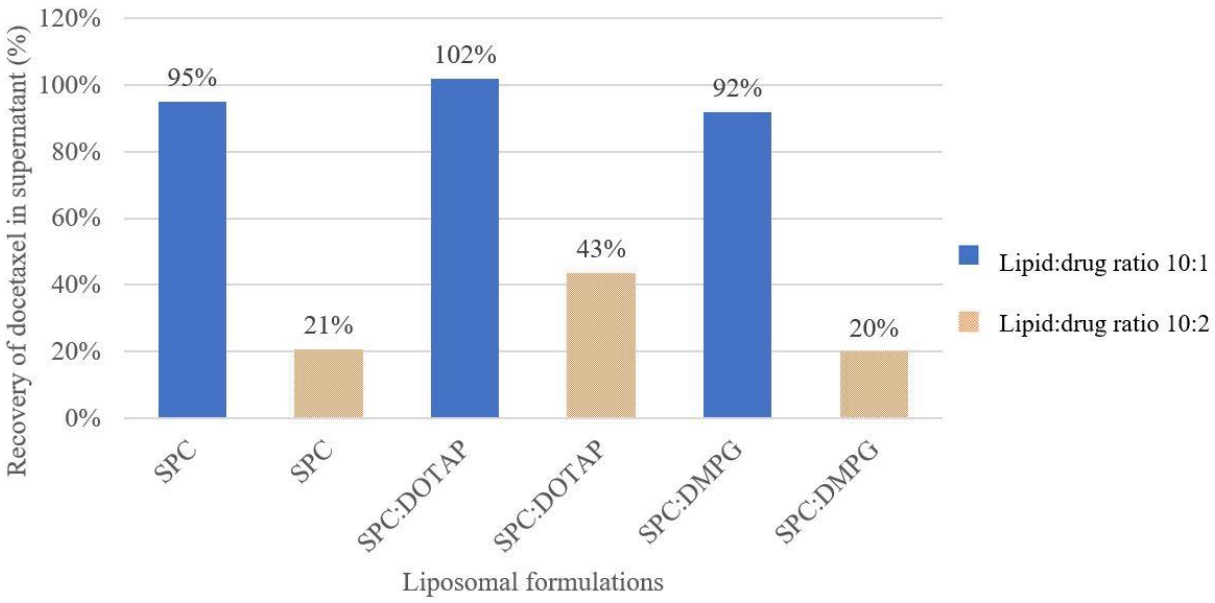

Figure 20: *DOC recovery after centrifugation.*

Thus, in this third part of the study it was not executed a phospholipid assay. We assumed that the recovery of lipids was approximately 100 % based on the results from the Screening study, and hence calculated the drug:lipid ratio of the formulations in Table 15 to estimate how much DOC that was associated with the liposomes. The results are presented in Figure 21. The results were compared with the same formulations in the Screening study, but with different lipid:drug ratios: 20:1 and 10:1, respectively.

For the liposomal formulations with SPC lipid only, we can see that an increasing amount of DOC has a negative effect on the drug:lipid ratio. This trend was also seen in the two other formulations: SPC:DOTAP and SPC:DMPG, but not in the same extent for SPC:DOTAP liposomes, as for the two other liposomal formulations.

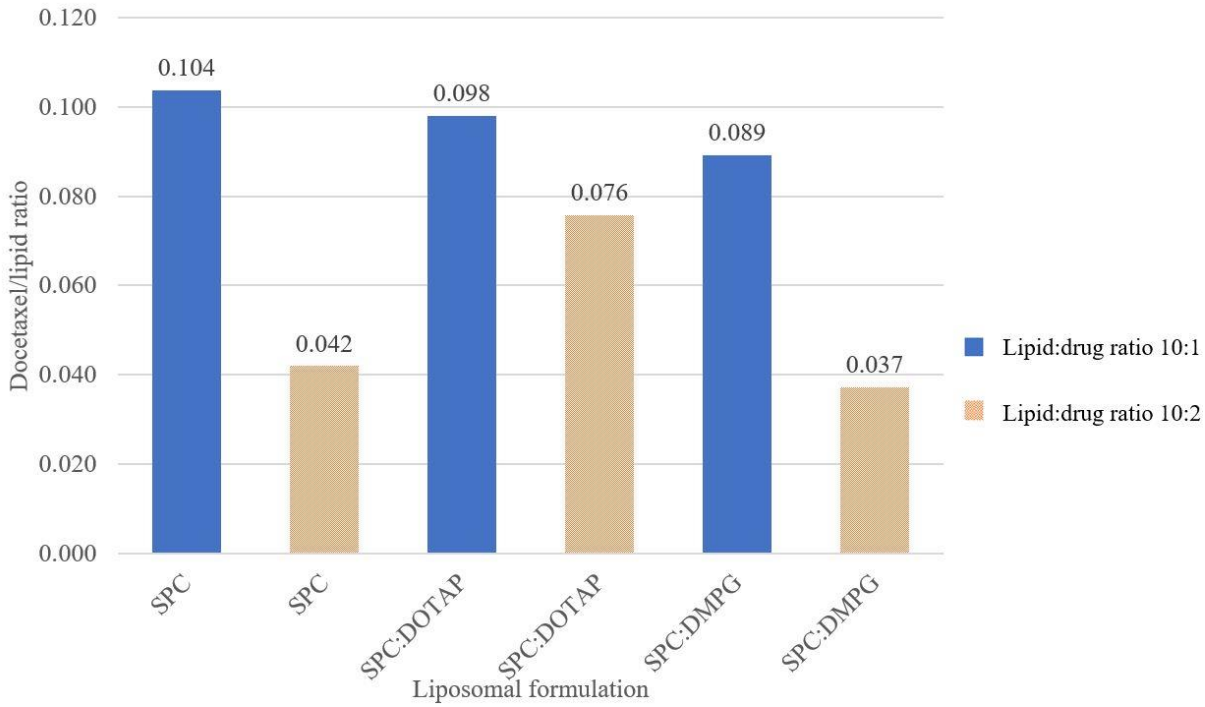

Figure 21: *Estimated DOC:lipid ratio of the liposomal formulations from the Screening study (blue) and the Optimization study (beige).*

It is desirable to achieve a high entrapment of DOC, in other words, to achieve the highest drug:lipid ratio. The outcome of increasing the drug:lipid ratio is reduced amount of lipid administrated for a given dose. This is beneficial both with concerns of reducing the economic

cost of production (Straubinger and Balasubramanian, 2005). DOTAP is more expensive than other naturally occurring lipids (Sætern *et al*., 2004).

In this Optimization study, we investigated if DOC could be further solubilized in the liposomes, and from the results, it seemed like a 10:1 (w/w) lipid:drug ratio, used in the Screening study, was better for entrapment of DOC. The SPC:DOTAP liposomal formulation achieved a higher DOC recovery compared to the SPC and the SPC:DMPG liposomal formulation with a 10:2 (w/w) lipid:drug ratio, and was brought to the DOTAP study for further investigation.

5.4 DOTAP study: the effect of different DOTAP concentration on DOC entrapment

The SPC:DOTAP liposomal formulation showed a superior recovery of DOC compared with the other formulations in the third part of the study, the "Optimization study". DOTAP and cationic liposomes are particularly interesting because of the electrostatic attraction between the positive particles and negative charged components that covers cells (sulphated proteoglycans of glycocalyx) which leads to binding to cells (Steffes *et al*., 2017). A previous study demonstrated that cationic liposomes composed of DOTAP:DOPC:Paclitaxel have ability to accumulate in tumour blood vessels and increase antitumoral efficacy (Schmitt-Sody *et al*., 2003).

Sætern *et al*. (2004) compared the effect of varying the amount of DOTAP in egg PC:DOTAP and Camptothecin liposomes in order to find an optimal content of DOTAP for maximum incorporation of the drug. They reported that with increasing amount of DOTAP they reached a plateau for incorporation of the drug at approximately 20 mol% DOTAP (Sætern *et al*., 2004).

The fourth and final part of the project involved comparison of the effect of varying the concentration of DOTAP within SPC:DOTAP liposomes. This part involved making new liposomal formulations with DOC, where the amount of DOTAP was tested in increasing concentrations. The liposomes were prepared with a thin film-hydration method and sonicated for one minute. The liposomal characteristics of the five different formulations is presented in Table 16. All formulations contained 200 mg lipid and 30 mg DOC.

Table 16: *Liposomal characteristics of DOC:SPC:DOTAP liposomes.*

Liposomal formulation (DOTAP % (w/w) of total lipid)	**Lipid composition (lipid ratio w/w)**	**Average diameter (nm ± SD)**	**PI (AU ±SD)**	**Zeta potential ± SD**
1 (0 %)	SPC	98.10 ± 0.40	0.22 ± 0.01	-0.92 ± 0.39
2 (10 %)	SPC:DOTAP (9:1)	106.55 ± 0.72	0.27 ± 0.01	54.93 ± 1.75
3 (20 %)	SPC:DOTAP (8:2)	102.28 ± 0.53	0.26 ± 0.01	51.80 ± 1.57
4 (30 %)	SPC:DOTAP (7:3)	102.05 ± 0.47	0.23 ± 0.01	57.58 ± 1.74
5 (50 %)	SPC:DOTAP (1:1)	97.62 ± 0.57	0.22 ± 0.01	60.33 ± 2.98

Since sonication processing of SPC:DOTAP liposomal formulations in part 3, the "Optimization study", gave satisfactory size and PI, we decided to sonicate all the formulations for 1 minute. The average diameter was around 100 nm for all five liposomal formulations. Formulation 1 was composed of 100 % SPC. Formulation 2-5 were composed of increasing amount of DOTAP, from 10-50 % (w/w). It seemed like the average diameter decreased slightly with increasing amount of DOTAP.

The PI value of all five liposomal formulations were < 0.3. The zeta potential of formulation 1 (SPC) was slightly negative, as expected since the PC head group are neutral (Kraft *et al*., 2014). Formulation 2-5 all showed a highly positive charge, as expected based on previously results and the fact that DOTAP is a cationic lipid (Zhi *et al*., 2018).

The five different liposomal formulations were characterized with measurement of concentration before and after purification using centrifugation, as shown in Table 17.

Table 17: *Drug content in the liposomal formulations with increasing amount of DOTAP.*

Liposomal formulation (DOTAP % (w/w) of total lipid)	**Docetaxel concentration (µg/mL)**		**DOC recovery in supernatant (%)**
	Total sample	Supernatant	
1 (0 %)	298.19 ± 9.42	79.30 ± 1.14	27
2 (10 %)	305.18 ± 2.86	166.67 ± 5.32	55
3 (20 %)	318.92 ± 2.76	154.22 ± 1.34	48
4 (30 %)	317.23 ± 6.38	168.75 ± 1.38	53
5 (40 %)	282.83 ± 4.48	218.24 ± 5.73	77

The recovery of formulation 1, the one liposomal formulation only composed of SPC was quite poor with only 27 % DOC left in purified sample, as shown in Figure 22. This is interestingly since the DOC recovery of the SPC liposomal formulation with a 10:1 (w/w) DOC:lipid ratio from the "Screening study", shown in Table 12, was 95 %. It could seem like an optimal DOC:lipid ratio for SPC liposomes was 10:1 (w/w), as the DOC recovery for both SPC liposomes in this DOTAP-study, with a 10:1.5 (w/w) DOC:lipid ratio (Table 17), and in the "Optimization study" a 5:1 (w(w) DOC:lipid ratio (Table 15) was much lower.

Formulation 2-5 composed of SPC:DOTAP with increasing amount of DOTAP showed a recovery of DOC between 48 and 77 % as shown in Figure 22.

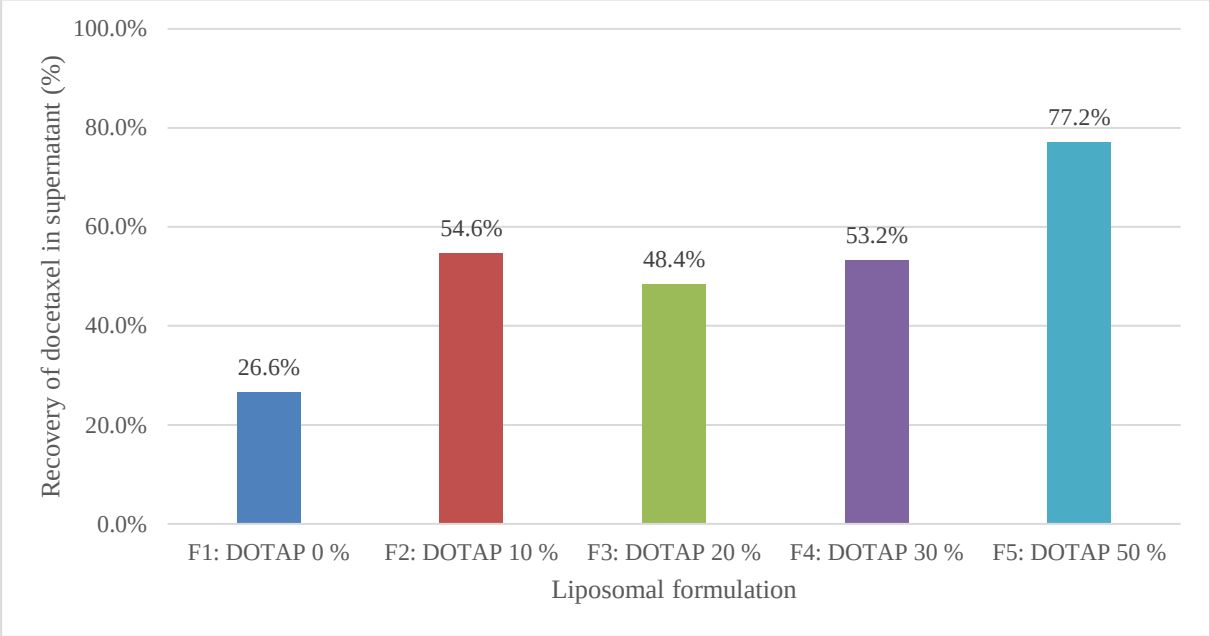

***Figure 22**: DOC recovery of the five different liposomal formulations in the DOTAP study.*

The highest recovery of DOC was at 50 % (w/w) (~50 mol%). There seem to be a trend that an increasing amount of DOTAP solubilize more DOC, although the recovery of formulation 3 and 4 was slightly lower than formulation 2. Compared to Sætern *et al*. (2004), it seems like we did not achieve an optimal DOTAP content for maximum incorporation of DOC (Sætern *et al*., 2004). It could be discussed that even more DOTAP content would solubilize even more DOC, but this needs further investigation.

In this part of the study we did not perform any recovery assessment of PL. The DOC recovery of the five liposomal formulations was varying, and it might be that the DOC concentration was too high, hence precipitated in the liposomal dispersion, and thereby the precipitated drug captured more liposome in the pellet under centrifugation. Since we did not perform any recovery assessment of PL, this cannot be confirmed but needs further investigation.

Formulation 3 (SPC:DOTAP) from this DOTAP study had a lipid ratio of 80:20 (w/w) with a DOC recovery of 48 %. The SPC:DOTAP liposomal formulation from the “Screening study”,

shown in Table 12, with a DOC:lipid ratio of 10:1 showed a recovery of DOC near 100 %. The SPC:DOTAP liposomal formulation from the “Optimization study”, shown in Table 15, with a DOC:lipid ratio of 5:1 (w/w) showed a DOC recovery of 43 %. It could seem like the optimal DOC:lipid ratio of the SPC:DOTAP formulation was achieved at 10:1 (w/w).

6 Conclusion

The present research work focused on the effect of lipid composition on the DOC entrapment. We established a small scale screening method for preparing DOC-liposomes and for separating the unentrapped DOC from the liposomes in order to determine the DOC entrapment efficiency.

In the Pilot project, we established the method used throughout the whole laboratory experiment period. In the screening study, 14 different liposomal formulations with different lipid compositions were prepared to investigate how the lipid compositions affected the DOC entrapment efficiency. Different DOC entrapment values, in the range between 18 and 115 % were obtained. No correlation between lipid saturation and DOC entrapment efficiency was observed.

However, three of the liposomal formulations showed entrapment efficiency near 100 %, with 110, 115 and 95 % for SPC, SPC:DOTAP and SPC:DMPG liposomal formulations, respectively. These formulations were brought to the Optimization study to investigate if even more DOC could be entrapped in the liposomes. The results showed that a 10:1 (w/w) lipid:drug ratio gave better entrapment efficiency than a 10:2 (w/w) lipid:drug ratio. The only cationic liposomal formulation in this project, SPC:DOTAP, showed the best entrapment efficiency and was further investigated with varying the concentration of the positively charged lipid, DOTAP, to see if the DOTAP concentration affected the DOC entrapment. Our results indicate that an increasing amount of DOTAP affects the entrapment efficiency positively.

7 Perspectives

The results of this thesis represent a preliminary Pilot project and the development of liposomal formulation of DOC is only in its infancy. The SPC:DOTAP liposomes with DOC should be further explored. The formulation should be further investigated to evaluate its stability, and potential to be prepared sterile. Moreover, its stability in biological fluids should be confirmed.

Short-term perspective

- Deeper insight and further investigation on solubilizing DOC in the liposomal formulation.
- Investigate if a smaller amount of Chol could contribute to a better entrapment efficiency.
- Run more parallels of SPC and SPC:DOTAP to see if the results are reproducible and perform PC-assay to evaluate entrapment efficiency and drug load capacity accurately.
- Evaluate other methods such as dual asymmetric centrifugation (DAC) as a processing method for preparing liposomes.
- Evaluate the of stability of the liposomal formulation.
- Evaluate *in vitro* efficacy (in cytotoxicity assays using human cell lines).

Long-term perspective

- Evaluation of safety and efficacy in animal studies after administration of the liposomal formulation parenterally.

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