THESIS FOR THE DEGREE MASTER OF PHARMACY

INVESTIGATION AND OPTIMIZATION OF LIPOSOME FORMULATION FOR USE AS DRUG CARRIER FOR THE ANTICANCER AGENT CAMPTOTHECIN

BY

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ABSTRACT

In this thesis, the method development and investigation of different liposomal formulations to incorporate and retain Camptothecin (CPT) is described. CPT is a potent anticancer drug that has shown to be active against a broad spectrum of cancers. However, due to its challenging physicochemical properties, like poor water solubility, severe toxic effects to normal tissues and instability, its clinical development has been limited for nearly 40 years. A strategy to overcome CPT's challenging properties is to use liposome-based carrier system. By taking advantage of this carrier system, we may solubilise CPT in the phospholipid bilayer of liposomes, protect it from blood proteins and achieve a selective drug accumulation in tumor tissues or tumor-associated cells by enhanced permeability and retention effect (EPR).

A good liposome formulation of clinical utility must fulfil two important criteria. The liposomal drug carrier must incorporate CPT in the liposomal bilayer in a relevant therapeutic concentration and be able to retain the drug within the liposome to make it bioavailable at the target site after i.v. administration. The focus of this thesis was to study different liposomal formulations and their ability to incorporate and retain CPT. Screening of eight different liposome formulations with respect to association with CPT was performed. The 1,2-di-oleyl-3 trimethyl-ammonium-propane (DOTAP) containing formulations showed superior incorporation capacity, giving an CPT incorporation of 250 µg/130 µmoles lipid. The DOTAP containing formulations exhibited as well a trend toward higher retention ability in serum compared to the other formulations. Although they showed better retention ability, only 25 % of the drug was associated with the liposomes, which is far from being optimal. One of the important criteria mentioned above for liposomes as drug delivery systems is their ability to remain stable in blood circulation for prolonged time in order to reach the specific target and to avoid rapid clearance by RES after i.v. injection. To achieve this, PEG decoration on the liposome surface can be employed. We chose to PEGylate DOTAP formulations in order to get a better understanding of this system. PEGylation lead, as expected, to increased stability of the liposomes, however a reduced incorporation capacity was observed. The presence of 1 % and 10 % PEG gave better retention and slower leakage from the liposomes. We conclude that DOTAP inclusion in our liposomes increased the incorporation of CPT into the lipid bilayer, that liposomal retention in our current formulations must be improved, and while PEGylation is necessary in order to prevent rapid *in vivo* clearance, the inclusion of PEG reduces incorporation, and therefore further studies are needed in order to improve incorporation of CPT in PEGylated liposomes.

ABBREVIATIONS

AcCN	Acetonitrile
Chol	Cholesterol
CPT	Camptothecin
CPT-11	Irinotecan
DMSO	Dimethyl sulphoxide
DOPG	Dioleoylphosphatidylglycerol
DOTAP	1,2-di-oleyl-3 trimethyl-ammonium-propane
DPH	1,6-Diphenyl-1,3,5-hexatriene
EPC	Egg phosphatidylcholine
EPR	Enhanced permeability and retention
HPLC	High performance liquid chromatography
HSA	Human serum albumin
i.v.	Intra venous
LC	Liquid chromatography
LUV	Large unilamellar vesicle
MLV	Multilamellar vesicle
PA	Phosphatidic acid
PB	Phosphate buffer
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCS	Photon correlation spectroscopy
PE	Phosphatidylethanolamine
PEG	Poly(ethylene glycol)
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid
PS	Phosphatidylserine
RES	Reticuloendothelial system
SD	Standard deviation
SUV	Small unilamellar vesicle
Topo-I	Topoisomerase I
UV	Ultraviolet
v/v	Volume ratio

1. INTRODUCTION

Over the past thirty years, liposomes are becoming important as a delivery system for therapeutic agents, chemotherapeutics, antigens, immunomodulators, imaging and genetic materials. A large number of liposome based drugs are in preclinical and clinical research. Cosmetic industry has also shown great interest in liposomes, and today there are many liposome-based cosmetic formulations e.g. skin-care products available on the market (Garidel et al., 2000).

1.1 Camptothecin

Camptothecin (CPT) is a potent anticancer agent that has shown significant cytotoxic activity (Watanabe et al., 2008). CPT is an alkaloid derived from the bark of the Chinese tree *Camptotheca acuminate,* and was isolated already in 1966 by the group of Wall and Wani (Wall et al., 1966). But due to its limitations, like poor water solubility and the undesired physical and chemical properties described below, their pharmaceutical development and clinical implementation has been impaired and research on other chemotherapeutic agents prioritized. In the 1980s the molecular target for CPT was however identified, again raising researchers' interest in and attention to CPT as an anticancer agent (Hsiang and Liu, 1988) (Mattern et al., 1987).

CPT's anticancer effect lays in its ability to bind to DNA and inhibit Topoisomerase I (Topo-I) at physiological pH as shown in Figure 1.1. Topo-I is a central enzyme in the process of DNA replication. It is responsible for winding and unwinding of the supercoiled DNA composing the chromosomes. Transcription of DNA cannot occur, if the chromosomes are not unwound. Further protein synthesis is inhibited, inducing apoptosis (Venditto and Simanek, 2010). The primary mechanism of CPT is S-phase specific, which means that it is very toxic to cells undergoing DNA synthesis (Burke and Bom, 2000).
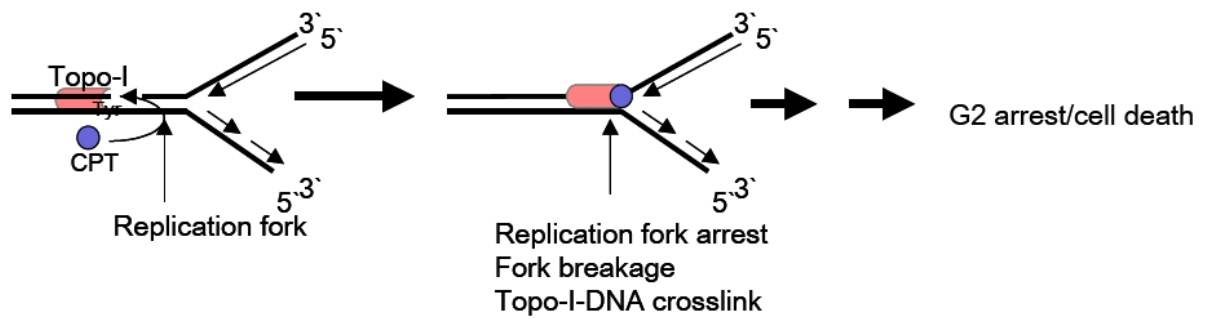
Figure 1.1: CPT is binding to Topo-I and arrests the replication fork leading to cell death (reprinted with permission from Dr. Holsæter (Saetern, 2004))

It is known that CPT exists in a pH dependent equilibrium between a lactone form and a carboxylate form (Figure 1.2). CPT is present in its active lipophilic lactone form at pH below 6. At physiological pH, CPT is largely present as its significantly less biologically active and more water soluble carboxylate form. Under physiological conditions and in human plasma CPT-lactone is rapidly hydrolysed to the open carboxylate form. The carboxylate form of CPT binds with a 100-150 fold higher affinity to human serum albumin (HSA) compared to the lactone form, which drives the equilibrium toward the open-ring carboxylate form (Figure 1.3). HSA-CPT binding makes the inactive carboxylate form inaccessible for cellular uptake. Moreover, the inactive carboxylate form is excreted by the kidneys, and causes several toxicity problems like haemorrhagic cystitis and myelotoxicity. Red blood cells/cell membranes on the other hand stabilize the lactone form, also shown in Figure 1.3 (Mi and Burke, 1994) (Saetern et al., 2004a). The biologically active form of CPT has a very short half-life (approximately 12 min), and in presence of human plasma 99% of the drug is converted to its less active and potentially toxic carboxylate form (Burke, 1996).
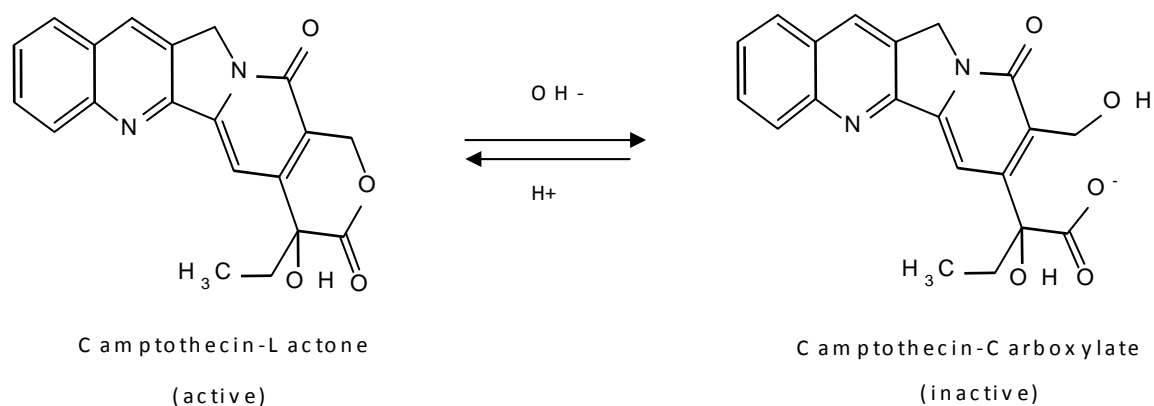
Figure 1.2: Equilibrium between the carboxylate and lactone form of CPT (reprinted with permission from Dr. Holsæter (Saetern, 2004)).

Currently, there are only two CPT-analogues that have passed the clinical trials. Irinotecan (Campto®) and topotecan (Hycamptin®) are the two derivatives and have been approved for treatment of the ovarian carcinoma, small-cell lung cancer and colorectal cancers. These two CPT derivatives are both water soluble, due to molecular modifications, and also exhibit a reduced binding affinity for HSA (Li et al., 2006). However, there are still problems with these derivatives' unstable E-ring, which is converted to the carboxylate form at physiological pH (Emerson, 2000). Researchers are still interested in finding new and better ways to solve CPTs' stability problems, and this may be achieved by incorporating this drug in liposomal drug carrier, thereby overcome some of CPT's challenges and keeping it in its active lactone form (Watanabe et al., 2008). This can be achieved by the fact that lipids can dissolve the lipophilic drug and the pH can be controlled inside the liposome in such a way that the equilibrium is forced toward the active lactone form (see Figure 1.3). The lactone form has also shown to be stable when harboured in the liposome bilayer (Burke et al., 1992). The drug is in addition protected from HSA and the complexing with the carboxylate form is avoided (Emerson, 2000).

Liposome pH 6.0   **Liposome membrane**   **Blood pH 7.4**

Carboxylate ⇄ Lactone ⇄ Lactone ⇄ Lactone ⇄ Carboxylate

Lipid compartments   HSA stabilisation
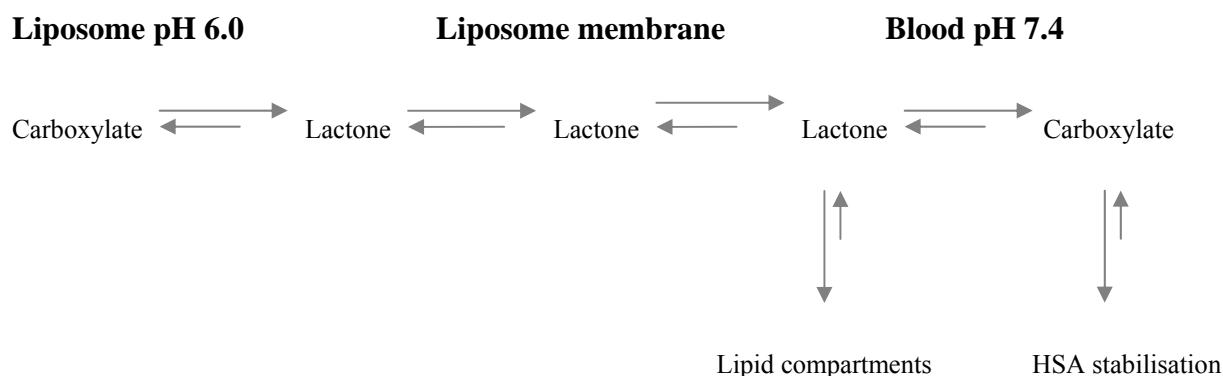
Figure 1.3: Schematic description of lactone and carboxylate equilibrium in both liposome membrane and in blood circulation

1.2 Liposomes

1.2.1 Definition and background

Liposomes are self-assembling spherical vesicles with a size ranging from 20 nm to 10 μm. Liposomes may exist as uni- and multilamellar vesicles. Unilamellar vesicles consist of a lipid bilayer separating the aqueous core from an outer aqueous environment, while multilamellar vesicles have multiple lipid bilayers separating the different aqueous environments (Brandl, 2001). Liposomes normally consist of different types of naturally occurring phospholipids, but other lipids such as cholesterol can be included to tune the liposome properties. In order to obtain liposomes of mixed composition, the lipids are dissolved in an organic solvent, which is subsequently evaporated from the lipid mixture and a dried lipid film is obtained. Liposomes are formed spontaneously when dry lipids is dispersed in an aqueous media. They have the ability to function as drug carriers for both hydrophilic, lipophilic and amphiphilic drugs. Hydrophilic drugs can be encapsulated in to the aqueous core, while lipophilic and amphiphilic drugs can be incorporated within the lipophilic bilayer (see Figure 1.4) (Chrai et al., 2002) (Brandl, 2001).
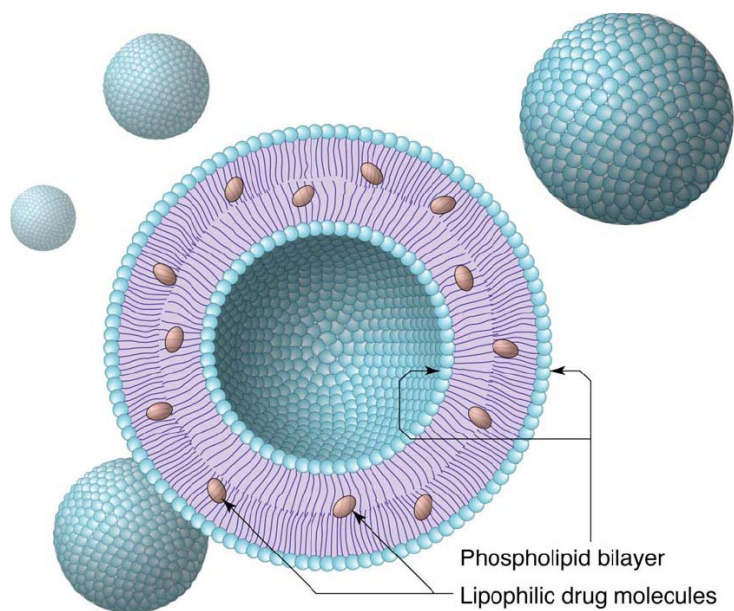
Figure 1.4: Liposomes with lipophilic drugs incorporated in the phospholipid bilayer (reprinted with permission from Dr. Holsæter (Saetern, 2004))

By using liposomes as drug-delivery carriers, we may keep CPT in its active lactone form by protecting CPT from degradation and HSA binding in the blood stream (Emerson, 2000). And because liposomes themselves are formed from naturally occurring lipids of low intrinsic toxicity, they are non-immunogenic and biodegradable in the body (Chrai et al., 2002).

1.2.2 Lipids in liposome products

1.2.2.1 Phospholipid (PL)

Phospholipids (PLs) typically found in high proportions in cell membranes of living matter, are an important component in liposome formulations. PL consists of two fatty acids linked to a polar head group, and they have either glycerol (Figure 1.5) or sphingomyeline as the back bone. PLs are amphipathic molecules, and have both hydrophobic and hydrophilic groups. The two hydrocarbon chains constitute the hydrophobic tails, while the phosphate group and its polar attachment constitute the hydrophilic group (Cooper and Hausman, 2009).

PLs can consist of different head and tail groups that affect the surface charge and bilayer permeability of the liposomes (Perrie and Rades, 2010).
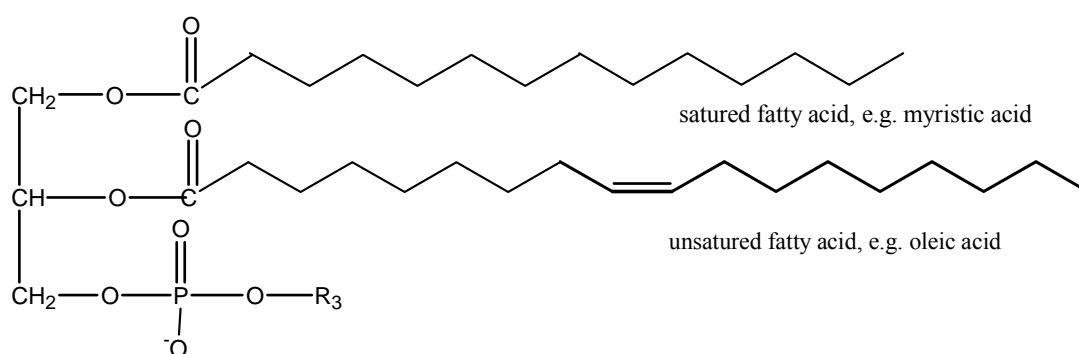
Figure 1.5: Structural formula of glycerophospholipid (reprinted with permission from (Flaten, 2003))

Phosphatidylcholine (PC) is the most commonly phospholipids employed in liposomes, and can be obtained from both natural and synthetic sources. PC is zwitterionic and consists of a hydrophilic headgroup with a quaternary ammonium moiety choline, which is linked to a glycerol via a phosphoric ester (Brandl, 2001).

The stability of the liposome membrane depends on the packing of the hydrocarbon chains of the lipid molecules. The hydrocarbon chain length and degree of saturation of the acyl chains influences at which temperature, the main transition temperature (Tm), the membrane transforms from a fully extended and closely packed "gel phase" to a liquid crystalline disordered "fluid phase". In general, fluid membranes are more permeable to solutes than rigid bilayers (Brandl, 2001).

Table 1.1: The most common glycerophospholipids

The esterified group	Name of the phospholipid	Abbreviation	Net charge at pH 7
-H	Phosphatidicacid	PA	Negative
$-CH_2CH_2N^+(CH_3)_3$	Phosphatidylcholine	PC	Zwitterionic
$-CH_2CH_2NH_3^+$	Phosphatidylethanolamine	PE	Zwitterionic
$-CH_2CHOHCH_2OH$	Phosphatidylglycerol	PG	Negative
$-HC_6H_5(OH)_5$	Phosphatidylinositol	PI	Negative
$-CH_2CHNH_3^+COO^-$	Phosphatidylserine	PS	Negative

The charge of the lipid used in liposome formation dictate the surface charge of the liposomes. The surface charge of liposomes can be tailored by replacing phosphatidylcholine (PC) partly with negatively or positively charged (phospho)lipids (Table 1.1), which induces electrostatic repulsion and stabilization against liposome fusion (Ogihara et al., 2010). The surface characteristics of liposomes may also be altered by modifying lipids with hydrophilic moieties e.g. polyethylene glycol (PEG) to membrane bilayers which is described more below (Brandl, 2001).

1.2.2.2 Cholesterol (Chol) and other employed lipids

Cholesterol (Chol) is one of the commonly used lipids in liposome formulations, and its incorporation into the lipid bilayer has a major effect on the liposome properties. The presence of Chol in the lipid bilayer enhances the stability and form highly ordered and rigid membrane with fluid like characteristics (Lee et al., 2005).

Chol molecular structure (Figure 1.6) with the four hydrocarbon rings makes it strongly hydrophobic. The presence of the hydroxyl group (OH) attached to the end of Chol makes that part weakly hydrophilic (Cooper and Hausman, 2009). Chol can be incorporated into the

lipid bilayers at concentrations up to 1:1 molar ratio, and does not form a bilayer on its own. Therefore other phospholipids are needed to form a bilayer. Due to its amphiphatic properties, Chol inserts itself in the bilayer with its OH-group oriented towards the aqueous core, and the rigid hydrophobic tail toward the phospholipid bilayers (Perrie and Rades, 2010).
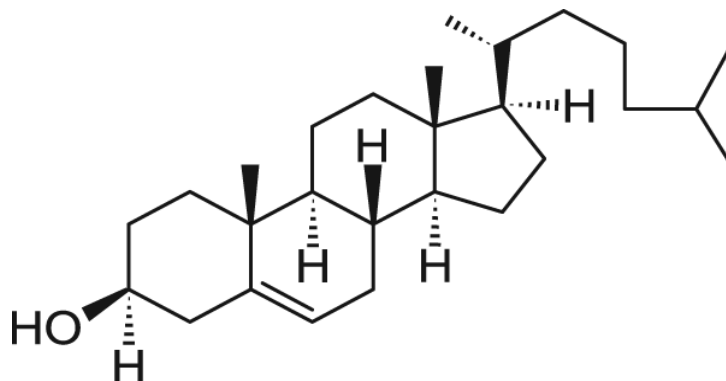
Figure 1.6: General structure of cholesterol (Chol) (www.avantilipids.com)

1,2-di-oleyl-3-trimethyl-ammonium-propane (DOTAP) is another example of lipids used in liposome formation. DOTAP is a cationic lipid with two unsaturated fatty acids. It consists of propane as backbone and trimethylammonium as the hydrophilic head group as shown in (Figure 1.7).
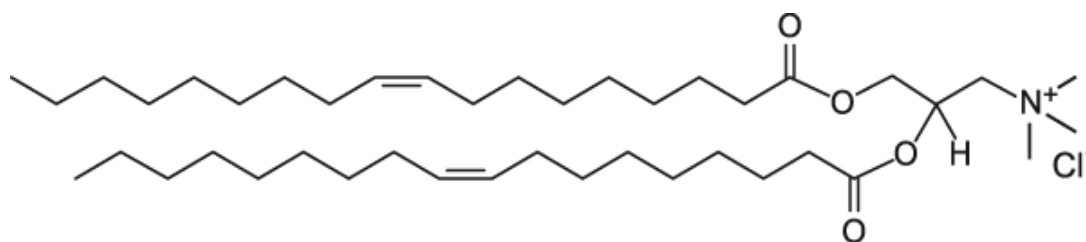
Figure 1.7: General structure of DOTAP (www.avantilipids.com)

1.2.3 Characterization of liposomes

Classifications of liposomes are based on their size and lamellarity. Different size and lamillarity depends on their composition and their method of preparation.

Liposomes are usually categorized in to three main types, based on the size and lamellarity, as follows.

- Multilamellar vesicles (MLVs) is one of the three categorizes. These are vesicles with a size ranging from 100 nm to several micrometers, depending on the method of preparation. They consist of a large number concentric lamellar, and due to their large lamellarity they are more suited to incorporation of lipophilic molecules compared to hydrophilic substances

- Small unilamellar vesicles (SUVs) are vesicles consisting of single bilayer and can theoretically be as small as about 20 nm. They are more suitable for parenteral administration than MLVs, because of their homogeneity in size. Their small size results in lower amount of encapsulation of hydrophilic drugs.

- Large unilamellar vesicles (LUVs) are vesicles generally with size in the order of 100 nm, consisting of one single lamellar. They can entrap a higher amount of hydrophilic drugs due to their larger aqueous core compared with SUVs (Perrie and Rades, 2010).

1.2.3.1 The role of liposome size

The rate of the opsonisation and clearance by the reticuloendothelial system (RES) of the injected liposomes from the blood circulation is dependent on the composition and size (Liu et al., 1995). RES is part of the immune system and their main function is to eliminate foreign materials from the body (Harashima et al., 1994) (Perrie and Rades, 2010). RES consists of cells such as blood monocytes and macrophages found mainly in the Kupffer cells in liver, the lung and the spleen. Shortly after i.v injection, the liposomes become coated by serum proteins called opsonins. Once they are opsonised, they will rapidly be phagocyted by the RES cells, and the major part of the injected liposomes will be accumulated in the liver and spleen (Maurer et al., 2001).

Large liposomes (>200 nm in diameter) are rapidly opsonised and taken up by the (RES) disappear from the blood circulation within short time and primarily end up in the spleen. Opsonisation decreases with a decreasing in liposome size. Small liposomes have a relatively larger surface area, and will have a lower density of opsonins on the membrane surface which results in lower uptake by the macrophages (Liu et al., 1995). Liposomes with a size of 70 to 200 nm will have a greater chance to escape from RES and remain in the circulation longer and then reach the target. Due to extravasations through the fenestrated capillary walls in the liver, the small liposomes (< 70 nm in diameter) show shorter circulation time. The structure and architecture of the blood capillary walls varies in different organs and tissues. There are structure differences between healthy and tumour capillaries and blood supply to the organs and tissues is somewhat different (Brandl, 2001).

1.2.3.2 The role of the surface charge and membrane characteristics

Lipid organization in the liposome membranes has a major role on the physical membrane properties such as permeability, membrane elasticity, surface charge and binding properties of proteins, and is of equal importance for clearance as compared to liposome size (Garidel et al., 2000).

Neutral-charged liposomes with tightly packed membranes tend to remain longer in the circulation and exhibit increased drug retention, compared to charged systems. Protein opsonisations onto the liposome surface are reduced due to the tightly packed and rigid membrane. The presence of Chol in liposome formulations may change the packing of the phospholipids to a more ordered and rigid membrane and may stabilize to avoid drug leakage. Moreover, this may reduce binding of opsonins on the liposomes and may improve stability and retention of liposomes *in vivo* (Maurer et al., 2001).

Certain plasma proteins have an affinity for liposomes, and the affinity is enhanced if the liposome is charged. In particular cationic systems are expected quickly interaction with various components in systemic circulation and thus having shorter half life *in vivo* (Maeda, 2001). It is also known that anionic liposomes containing negatively charged lipids such as phospatidylserine (PS), phosphatidicacid (PA) and phosphatidylglycerol (PG) are quickly taken up by macrophages and thus disappear from the circulation in short time (Liu et al., 1995) (Massing and Fuxius, 2000).

1.3 Long circulating liposomes

Liposomes for use as drug delivery systems must be stable in the blood circulation for prolonged time to reach the specific target other than RES. In order to avoid rapid clearance by the RES after i.v. injection and thus allowing them to remain in the circulation for prolonged periods, PEG attachment on the liposome surface can be used. PEG is a hydrophilic polymer with varying in molecular weight due to the number on monomer repeat units. The polymer acts as a steric barrier with the flexible chains forming "brushes" which extending out from the surface (se Figure 1.8), thereby preventing interaction of opsonins and uptake by phagocytic cells. These liposomes are known as "stealth liposomes", and have good solubility properties in aqueous media (Torchilin and Papisov, 1994) (Allen, 1994). Although PEG is non-biodegradable, it does not form any metabolites, has a very low toxicity profile and does not accumulate in the RES (Perrie and Rades, 2010).

Hydrophilic molecules (e.g PEG)
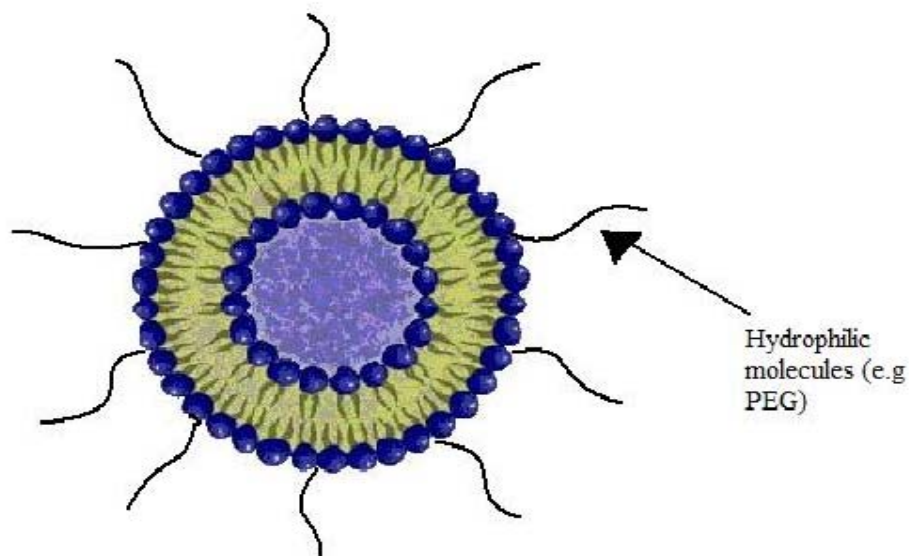
Figure 1.8: Modified illustration of sterically stabilized liposome surrounded with PEG (www.uni-magdeburg.de)

1.3.1 Liposomes in cancer therapy

Liposomes are used for drug delivery in cancer therapy due to their unique properties. They have the distinct advantages of being non-toxic and degradable in the body because of their naturally occurring lipids as main content. Liposomes have also a unique ability to entrap both hydrophilic and lipophilic drugs to its compartment and lead to a controlled release effect (Massing and Fuxius, 2000). Drug entrapment in the liposomes has also shown reduced drug toxicity due to minimized uptake in other tissues such as heart, kidneys and gut. Beside their ability to protect the entrapped drugs from degradation in the blood stream, their most important properties is the ability to accumulate in the tumors by passive targeting due to the enhanced permeability and retention effect (EPR) (Figure 1.9). The EPR effect is due to the differences between the vasculature in tumors and healthy tissues. Because of the angiogenesis, the blood vessels in tumor are more leaky and have less perfect cellular packing leading to bigger gaps between the cells. Furthermore, the lymphatic system which is responsible for removing substances such as liposomes or other nanoparticles from the tissues is marginally expressed compared to normal tissue (Jain, 1987). By utilizing the EPR effect, small liposomes (< 70 nm) are able to escape vasculature within tumors and accumulate there via passive targeting effect (Brandl, 2001).
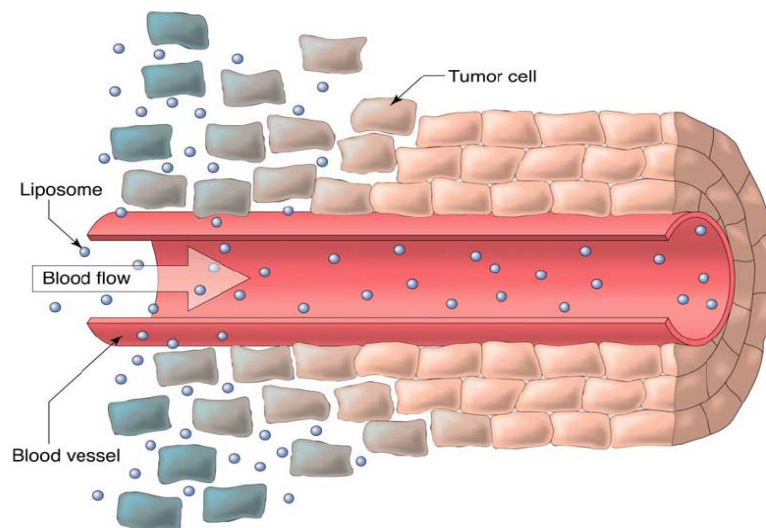
Figure 1.9: Accumulation of liposomes in tumour tissues due to EPR effect (reprinted with permission from Dr. Holsæter (Saetern, 2004)).

A range of water soluble, low-molecular weight anticancer drug compounds such as e.g. doxorubicin, have as said above been demonstrated to show significantly enhanced accumulation within solid tumors upon entrapment in liposomes when administered i.v. due to the EPR effect. Unfortunately such tumor-targeting by liposomal carriers so far could not be achieved to the same extent for other cytostatics, especially for the class of poorly water soluble compounds. We hypothesize that a premature loss of the anticancer compound from the liposome carrier is the reason for this (Fahr et al., 2006). A central prerequisite for successful delivery of the anticancer drug, namely that the drug remains associated with the liposome carrier during transit in the blood stream and is only released upon arrival at the target site, may not have been sufficiently fulfilled with the so far investigated liposome formulations of such drugs.

The first and the most important aim of this thesis was to investigate CPT incorporation as well as the retention ability of different liposome formulations in order to identify which factors are crucial for obtaining the optimal liposome formulation for *in vivo* CPT delivery. The second aim was to come up with a formulation exhibiting surface characteristics that makes the liposomes likely to circulate over longer time periods in the blood. In order to perform these studies, appropriate methods were needed, thus a third aim was to establish suitable protocols for our purposes.

2 MATERIALS & METHODS

2.1 Materials

Table 2.1: Chemicals

Chemicals	Purity	Quality	Producent
Acetic acid	100 %	Glacial, p.a.*	Merck, Germany
Acetone	Min. 99.5 %	p.a.*	Merck, Germany
Acetonitrile	≥ 99.5 %	Gradient grade for LC	Sigma-Aldrich chemie, GmbH, Germany
(S)-(+)-Camptothecin	96.1 %	For laboratory use only	Sigma-Aldrich chemie GmbH, Germany
Chloroform	99.0 %	For analysis	Merck, Germany
Chromatography Agarose Beads	GFU-04-500 4 % ACL Agarose Beads		Sooner Scientific.Inc., USA
di-Sodiumhydrogenphosphate-dihydrate	Min. 99.0 %	Extra pure	Merck, Germany
DMSO	99.5 %	GC	Sigma-Aldrich chemie GmbH,Germany
Ethanol	96 %	For analysis	Sigma-Aldrich chemie, GmbH, Germany
Methanol	Min. 99.9 %	For high performance liquid chromatography	Sigma-Aldrich chemie, GmbH,Germany
Phospholipids B test kit			Wako Chemicals, USA

Potassium dihydrogenphosphate	Min. 99.5 %	p.a.	Merck, Germany
Sodium Chloride	Min. 99.8 %	p.a.	Sigma-Aldrich chemie GmbH, Germany
Triton X-100	97 %		Sigma-Aldrich Chemie GmbH, Germany
Triethylamin	Min. 99 %	For synthesis	Merck, Germany

p.a.= pro analysis

Table 2.2: Lipids

Name	Abbreviation	Charge	Producer
1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol	DOPG	-	Avanti polar, USA
1,2-di-oleyl-3-trimethylammonium-propane	DOTAP	+	Avanti Polar, USA
Cholesterol	CH	No charge	Sigma-Aldrich GmbH, Germany
Egg phosphatidylcholine	E PC	+/-	Lipoid GmbH, Germany
Poly(ethylene glycol)2000 Da	PEG	No charge	Lipoid GmbH, Germany

Table 2.3: Equipment

Equipment	Type	Producer
Bath sonicator	Branson®1510	Branson ultrasonics, USA
Bath sonicator	Model G112SPIT	Laboratory supplies Co., Inc., USA
Centrifuge tubes	Polycarbonat tubes, thick-wall	Beckman Instrument, USA
Centrifuge	Biofuge Startos	Heraeus Instruments, UK
Chromatography columns	Bio-Spin®Disposable chromatography columns	Bio-Rad Laboratories, USA
Dialyse cassette	Slide_A-Lyzer Dialysis cassette 10.000 MWCO 0.5-3.0 ml capacity	Thermo scientific, USA
Eppendorf-tube	Safe-Lock tubes 2.0 ml	Eppendorf AG, GmbH, Germany
Rotary evaporator	Büshi R-124 rotary evaporator with vacuum pump v-500-system	Büshi, Switzerland
HPLC	Waters 2690 Separation module Waters 474 Scanning Fluorescence detector Waters 2487 Dual λ Absorbance detector Symmetry C18-columm (3,9x150 mm)	Waters,USA
Filter	0.22 μm non-sterile syringe filters	Pall Life Sciences, USA

Filter	0.22 µm cellulose acetate filter	Sartorius AG, GmbH, Germany
Fluorescence microscope	Leica CTR 6000 microsystem	Leica, Germany, GmbH
Fluorescence spectrophotometer	Perklin Elmer LS 55 Fluorescence spectrometer	Perklin Elmer, UK
Microtitre plates	Costar® UV 96-well plate with UV transparent flat bottom, Acrylic	Costar®, USA
Microtitre plate reader	Spectra Max 190 Microplate Spectrophotometer	Molecular devices, USA
PCS	Submicron Particle Sizer, model 370	Nicomp,USA
pH meter	744 pH meter Metrohm	Metrohm Ltd, Switzerland
Probe-Sonicator	Ultrasonics Vibra Cell VC 754 750 Watt ultrasonic processor CVR 234 converter Needle probe 19 mm	Sonics and Materials, USA
Probe-Sonikator	Sonics high intensity ultrasonic processor 500 Watt model Needle probe 13 mm	Sonics and Materials, USA
Ultracentrifuge	Optima LE-80	Beckman,USA
Vortex	MS2 Minishaker IKA	Chiron AS
Zetasizer	Nano series	Malvern instruments, UK
Zetasizer capillary cells	Folded capillary cells	Malvern instruments, UK

2.2 Methods

Buffer solutions

Isotonic phosphate buffered salines (PBS) 0.025 M

1. 0.025 M di-sodiumhydrogenphasphate-dihydrat

    I.   di-sodiumhydrogenphosphate-dihydrat   8.90 g

    II.   Sodium chloride   15.16 g

    III.   Distilled water   ad 2000.0 ml

  I and II are dissolved in III

2. 0.025 M Potassium dihydrogen phosphate

    I.   Potassium dihydrogen phosphate   6.804 g

    II.   Sodium chloride   15.0 g

    III.   Distilled water   ad 2000.0 ml

I and II are dissolved in III

Solution 1 and 2 are filtered through a 0.22 μm filter (cellulose acetate filters, Sartorius AG, Germany) and mixed to achieve the desired pH which is pH 6.0.

Phosphate buffer (PB) 0.025 M pH 3.0

    I.   Ortho-Phosphoric acid 85 %   1.038 g

    II.   Sodium hydroxide   ad pH 3.0

    III. Distilled water   ad 1000.0 ml

I is dissolved in 250 ml III, II are added to the solution. Rest of III is added up to 1000.0 ml. The solution is filtered through a 0.22 μm filter (cellulose acetate filter, Sartorius AG, Germany).

Triton- solution 10 % (w/w)

    I.   Triton X-100   10.0 g

    II. PB 0.025 M pH 3.0   90.0 g (ad 100 g)

I is dissolved in II.

Mobile phase for HPLC analysis

TEAA buffer 1 v/v %

    I.   Triethylamine   20 ml

    II.   Distilled water   ad 2000.0 ml

    III.   Acetic acid   ad pH 5.5

I and 1500 ml II are mixed by a magnetic stirrer. III is added to obtain pH 5.5. Then the volumetric bottle is filled to 2000.0 ml with II. The pH value is controlled to be 5.5 using a pH-meter. The solution is filtered through a 0.22 μm filter (cellulose acetate filter, Sartorius AG, Germany)

2.3 Liposome preparation

Liposomes can be prepared by several techniques (Torchilin and Weissig, 2003). We employed both film hydration and freeze drying method, in order to determine if there are any differences in the incorporation of CPT in liposomes. The most appropriate method was then used further.

2.3.1 Lipid-CPT film preparation

Lipid-CPT films were prepared by mixing the lipid solutions in the desired composition with solution of the active drug CPT in a round bottom flask. Stock solutions of 100 mg/ml were made with different lipids in chloroform or a chloroform:methanol mixture. Stock solutions of CPT in a mixture of chloroform:methanol (4:1 volume ratio) with a concentration of 2 mg/ml were also prepared. After mixing the desired components, containing totally 1 mg CPT/130 μmoles lipid, the solvents were removed on a Büshi R-124 rotary evaporator with vacuum pump 500-system (Büshi, Switzerland) for 45 minutes at 200 mPa on a water bath at 45 ˚C. After 45 minutes, the round bottom flask was removed from the water bath and the pressure was adjusted to 50 mPa for about 3 hours to remove traces of solvent and obtain a dry film. The lipid compositions of the different formulations which were prepared are given in Table 2.4.

Table 2.4: The lipid compositions (mol %) of the different formulations

Lipids	Formulations							
	1	2	3	4	5	6	7	8
Chol			10					
E PC	100	90	90	85	80	85	85	85
DOPG		10						
DOTAP				15	20	15	15	15
PEG						1	5	10

2.3.2 Lipid-CPT freeze-drying preparation

The freeze-dried samples were prepared by mixing solutions of the desired lipid EPC and the active ingredient CPT in injection vials. 1 ml of lipid-CPT solution containing 100 mg lipid and 1 mg CPT stock solution was used for freeze-drying. The vials with the mixture were shock-frozen in liquid nitrogen for 1 minute and placed in a freeze dryer (beta 2-16 equipped with an LMC-2 controller, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Herz, Germany) with opened stoppers. The freeze-drying method are described earlier (Saetern et al., 2004b) and was performed for 65 hours at temperatures from - 40 °C to 45 °C with pressure declining from 800 mbar to 0.008 mbar. After freeze-drying the PL-CPT vials were sealed with aluminium closures and stored at – 80 °C.

2.3.3 Rehydration

The lipid-CPT samples from freeze-drying or film preparation were brought to room temperature for about 15 minutes before rehydration. Subsequently 3 ml 0.025 M PBS with pH 6.0 was added to the lipid film and 1ml to the freeze-dried cake for hydration. The lipid-CPT mixture was vortexed and shaken to ensure that all the lipids were dispersed in the buffer. The dispersion was then ready for further size reduction.

2.4 Size reduction of liposomes

After rehydration with buffer, it is assumed that the liposomes are present in multilamellar vesicles (MLVs). To reduce the size and lamellarity of liposomes high energy must be delivered to the liposome dispersion. There are several methods to reduce MLVs to SUVs, and some of the most frequently used techniques are bath and probe-sonication (Brandl, 2001).

2.4.1 Bath sonication

The hand shaken MLVs film were sonicated in 3 ml portions in 15 minutes intervals using a bath sonicator G112SPIT Special Ultrasonic Cleaner (Laboratory supplies Co.,Inc.,USA). The sonicator and the samples were allowed to cool for ten minutes between each interval.

2.4.2 Direct probe sonication

Sonication of liposomes made by the film hydration method

The hand shaken MLVs film were placed in an ice bath and sonicated in 3 ml quantity with an ultrasonic vibra cell (Sonics and Materials, USA) using a 19 mm needle probe tip and an output of 40 % max. The different formulations were sonicated in intervals of two minutes until the desired size was reached. The probe and the dispersion were allowed to cool for ten minutes between each interval. Upon sonication, the sample were placed in the fridge at 4 °C for equilibration overnight before further experiments were performed.

Sonication of liposomes from the freeze-drying hydration methods

The freeze dried samples were sonicated in a similar procedure as the hydrated liposome films. The sonication intervals were the same, but due to the smaller 1 ml quantity, the samples were sonicated using a Sonics high intensity ultrasonic processor (Sonics and Materials, USA) with a 13 mm needle tip that can accommodate a volume of 1 ml. Upon sonication, the sample were placed in the fridge at 4 °C for equilibration overnight before further experiments were performed.

2.4.3 Size determination by photon correlation spectroscopy (PCS)

The particle size and distribution of the sonicated liposomes can be measured by photon correlation spectroscopy (PCS), which is based on dynamic light scattering. The principle is based upon Brownian motion of particles in the solution. Small particles diffuse much faster than large particles, affecting the rate of fluctuation of scattered light intensity. The PCS instrument focuses laser light to the sample, and registers any movement from particles in solution (Torchilin and Weissig, 2003) (User manual, Nicomp Model 380,1997).

PCS measurements of particle size and distribution were performed on Nicomp TM model 380 particle sizing system (USA). In order to avoid impurities in the sample, sample preparation was measured in clean environment using particle free equipments. The cuvettes (borosilicate glass) were bath sonicated (Branson® 1510) for 10 minutes in freshly filtered PBS pH 6.0. Then all the samples and equipment needed were carried out in a laminar air-flow LAF bench prior to use. The test tubes were rinsed with PBS pH 6.0 filtered through a 0.22 μm pore size syringe filter. For measurement, the sample was diluted in filtered PBS pH 6.0 until a stable intensity of approximately 250-350 kHz was achieved (User manual, Nicomp Model 380, 1997).

The following instrument parameters described below were used in accordance with (Ingebrigtsen, 2001) with some exceptions. The buffer used was 0.025 M PBS pH 6.0.

- Nicomp distribution

- Automatic choice of channel width

- Number weighting

- Temperature 23 ˚C

- Liquid index of refraction: 1.333

- Laser wavelength : 632.8 nm (Helium-Neon)

- Liquid viscosity: 0.933 CP

- Scattering angle: 90˚ (Fixed angle)

- Number of cycles: 3

- Run time: 15 minutes

2.5 Ultracentrifugation

To separate excess CPT, crystals, titanium particles from the sonication probe and lipid aggregates from the liposomes, an ultracentrifuge was employed. CPT crystals and lipid aggregates have a higher density than the small probe sonication liposomes, and will settle in the pellet upon ultracentrifugation. The SUVs with associated CPT will be present in the supernatant. 500 µl PBS pH 6.0 was added to 2 ml of the liposomal dispersion in a 3-ml thick wall polycarbonate centrifuge tube to raise the volume enough to fill the centrifuge tube. The samples were vortexed for 30 seconds prior to centrifugation, and were then centrifuged using Beckman Optima L8-M centrifugation with SW60Ti rotor (Beckman Inc.,USA). The centrifugation speed was 100 000 g, the temperature 10 ˚C and duration was optimized to separate free CPT crystals and lipid aggregates from the liposomes (Saetern et al., 2004b). Totally 900 µl of the supernatant was then carefully withdrawn for further determination of amount of CPT and PC as well as further studies on retention ability.

2.5.1 Fluorescence microscopy

In order to ensure that the ultracentrifugation method was optimized so that there were no CPT crystals in the supernatant, the supernatant was examined using a fluorescence microscopy Leica CTR 6000 microsystem (Germany). CPT is itself fluorescent so no external labelling of the CPT crystals was needed. The supernatant was examined by applying a drop on a glass clean slide and put a cover glass on top. It was important to avoid air bobbles between the glass slide and the cover glass. The preparation was examined under the microscope using a 20 x objective and filter set A, yielding an excitation wavelength of 360 nm with a bandwidth of 40nm and recording fluorescence at 470 nm (bandwidth 40 nm) with a dichromatic mirror at 400 nm. Images were recorded using Leica Application Suite version 2.5.0 R1 (Germany).

2.6 CPT liposomes retention ability

The different formulations ability to retain CPT after incorporation was investigated using the spin column method. The principle behind the column filtration is that liposomes do not penetrate into the pores of the beads packed in the column, but instead percolates through the interbead spaces. Proteins as well as free drug are smaller in size and will be retarded in the pores of the bead pack. It is therefore assumed that CPT associated liposomes will be separated from the serum proteins as well as free CPT and will be collected in the early fractions. The formulations' retention ability both in buffer and serum could be obtained using this method.

2.6.1 Spin column method

The different formulations' ability to retain the drug in buffer and serum was investigated by using the spin column method (Torchilin and Weissig, 2003). 2.5 ml gel SeparatorGel Agarose Beads ACL 4% (Sooner Scientific) was packed in spin columns (Bio-Rad) using centrifuge Biofuge Startos (Heraeus Instruments, UK). The centrifuge was stopped manually when speed of 400 rpm was reached.

300 µl of supernatant was diluted 1:2 v/v with PBS pH 6.0 and incubated at room temperature. Another 300 µl of supernatant was diluted 1:2 v/v with serum and incubated at 37 ˚C. Separations of the incubated samples were measured at 0 hours, 5 hours and 24 hours and the samples were separated into 4 different fractions. Separations were done by adding 100 µl of CPT-liposome dilution to a column. First fraction was collected in an eppendorf-tube by centrifugation of the column until a speed of 400 rpm was reached. Further, the second fraction was collected in a new eppendorf-tube by adding 100 µl PBS buffer pH 6.0 to the column and performing the same centrifuge procedure as describe above. The same process was repeated twice more using 100 µl PBS buffer pH 6.0 and the fractions collected in eppendorf- tubes. Above, every fraction was diluted 1:2 v/v with Triton 10 % for further PC and CPT determination.

2.7 Quantification of Camptothecin

High performance liquid chromatography (HPLC) was used to quantify Camptothecin (CPT) in the liposomes. The method described by Warner and Burke (Warner and Burke, 1997) was used with some modifications. Samples were diluted 1:2 with 10 % triton solution to dissolve the liposomes and release the incorporated CPT from the liposomes prior the analysis. Each sample was analyzed in triplicates.

Quantification was achieved using CPT standards both in lactone and carboxylate form within a concentration range from 0.5 µM to 5.0 µM. The standard curve exhibited good linearity with a correlation coefficient of 0.997 ± 0.001 (n = 3).

Following HPLC-method was used:

- Mobile phase:

  A: 25% Acetonitrile in 1% (v/v) triethylamine acetate buffer pH 5.5 and adjusted to

    pH 5.99

  B: 35% Acetonitrile in 1% (v/v) triethylamine acetate buffer pH 5.5 and adjusted

    pH 6.32

  C: 95% Acetonitrile in 5% distilled water (v/v)

- 474 scanning fluorescence detector

- Detection Wavelengths: Excitation $\lambda=360$ nm, Emission $\lambda=440$

- Column: Waters Symmetri C18-column (3.9x150 mm)

- Injection volume: 10 µl

- Flow rate: 1.0 ml/min

- Run time: 15 min

- Sample temperature: 25˚C

- Column temperature: 30˚C

2.8 Quantification of phosphatidylcholine

Quantification of amount of phosphatidylcholines in liposomes was performed by using an enzyme assay, Wako LabAssay Phospholipid B test kit (USA). The assays are based on phospholipids (lecithin, sphingomyelin, lysolecithin) being hydrolyzed by phospholipase D enzyme yielding choline as the product. Choline is further oxidized by choline oxidase in a reaction which forms hydrogen peroxide. The latter takes part in a peroxidase-catalyzed coupling which produces a blue pigment. The amount of phospholipids in the sample can be determined by measuring the absorbance of the blue colour (Grohganz et al., 2003) (User manual, Wako Chemicals).
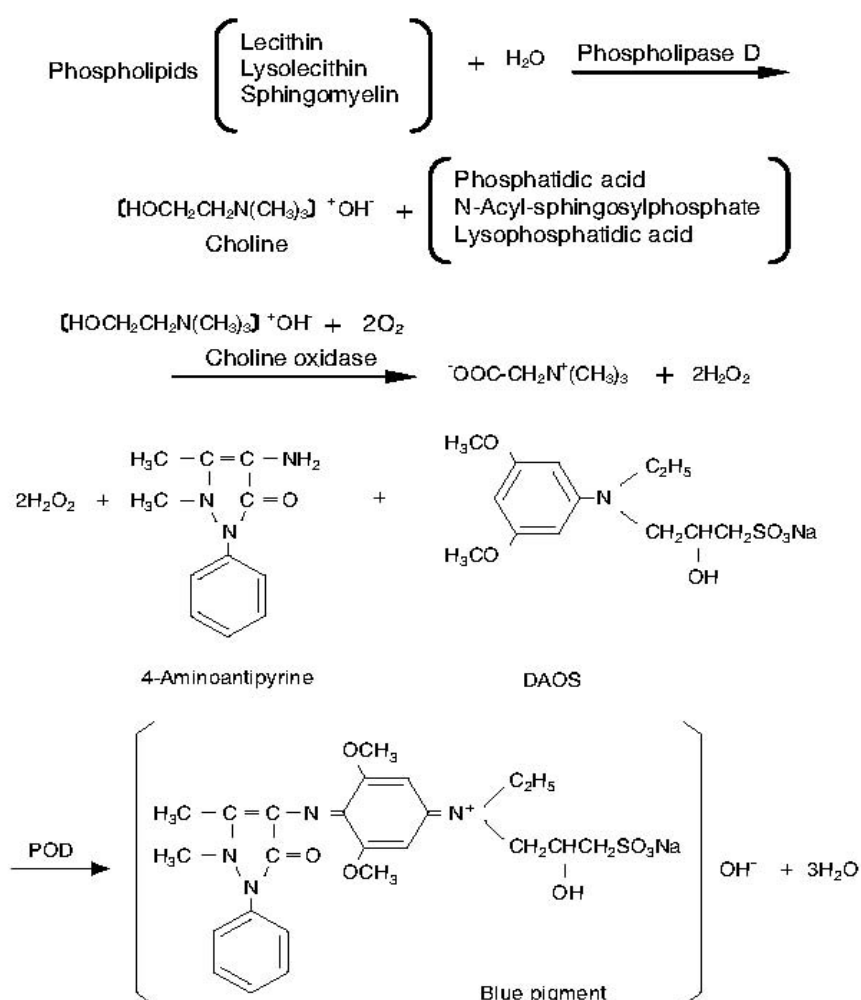

Figure 2.1: Enzymatic quantification of phosphatidylcholine (PC) (User manual,WAKO LabAssay Phospholipid, Wako Chemicals)

The assay was performed using a microtiter plate. Each microtiterplate was filled with 25 μl of the sample and 275 μl of colouring reagent. The plate was subsequently incubated at 37 ˚C for 15 minutes prior the absorbance measurements at 600 nm performed with a microtiterplate reader. All samples and standards were prepared and measured in triplicate (Grohganz et al., 2003).

This method was used after ultracentrifugation of liposomes to quantify phosphatidylcholine (PC) in the supernatant and pellet as well as to quantify the amount of PC in the fractions from the retention study.

PC recovery in the supernatant after ultracentrifugation was calculated as follows:

- PC recovery = (Mean amount of PC in supernatant / mean amount of PC in total dispersion)

The results from the incorporation study were adjusted based on the recovery to make the comparison easier.

PC content in fractions collected from the columns were used to determine the percentage of liposomes contained in each fraction compared to the original sample (either diluted in buffer or serum) used for the retention analysis. For each sample, the fraction with the highest PC content was used for CPT determination. If the PC content in this fraction was 40% of the original sample, it was assumed that 40% of the liposomes were eluted in this fraction. The results from the retention study were adjusted according to the percentage of liposomes in the fraction to relate to the amount of lipids in the original sample.

2.9 Determination of zeta Potential of liposomes

Surface properties of liposome formulations can vary depending on the composition of the lipid. There are cationic, anionic and neutral lipids, which can be used for preparation of liposomes. Zeta potential can be used to identify any correlation between the liposome incorporation and retention of CPT and surface charge properties of the liposomes (Brgles et al., 2008). The zeta potential is charge at the slip plane of the particle surface, and although it is not a direct measurement of the surface charge, it is a good estimation. Zeta potential is one of the important factors affecting liposomes stability, incorporation efficiency and interactions

with biological system *in vivo*(Gjelstrup Kristensen, 2000) (Labhasetwar et al., 1994). The zeta potential of liposomes was measured using a zetasizer Nano ZS (Malvern, UK).

The samples were thus analyzed using the zetasizer. Prior to analysis the samples were diluted 1 in 10 with PBS pH 6.0. The zetasizer capillary cell was also rinsed with 96 % ethanol and distilled water using a 1-ml syringe prior to analyses as recommended by the manufacturer. The diluted samples were then analyzed for ten cycles with a voltage of 4 mV.

2.10 Fluorescence anisotropy

To evaluate distribution of CPT in the liposomes, we used intrinsic fluorescence of CPT by fluorescence polarization measurements to examine mobility of CPT in the liposomal bilayer. Polarization measurements were performed on a fluorescence spectrophotometer (Perklin Elmer, UK) and a number of liposomal compositions were investigated. The samples were analyzed in a rectangular quartz fluorometer cell, and the excitation and emission wavelengths were set at 360 and 440 nm, respectively.

3. RESULTS AND DISCUSSIONS

3.1 Method development

In this study the method of incorporation capacity screening presented by Saeterns group (Saetern et al., 2004b) was used. However, due to different type of equipment and a desire to improve the feasibility of the method some changes were done as described below.

3.1.1 Sonication procedure

It is well known that the sonication process may influence the size and size distribution of liposomes (Woodbury et al., 2006). The goal was to obtain a sufficient size reduction and monodispersed liposomal size. Since small liposomes is preferred for i.v. application, the liposomal size was set to be <200 nm in diameter (Saetern, 2004). In the study by Saeterns group (Saetern et al., 2004b) they used probe sonication with a (Labsonic U,B.Braun Biotech International, Leverkusen, Germany) at 50 W, but this equipment was not available for us so we had to find another way to prepare SUVs. In order to determine the optimal sonication process for our purpose, size determination by PCS was performed upon sonication. Due to the toxicity of CPT, we wanted to keep the CPT containing liposomes in closed containers for not to expose the environment. We therefore chose to employ the bath sonicator to obtain SUVs. The result of bath sonicated liposomes is displayed in Table 3.1.

Table 3.1: Bath sonicated EPC liposomes with duration of 5 x 15 minutes

Liposomal composition	Mean particle size (nm ± SD)	Polydispersity Index (P.I.)*
EPC	704.3 nm ± 41.5	0.755

*P.I. represents the polydispersity index used as indication of size distribution of vesicles.

Lower values of P.I. indicate more homogeneous liposomal sample.

As described in Table 3.1 the measured size of the liposomes was found to be very large even after sonication for five intervals of fifteen minutes. The P.I is as well quit high indicating that the efficiency of size reduction is low and the samples are containing highly polydispersed population of liposome.

The liposome size, standard deviation of the mean particle size and the large P.I suggests that bath sonicator was not appropriate for size reduction of our liposomes, in addition the process was quit time consuming. It was therefore decided to employ a more powerful sonicator to reduce the size in a more rapid and reproducible manner.

3.1.1.1 Probe sonication

Since bath sonication in a closed container showed not to be appropriate we had to choose probe sonication in an open container and place the sonicator in a closed box in an appropriate room (cytostatic laboratory) instead. The size reduction of the liposomes was performed using an Ultrasonics Vibra Cell (USA) with a needle probe of 19 mm diameter. In order to determine an optimal sonication process for size reduction of the liposomes, it was necessary to present number of trials and evaluate the impact of number of intervals and time duration on the liposomes.

Due to the high temperature during sonication, there is a risk of lipid degradation. It is therefore necessary to keep the sonication time short, keep the system cooled and include a break between the sonication cycles. Optimal sonication conditions were evaluated in regard to vesicle size and size distribution by using EPC and EPC/Chol (90%:10%). The reason why we chose a formulation with cholesterol in addition to pure EPC was that it is expected that the inclusion of Chol in the liposomal bilayer makes it more rigid and more resistance to size reduction (New, 1990).

The sonicated lipsomes was tested by PCS upon sonication and results are shown in Table 3.2. The liposomes obtained after 2x2min of sonication showed a satisfied size for the EPC formulation. In the case of EPC/Chol formulation the results show larger vesicle size in comparison with the EPC formulation. The sonication time was therefore increased for this formulation, and the results show an obvious size reduction for our purpose after three intervals of two minutes.

Table 3.2: Probe sonicated liposomes

Duration	Liposomal composition	Mean particle size± SD	Polydispersity Index (P.I.)
2 x 2 min	EPC	42.9 nm ± 2	0.328
2 x 2 min	EPC-Chol (90%/10%)	228.9 nm ± 5	0.712
3 x 2 min	EPC-Chol (90%/10%)	32.5 nm ± 2	0.392

3.1.2 Optimization of ultracentrifugation for separation of free CPT from liposomes

Separation of CPT crystals from liposomes by ultracentrifugation was used to be able to see how much drug is associated with the liposomes. CPT crystals and lipid aggregates have higher density than SUV liposomes, and will settle in the pellet upon ultracentrifugation. In order to ensure that the ultracentrifugation earlier described (Saetern et al., 2004b) was optimized for our liposomes, and that there were no CPT crystals in the supernatant, the supernatant obtained after 20 min of centrifugation at 100 000 g was examined using a fluorescence microscopy Leica CTR 6000 microsystem, as described in 2.7. Averages of 10 to 20 pictures were taken of each sample and several different formulations were examined. Figures 3.1 and 3.2 are representative examples.

Crystals of CPT could be visually distinguee from dissolved CPT because they appear as an intense spot while CPT in liposomes results in a less intense fluorescing background. The microscopy pictures (Figure 3.1) clearly show CPT-crystals in the supernatant upon ultracentrifugation of 100 000 g for 20 minutes.

Refocusing of the lens to see the liquid-air interface in the glass slide
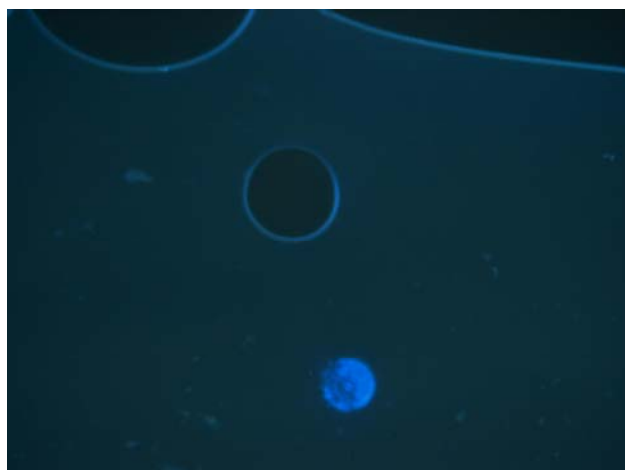
Figure 3.1: Fluorescence pictures from the examination under fluorescence microscope, Leica CTR 6000 microsystem, of the supernatant from the formulation number 1 after ultracentrifugation of 20 minutes. Observations were conducted using a 20 X objective.

In order to determine the optimal conditions for ultracentrifugation and to avoid CPT-crystals in the supernatant, it was necessary to increase the duration to 25 minutes. Further, samples of the supernatant were again examined using the fluorescence microscope and the results are presented in Figure 3.2.

Figure 3.2: Fluorescence pictures from the examination under fluorescence microscope, Leica CTR 6000 microsystem, of the supernatant from the formulation number 1 after increasing the ultracentrifugation duration to 25 minutes. Observations were conducted using a 20 X objective

Our microscopy analysis demonstrated that ultracentrifugation at 100 000 g for 25 minutes gave appropriate separation of CPT crystals from the liposomes, since no CPT crystals were visible in the supernatant after these ultracentrifugation conditions (see Figure 3.2).The latter centrifugation condition gave thus sufficient separation as revealed by the fluorescence microscopy and was chosen for further studies.

3.1.3 Comparison of film and freeze-drying method

As part of the development of a method to detect incorporation and retention of CPT in different liposome formulations, we compared CPT incorporation using two different methods for removing organic solvents from the lipid:CPT mix. We wanted to employ the film methods for our research, while the freeze-drying method had previously been employed for similar research in this lab. The purpose of the comparison of film and freeze-drying method was to determine if there are any significant differences in the incorporation capacity of CPT in the liposomes when using these two methods. The reason for this was that the freeze-drying method caused a lot of problems (M. Skar, personal communication) and we wanted to do it in a more appropriate way. The freeze drying method reported by Saetern and co-workers (Saetern et al., 2004b) is anyway a more suitable method when DMSO is used as the solvent for CPT. The method removes organic solvent by sublimation, and in order for this procedure to work properly the lipid mixture needs to be in the solid state at -40 ˚C . We employed organic solvents Chloroform:Methanol (4:1 volume ratio) in the CPT stock solution as recommended by the manufacturer. DMSO was not used as we expected better mixing of the lipids and the CPT in organic phase before drying, using the Chloroform:Methanol mixture. It is more difficult to remove chloroform to obtain a dry cake using freeze-drying. For this reason it was decided in advance to use the film method if there were no major differences between the incorporation capacities of CPT between these two methods.

However, due to drying problems with the Freeze dryer (beta 2-16 equipped with an LMC-2 controller, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Herz, Germany) we only achieved one parallel of formulation number 1 using this method. The result of the comparison is displayed in Figure 3.3. It appears that there are no significant differences in CPT-incorporation between these two methods. The variations are within the standard deviation that we expect from incorporation studies using lipids.
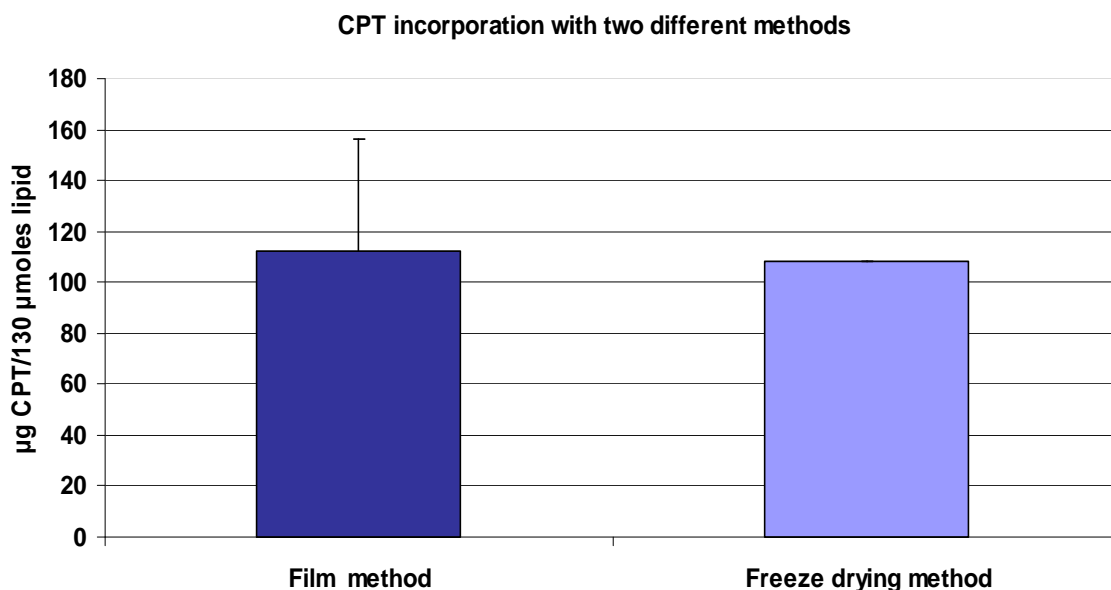
Figure 3.3: Camptothecin-incorporation capacity of EPC (formulation 1) by film (n=3) and freeze-drying method (n=1).

The freeze-drying problems is mainly related to our choice of solvents for preparation of CPT stock solution which was Chloroform:Methanol (4:1 volume ratio). Chloroform has a melting point of - 63 ˚C and by using the procedure as described in 2.3.2 it appeared some difficulties to sublimate the solvent to vapour. The melting point of chloroform might indicate that the solvent did not pass through the solid state and become sublimated, but rather evaporated when raising the temperature from – 40 ˚C to 45 ˚C and lowering the pressure from 800 to 0.08 mAtm within 65 hours. Moreover, CPT-incorporated liposomes approach performed earlier by Saetern and co-workers (Saetern et al., 2004b) gave reproducible data in their studies, probably due to their choice of solvent. Saetern et al (Saetern et al., 2004b) employed DMSO for preparation of CPT stock solution, and gained dry lipid cakes. DMSO has a melting point of 18.4 ˚C, and will be in the solid state and further become sublimated when raising the temperature from – 45 ˚C to 45 ˚C, while reducing the pressure.

However, it looks like these two methods is comparable as was expected and the film method was chosen as the method for further liposome preparation due to less troublesome preparations.

3.2 Characterization of how lipid composition influences CPT incorporation and retention

Earlier studies in our research group has revealed that the lipid composition of the liposomes have an effect on the CPT incorporation capacity (Saetern et al., 2004b) (unpublished results). In this study we wanted to investigate these effects in more detail by changing the lipid composition based on the previously studies. We also wanted to look at the different formulations retention ability since it is not enough that the drug is incorporated it also needs to stay with the liposomes in circulation.

3.2.1 CPT liposome incorporation study

Five liposome formulations with lipids of varying carbon chain length, saturation and charge as shown in Table 2.4 were prepared with a total concentration of 1 mg CPT/130 μmoles lipid, and the content of CPT in the liposomes was quantified after ultracentrifugation. As the molecular weight of the lipids varied significantly, especially later when PEG lipids were used, we based the formulations on 1 mg CPT/130 μmol lipid which is equivalent to 1 mg CPT/100 mg of EPC. Results from incorporation in the tested formulations are presented in Figure 3.4.
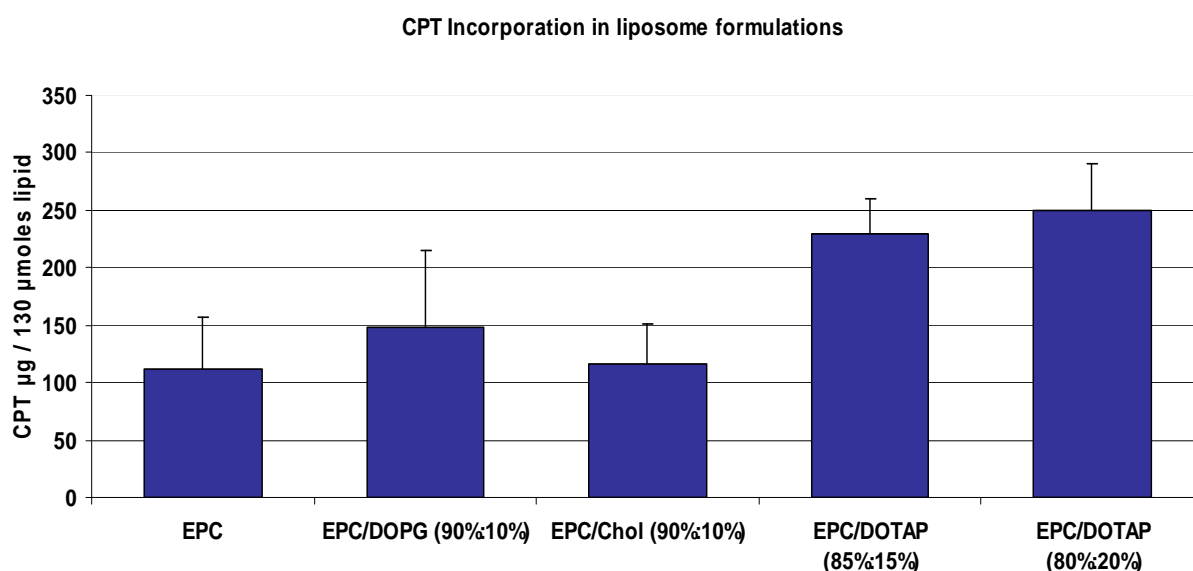

Figure 3.4: Camptothecin-incorporation capacity of the different liposome formulations (n = 3)

The most promising formulations in this study were formulation 4 and formulation 5, which exhibited a significantly higher CPT-incorporation capacity compared to the other liposome formulations displayed in Figure 3.4. Both these formulations contain DOTAP and this is likely the main reason for the higher incorporation capacity. As we can see in Figure 3.4 the incorporation efficacy increases with a raising DOTAP content from 15 mol % to 20 mol %, although the two are not significantly different. Increased incorporation capacity observed in our results, corresponds with earlier studies and observations (Saetern et al., 2004b). Correlation between presence of DOTAP in liposomes and increased incorporation capacity previously studied by Saetern and co-workers (Saetern et al., 2004b), indicates that CPT have a higher affinity for cationic lipids such as DOTAP as compared with other non-charged lipids. Since CPT exists in a pH dependent equilibrium between its lactone isomer and negatively charged carboxylate isomer, the latter may bind to the cationic liposomes through electrostatic interactions and lead to increased incorporation of CPT within the cationic liposomes.

The non-charged formulations, 1 and 3 do show lower incorporation capacities compared to the negative charged formulation. In comparison with literature, Cortesi and co-workers (Cortesi et al., 1997) reported that addition of Chol seems to raise the incorporation from 50 % to 57 %. In the contrary, other studies done by Saetern and co-workers (Saetern et al., 2004b) and Daouds group (Daoud et al., 1995) observed that addition of Chol presented in the membrane reduced CPT-incorporation capacity. However, a direct comparison of these results can not be made due to differences in experimental approach, as well as the higher amount of Chol in the liposomes from Saeterns group compared to those in our research.

A slight tendency toward increased incorporation was observed in formulation 2, when the negative charged lipid DOPG was presented in the PC membrane. This formulation contained 10 % DOPG and 90 % EPC, and as we can see in Figure 3.4, addition of DOPG raised the incorporation capacity as compared with formulation 1 containing 100 % EPC. This result are corresponding with the study done by Sugarmans group (Sugarman et al., 1996), which suggested that use of DOPG solubilised the drug-lipid particles to a higher degree. Also Saeterns group reported increased incorporation capacity when negative charged DPPG was present in the liposome formulation (Saetern et al., 2004b). Any effect by using the longer and more unsaturated oleyl fatty acid chain instead of palmitoyl was however not observed compared to what has been reported earlier (Saetern et al., 2004b).

3.2.2 CPT liposomes retention ability

A liposome formulations' ability to retain the incorporated drug is a prerequisite for successful delivery of drug to the target. The retention abilities for the different formulations were therefore investigated.

The retention ability of CPT of the five formulations shown in Table 2.4 was investigated over time, with withdrawal of samples after 0, 5, and 24 hours of incubation both in buffer and serum as described in section 2.6.1.

Figure 3.5: The different liposome formulations ability to retain CPT, associated with the liposomes over time in buffer at room temperature (n = 3)

The results of retention incubated in buffer at room temperature are displayed in Figure 3.5, and show different retention ability depending on the liposome formulations. There are no significant differences between the formulation containing EPC (formulation 1, Table 2.4) and EPC-DOPG (formulation 2, Table 2.4), which exhibit a slightly lower and decreasing retention ability during the incubation at room temperature. However, the most prominent results observed was the formulations containing EPC-Chol (formulation 3, Table 2.4) and

EPC-DOTAP 15% (formulation 4, Table 2.4), which leads to less drug leakage from the liposomes as compared to the other formulations. In general, none of the formulations (Figure 3.5) appears to retain CPT within the liposomes to high degree. However, the results exhibit high standard deviations. We assume that the high standard deviations are due to the packing of the gel (SeparatorGel Agarose Beads ACL 4%) in the columns. The columns were packed using a centrifuge without set time function, and had to be stopped manually when it reached the desired rpm. We assume that this resulted in different packing of the gel material in the columns, which may have led to incomplete or varying elution of the liposomes of the columns.

Liposomes in serum are most likely disposed to interact with blood components. One effect of this is drug loss to aggregates such as lipoproteins (Silvander et al., 1998).

The retention abilities of the formulations in serum were also investigated by the column method as described above. The results are presented in Figure 3.6, and summarize the different formulations' CPT retention ability over time when incubated in serum at 37 ˚C.
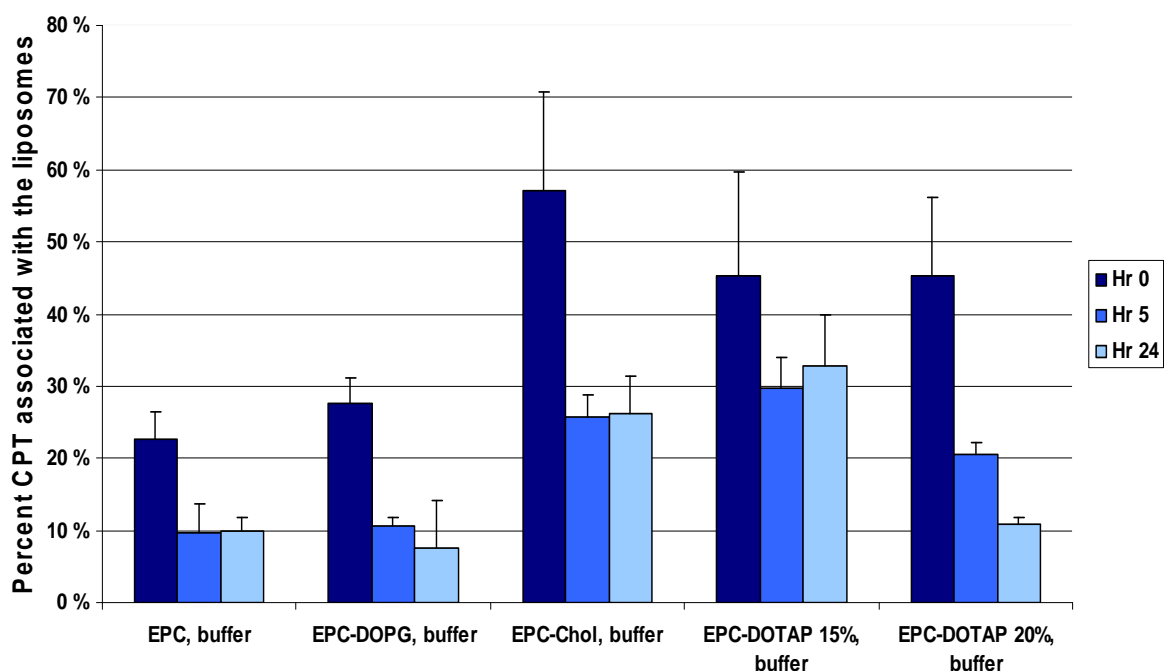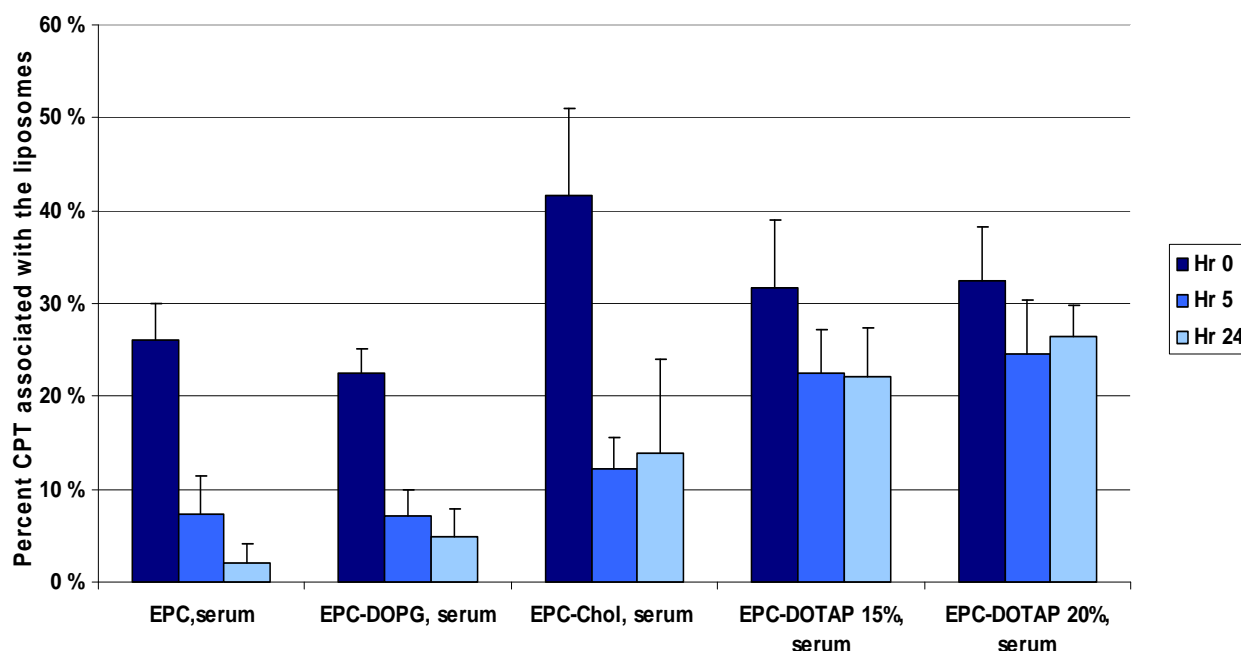

Figure 3.6: The different liposome formulations ability to retain CPT, associated with the liposomes over time in serum (n = 3)

As we can see in Figure 3.5 and 3.6 there are no significant differences between retention ability in buffer and serum for the three first formulations right after the dilution i.e. t = 0. Moreover, the liposomes retention ability seems to decrease when these three formulations were incubated in serum compared to in buffer. The most promising results are the DOTAP containing formulations incubated in serum, which exhibit a trend toward higher liposomal retention compared to the other liposome formulations. Although formulation 4 and 5 shows better retention ability, only 25 % of the drug is associated with the liposomes, which is far from being optimal.

In the case of serum containing samples, variations in the column production may have led to incomplete separation of liposomes and serum proteins. This might be the major reason of the high standard deviations. Due to the high standard deviation as described, more studies have to be done before any conclusion can be made. Another separate method to analyse retention, based on dialysis, is being developed so that the above results can be confirmed.

3.2.3 CPT liposomes incorporation ability and the influence of the zeta potential

Zeta potential of the different liposome formulations were measured to identify if there are any correlations between incorporation and retention ability and surface charge properties of the liposomes. The result is given in Figure 3.7 and shows a plot of measured zeta potential of the different liposomes.
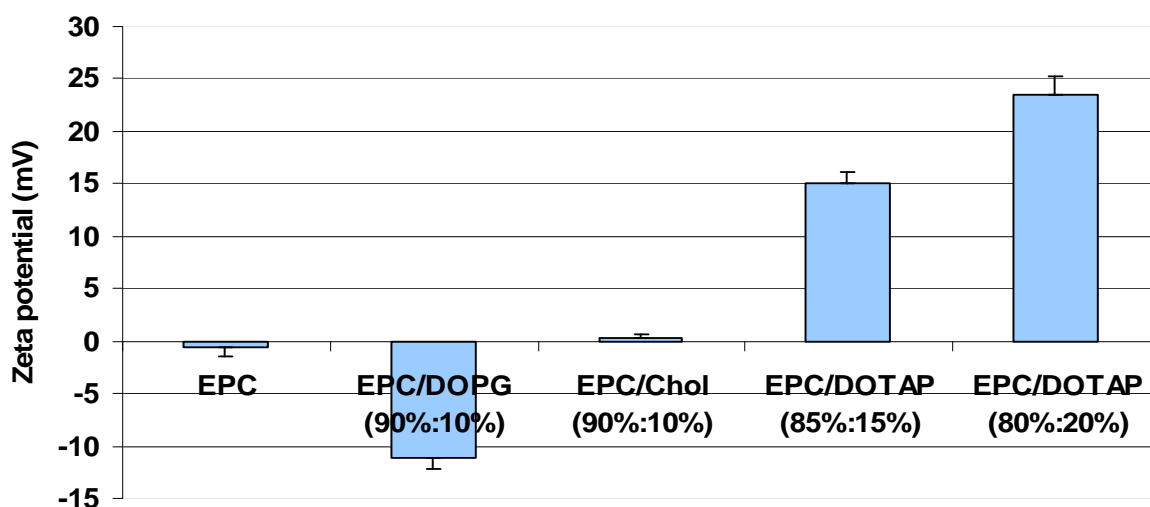
Figure 3.7: Zeta potential (mV) of the different liposome formulations (n = 3)

Liposomes which consist of EPC and EPC-Chol show a zeta potential of around 0 mV. A decrease of zeta potential is seen for the anionic lipid DOPG included in the liposome formulation 2. Further, the zeta potential starts to increase when the cationic DOTAP is employed in the liposomes, which is all according to what was expected.

A correlation between absolute zeta potential and incorporation efficacy, are presented in Figure 3.8 and a trend toward increased CPT retention ability is seen when the absolute surface charge of the liposomes becomes higher. However it is hard to draw any clear conclusions since increasing concentration of DOTAP also has shown increased CPT incorporation.

Figure 3.8: Correlation between liposomal Zeta potential (mV) and incorporation efficacy. The incorporation efficacy is plotted against absolute value of the zeta potential

3.3 Preliminary studies on PEGylated liposomes

As mentioned earlier, PEGylation of parenteraly administered liposomes are important to avoid opsonisation and rapid clearance from the blood circulation *in vivo*. The aim of the PEG inclusion 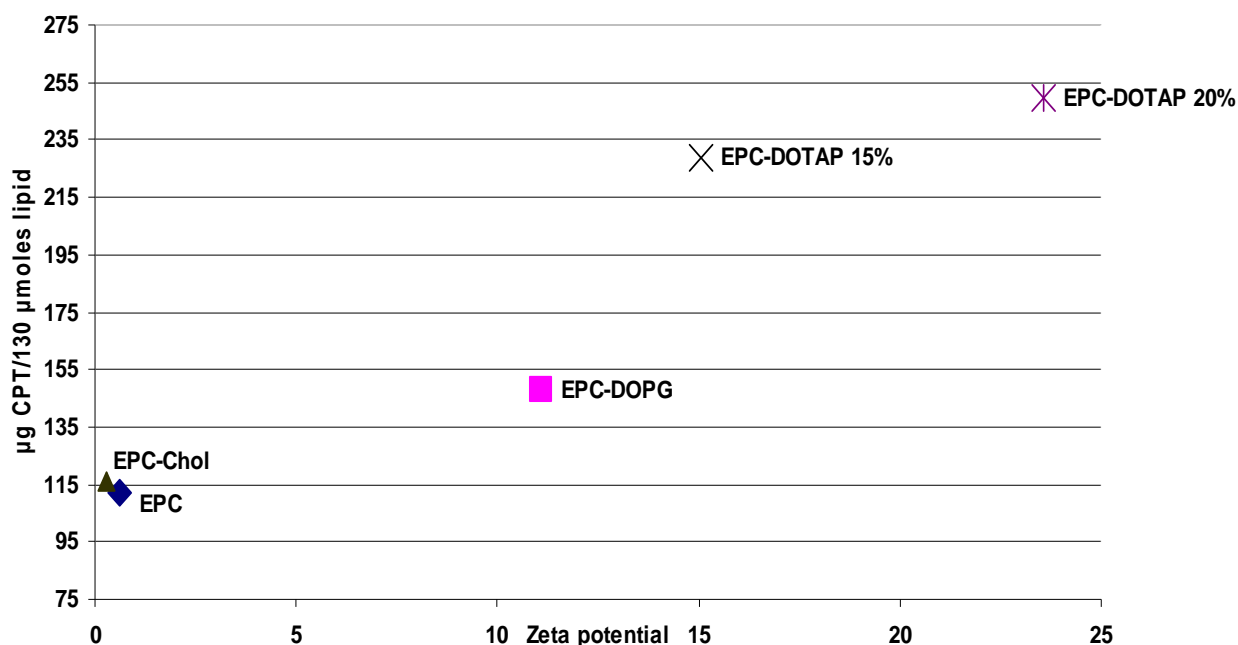to these liposomes was therefore to investigate whether the PEG had an effect on the CPT-incorporation capacity as well as retention ability, and to avoid rapid *in vivo* clearance in future studies.

3.3.1 PEGylated CPT liposomes incorporation study

As the EPC-DOTAP formulations showed increased CPT- incorporation in comparison to the other formulations displayed in Figure 3.4, these formulations were chosen for further PEG studies. Moreover, since the incorporation capacity of formulation 4 and 5 was quite similar, formulation 4 containing EPC-DOTAP 15 % was chosen for further PEG studies. Furthermore, DOTAP is more expensive than the other naturally occurring lipids and has a higher toxicity *in vivo*. The option was therefore to start with the lowest DOTAP content, which was 15 %, and see if same incorporation results could be obtained with inclusion of different ratios of PEG.

Incorporation capacity of PEGylated liposomes (formulation 6-8 in Table 2.4) were prepared with a total concentration of 1 mg CPT/130 μmoles lipid, and the content of CPT in the liposomes was quantified after ultracentrifugation as described in section 2.5. We utilized DSPE-PEG lipids where the PEG had a molecular weight of 2000 Da. The results of the incorporations are displayed in Figure 3.9.
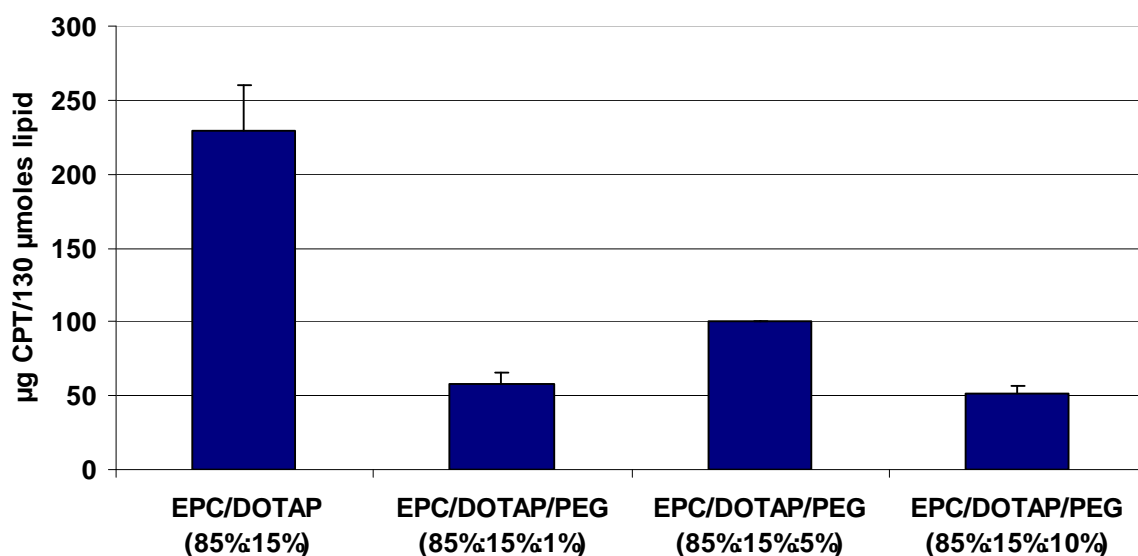
Figure 3.9: Comparison of Camptothecin-incorporation between the PEGylated and non-PEGylated liposomes. Formulation 5 (n = 3), formulation 6 (n = 4), formulation 7 (n = 1), formulation 8 (n = 2)

The use of PEG resulted in an important observation. Reduced CPT incorporation capacity was observed when different ratios of PEG were included in the DOTAP/EPC formulation compared to the EPC/DOTAP formulation whiteout PEG (see Figure 3.9). As we observed, there are no significant differences between formulation 6 which contains 1 % PEG and formulation 8 containing 10 % PEG. Formulation 7 shows a relatively higher incorporation capacity as compared with formulation 6 and 8. Due to technical problems that appeared during the preparation, unfortunately we obtained only one parallel of formulation 7. This makes the comparison with the other formulations more difficult. Overall, PEG inclusion into the cationic liposomes reduced the incorporation capacity. The reason for the reduced incorporation when PEG is included is not completely understood, but we hypothesize that the DSPE-PEG chains might affect the fluidity of the liposomes, decreasing the incorporation of the drug into the membrane. PEG has a neutralization effect on the liposomes, and as seen in Figure 3.11 PEG also reduces the zeta potential as expected, thereby possible diminish the favourable effect of the cationic lipids. The neutralization effect of PEG was linearly proportional to the molar inclusion of the PEG lipids.

3.3.2 PEGylated CPT liposomes retention ability

The CPT retention ability of the PEGylated formulations shown in Table 2.4 was investigated over time, with withdrawal of samples after 0, 5, and 24 hours of incubation both in buffer and serum as described in section 2.8 and results are presented in Figure 3.10.
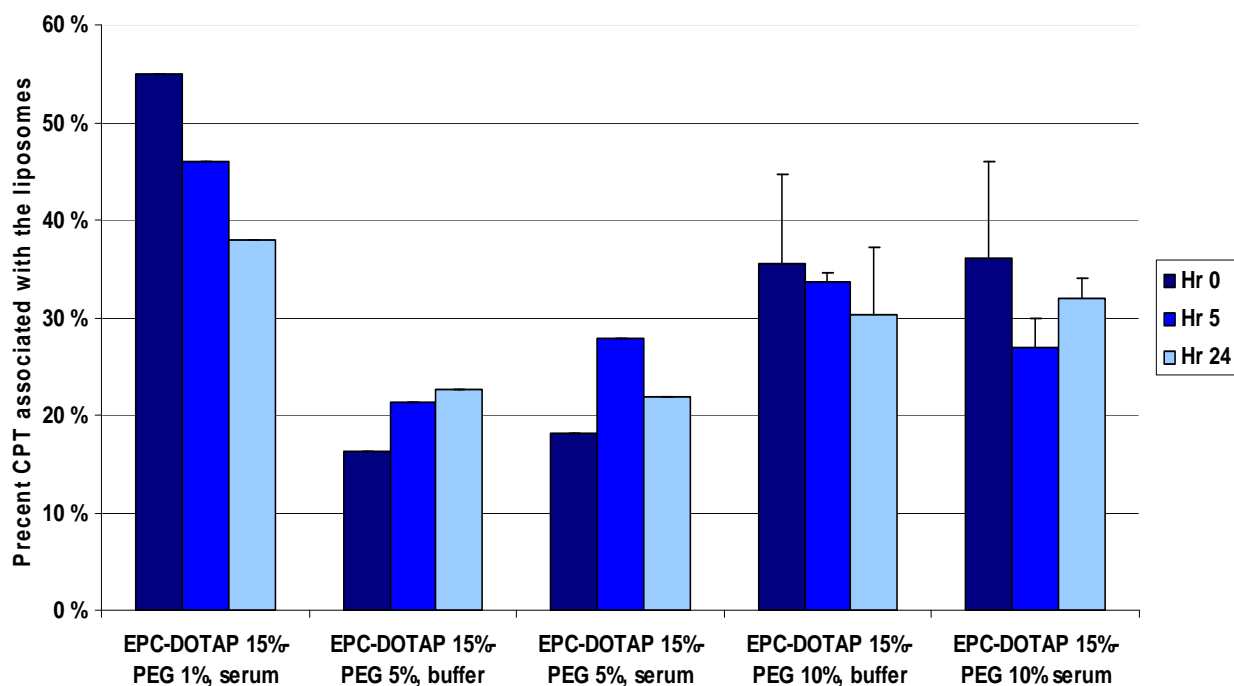
Figure 3.10: The different PEGylated liposomal formulations ability to retain CPT, associated with the liposomes over time in buffer and serum (n = 1 for PEG 1 % and 5 %, and n = 2 for PEG 10 %)

Depending on PEG ratios employed in the liposomes, different leakage profiles were observed. The results show that inclusion of different ratios of PEG to the liposomes influences the formulations' ability to retain the drug. The presence of 1 % and 10 % PEG show better retention and slower drug leakage from the liposomes compared to 5 % PEG formulation as displayed in Figure 3.10. Although we do not have results from several parallels of 1 % and 5 % PEG formulations, the results indicate that inclusion of PEG influence the formulations' ability to retain the drug and a tendency toward improved retention ability is observed especially when 1 % PEG is included. Leakage of drugs from the bilayer is thought to be related to fluidity and packing order of the membrane. It seems like inclusion of PEG gives higher degree of retention which is good. PEG inclusion in liposomes

is assumed to alter the surface characteristics and reduce the leakage from the liposomes (Brandl, 2001)

3.3.3 PEGylated liposomes CPT incorporation and the influence of zeta potential

Zeta potential of the different PEGylated formulation was measured to see how PEG is influencing the zeta potential as well as how much one have to include to get a zeta potential around zero. The results are displayed in Figure 3.11.
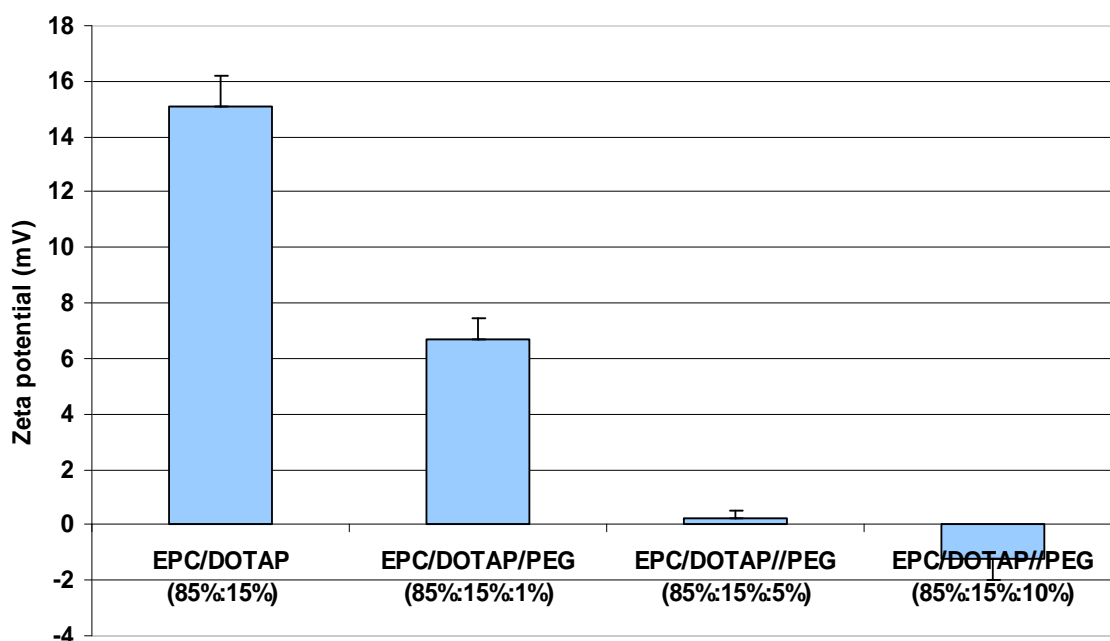
Figure 3.11: Zeta potential of PEGylated liposomal formulations (n = 3)

As expected, inclusion of neutral PEG into EPC/DOTAP liposomes resulted in decreasing zeta potential shifting towards zero. The zeta potential decreased with an increasing PEG inclusion leading to a neutralization of the liposomal surface charge.

The decreased zeta potential resulted in decreased CPT incorporation within the liposomes, thereby removing the favourable effect of the cationic lipids. Although the zeta potential decreases with an increasing PEG ratio, this seems not to decrease the liposomes retention

ability further. The research on PEGylated liposomes so far indicates that the PEG inclusion could have a favourable effect on CPT retention.

3.4 Fluorescence anisotropy

The fluidity of the liposomal bilayer membrane was investigated by fluorescence depolarization anisotropy in an attempt to find a relation between drug incorporation and retention ability with the membrane fluidity. The incorporated CPT was employed as a fluorescent probe to investigate changes in mobility and packing of the hydrocarbon acyl chains in the bilayer. CPT's rotational freedom in the bilayer dictates the degree of polarization. An increased anisotropy value indicates less rotational freedom for the drug trapped in the liposome, which conserves the polarization of the incoming light. We used the intrinsic fluorescence of CPT to measure anisotropy, so that a lower anisotropy value would indicate that the CPT molecules had a more rotational freedom in the membrane, and were therefore more loosely trapped. A number of liposomal compositions were investigated, and Figure 3.12 summarizes the findings.
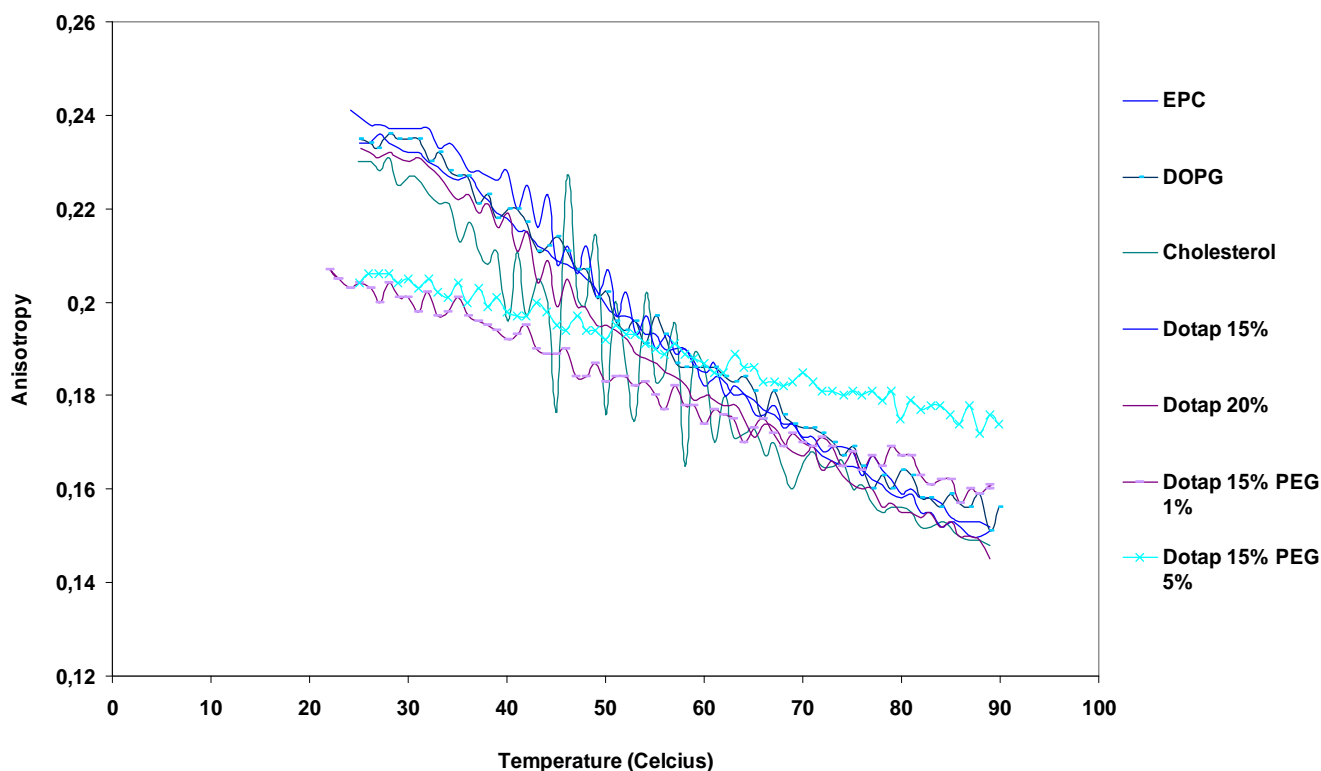

Figure 3.12: Anisotropy in different liposome formulations

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As shown in Figure 3.12 we saw little differences between the non-PEGylated formulations, most likely due to that these measurements are not specific enough toward retention to expose the small differences we discovered in the column experiments. We expected to see increased anisotropy with increased retention and decreased incorporation in liposomal formulations, hypothesizing that these drugs were more tightly trapped in the liposomal membrane. Contrary to what we expected, the PEG containing formulations as a group exhibited lower anisotropy while demonstrating an increased retention of the drug on the column experiments. In light of these results we conclude that anisotropy alone is an incomplete measure of the liposomes' ability to retain CPT over time. The reason for this can be manifold; I) The temperature dependent anisotropy measurements are done over a short time period just after ultracentrifugation, thereby not exposing the differences between the formulations after extended incubation with buffer. II) Leakage of CPT during the temperature dependent experiment will result in some background scattering of free CPT in solution, and it is unclear if this background scatter is linearly proportional to a reduction in anisotropy value. III) Different levels of CPT incorporation into the liposomes can result in different anisotropy values. It can be more optimal to measure anisotropy at different time point in the incubation period similar to the retention studies. The anisotropy measurements must be further optimized with another marker to yield meaningful data (see also section 4: Future perspectives)

3.5 Fusing of liposomes and size measurements using PCS

Charged liposomes are shown to be unstable in the presence of serum components, as indicated by rapid fusion, and leading to rapid clearance from the blood circulation (Mori et al., 1998). During the retention studies, we noticed that the sample solutions containing serum changed colour during the 24 hours of incubation to become whiter, more milk like. We hypothesize that fusion of liposomes is the reason of this. In order to check whether the hypothesis is correct or not, we analyzed some of the formulations on PCS. The results from the PCS analysis are displayed in Table 3.2.

Table 3.3: PCS results of some liposome formulations right after sonication (0 Hr) and after 24 Hr incubation in buffer and serum

Duration	Liposomal composition	Mean particle size (nm ± SD)	24 Hr buffer	24 Hr serum
		0 Hr		
2 x 2 min	EPC	42.9 nm ± 2	40 nm ± 2	259.6 nm ± 11
2 x 2 min	EPC-DOPG (90%:10%)	37.3 nm ± 1	50.2 ± 0.1	105.2 nm ± 2
3 x 2 min	EPC-Chol (90%/10%)	32.5 nm ± 2	36.7 nm ± 2	280.2 nm ± 23
2 x 2 min	EPC-DOTAP (80%: 20%)	30 nm ± 3	70 nm ± 2	120 nm ± 5
2 x 2 min	EPC-DOTAP (85%:15%)	28.9 nm ± 2	80.8 nm ±2	107.8 nm ± 3
2 x 2 min	EPC-DOTAP-PEG (85%:15%:1%)	75.1 nm ± 4	78.6 nm ± 4	77.3 nm ± 5
2 x 2 min	EPC-DOTAP-PEG (85%:15%:5%)	68.9 nm ± 1	71.3 nm ± 3	72.6 nm ± 4
2 x 2 min	EPC-DOTAP-PEG (85%:15%:10%)	48.5 nm ± 0.5	49.8 nm ± 1	49 nm ± 1

The results represent the mean of tree cycles (3 x 15 minutes)

*Hr = hours

Results displayed in Table 3.2 indicate that the size of liposome formulations without PEG attachment increases in presence of serum during the incubation time. This indicates that the milk like appearance is related to the liposome fusion which represents an irreversible process. As described above, it is known that charged liposomes exhibit instability and interacts with different serum components. This might be one of the reasons of the charged liposomes rapid fusion. It is also known that temperature conditions may influence liposome fusion, and since the serum containing samples were incubated respectively at 37 ˚C, this may have influenced the fusion rate of the neutral liposomes.

In order to use fusogenic liposomes in drug delivery systems *in vivo*, they have to be designed in such way to become stable in the circulation for an extend period of time, and to avoid opsonisation before they reach the desired target site.

The PCS results of PEG containing liposomes do not show any size alteration during the incubation period, and we conclude that PEG inclusion in the liposomes stabilizes their size under the incubation conditions by decreasing the fusion of liposomes. We also see that 1 % PEG is enough to prevent fusion, we wanted to minimize the effect of reduced incorporation when PEG was included. 1% PEG will therefore be used further in this on going project.

4. Conclusions

In the first part of this thesis establishment of appropriate methods for incorporation and retention studies were performed. A comparison of lipid film and freeze-drying method was done and no major differences were seen between the different methods according to incorporation capacity of CPT. The film method was thus chosen as the method for further liposome preparation. Further, bath sonication and direct probe sonication were compared in terms of preparation of SUVs. The latter method gave satisfied size reduction for our purpose and was used during the study. For separation of liposomes from CPT-crystals a centrifugation approach was used. Centrifugation at 100 000 g for 20 minutes was found not sufficient for removal of CPT crystals from the supernatant. Upon changing the duration to 25 minutes, all crystals were settled in the pellet and no CPT-crystals were visible under the fluorescence microscope. 25 minutes centrifugation was concluded to be the appropriate centrifugation condition for our study.

Second, CPT-liposomes with different lipid compositions were prepared and evaluated for incorporation capacity and retention ability. The incorporation capacity of CPT within the liposomes was found to be highest for positively charged DOTAP-containing liposomes as compared with other formulations containing zwitterionic or negatively charged phospholipids. The DOTAP-containing liposomes exhibited as well a trend toward higher retention ability in serum compared to the other formulations tested.

Third, in the preliminary PEGylation studies the PEG containing liposomes showed lower incorporation capacity compared to the liposomes without PEG, while better retention and slower leakage from the liposomes was seen when 1 % and 10 % PEG was included in the liposomes. In addition, PEG inclusion in the liposomes resulted in less fusion of liposomes during incubation in both buffer and serum. From the results it can be seen that 1 % PEG is enough to prevent fusion during the incubation, but further investigation are still needed to find optimal PEG ratios that can avoid opsonisation in further *in vivo* studies.

In summary we have made progress towards establishing a method for producing and testing of CPT-liposomes as well as indentifying a formulation that effectively can incorporate CPT and retain the drug in circulation. However, we have yet to arrive at the ideal formulation, and, as we are still evaluating elements that affect all areas of incorporation and retention, further studies are needed in order to reach the goal of a formulation appropriate for *in vivo* studies.

5. Future Perspectives

There is a need for further investigation and optimization of the protocol for the retention study to yield reproducible data. One method to measure the retention ability of the liposomes that is currently being tested is by employing dialysis. Dialysis is a process of separating small molecules from macromolecules such as proteins in a solution using a semi-permeable membrane. Dialysis operates by diffusion, which leads to movement of molecules from areas of higher to lower concentrations. By employing a dialysis membrane with the right molecular cut-off, the small CPT molecules will freely diffuse across the semi-permeable membrane into a second chamber of liquid until equilibrium is reached (Aulton, 2007). Both buffer and serum or protein solution could be used as acceptor medium to look at the influence of presence of proteins, and samples can be taken from the dialysis chamber at set time-points. Using this method no measurements of PC-recovery is needed for the retention studies, and liposome samples can be analyzed directly by the HPLC. By varying the different dialysis conditions, the liposomes' retention ability in different environments can be investigated.

The fluorescence anisotropy measurement must also be further investigated and optimized to yield more information. One way that may optimize this measurement is to employ a hydrophobic probe like1,6-Diphenyl-1,3,5-hexatriene (DPH) in the bilayer to investigate the bilayer fluidity and thus be able to predict CPT's rotational freedom in the bilayer. The advantage with DPH before CPT is that it is easy to dissolve in the lipid bilayer which will ensure the same concentration of a probe in all the different formulations (Silvander et al., 2000).

It is clear that further studies needs to be done to reach the goal of a formulation appropriate for further *in vivo* studies. It would thus be of great interest to continue developing selected formulations as well as introduce new elements into the formulations to achieve a desirable incorporation and retention of CPT. Studies done by Watanabe group (Watanabe et al., 2008) and Maitani group (Maitani et al., 2008) has shown that inclusion of various artificial lipids in liposomes, especially benzoic acid derivatives can increase the incorporation capacity and retention of CPT in liposomes both *in vitro* and *in vivo*.. This might be due to an interaction between a phenyl group on the lipids and CPT by a π-π interaction. Another interesting approach that may improve the incorporation and retention ability of CPT-liposomes is by including polymerizable diacetylene into the liposomal bilayer. Polydiacetylene molecules are

generally non-toxic molecules, and form polymerized vesicles under UV irradiation. Such polymerizable vesicles are found to be more stable in comparison with unpolymerized vesicles, and therefore more suitable prolonged release drug delivery. *In vitro* study done by Guo group (Guo et al., 2010) show that conjugated backbone of the molecules by the photopolymerization acts as a barrier for drug release. Higher polymerization degree is shown to lead to a more sustained release of the drug from liposomes. It would be of great interest to develop these formulations, and investigate CPTs' incorporation and retention ability within these liposomes.

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