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Development of Targeted Liposome Drug Delivery Vehicle

Thesis for the degree Master of Pharmacy

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AKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

Figure 4.4: Retention of CPT in the different formulations after 0, 5 and 24 hours in PBS pH 7.4 + BSA at 37 °C.

Figure 4.5: Retention of CPT in the different formulations after 0, 5 and 24 hours in PBS pH 7.4 + FBS (1:1) at 37 °C.

Figure 4.6: Retention of CPT in DTPEG after 0, 5 and 24 hours in PBS pH 7.4, PBS pH 7.4 + BSA, PBS pH 7.4 + FBS and erythrocytes at 37 °C.

Figure 4.7: Zeta potential measured at pH 6.0 for the different formulations.

Figure 4.8: Measured fluorescence anisotropy for EPCPEG, DTPEG, DBDTPEG and DDABPEG at 20 ˚C and 37 ˚C.

Figure 4.9: Mean particle size of the liposomes formulations with DSPE-PEG-MAL as determined by PCS-analysis.

Figure 4.10: Incorporation of CPT in DTPEGMAL, DTPEGMAL-BSA and DTPEGMAL-EGFR together with control.

Figure 4.11: Retention of CPT in DTPEGMAL after 0, 5 and 24 hours in PBS pH 7.4 and PBS pH 7.4 + FBS at 37 °C.

Figure 4.12: Retention of CPT in DTPEGMAL-BSA after 0, 5 and 24 hours in PBS pH 7.4 at 37 °C.

Figure 4.13: Zeta potential of DTPEGMAL and DTPEGMAL-BSA compared to the zeta potential of DTPEG.

Figure 4.14: Measured fluorescence intensity for DTPEGMAL and DTPEGMAL-BSA with control.

Figure 4.15: Measured fluorescence intensity for DTPEGMAL and DTPEGMAL-EGFR with control.

LIST OF TABLES

LIST OF EQUATIONS

x

ABSTRACT

The coating of the liposome surface with polyethylene glycol (PEG) has proven to prolong the circulation time of liposomes in the blood stream. PEG prevents the binding of opsonins and subsequent uptake of the liposomes by the mononuclear phagocytic system (MPS). The reduction in clearance of PEGylated liposomes from the circulation improve the bioavailability of the liposomes in the blood and increase the chance of liposomes being accumulated in tumor tissue by the enhanced permeability and retention effect (EPR), which is of particular interest for the liposomal delivery of anticancer agents.

The aim of this study was therefore to investigate the incorporation and retention ability of PEGylated liposome formulations of the highly lipophilic anticancer agent Camptothecin (CPT), and further try to develop an immunoliposomal formulation of CPT targeting the EGFR receptors on the surface of colorectal cancer cells. Incorporation and retention ability of CPT were investigated for five different PEGylated liposome formulations, and the effects of incorporating the cationic lipid dimethyldioctadecylammonium (DDAB) and the neutral lipid 4-(Dodecyloxy)-benzoic acid (DB) into the liposome membrane were concurrently explored.

The results from the incorporation and retention studies showed that the PEGylated formulations with DDAB and DB demonstrated lower incorporation ability and were slightly more unstable in regard to retention of CPT compared to the formulation with DOTAP. The formulation consisting of 79 % egg phosphatidylcholine (EPC), 20 % 1,2-di-oleyl-3-trimethylammonium-propane (DOTAP) and 1 % PEG conjugated with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG) clearly showed the highest incorporation of CPT (about 2 times higher than the formulation showing the second highest incorporation and more than 4 times higher than the control formulation) and the most stable retention ability in different media including medium containing erythrocytes. Based on the promising incorporation and retention results for the PEGylated formulation with DOTAP, the formulation was chosen as the basis for the development of the immunoliposomal formulation for targeted delivery to cancer cells. Both bovine serum albumin (BSA) and EGFR antibodies were conjugated to the liposome surface by the inclusion of 1 % maleimide terminated DSPE-PEG into the liposome membrane. The loss of CPT from the liposomes observed during conjugation was however significant.

In conclusion the presence of PEG on the liposome surface and DOTAP in the liposome bilayer seems to give the most promising PEGylated CPT formulation, which could possibly be a candidate for further in vivo studies. For the immunoliposomes, the attachment of antibodies on the surface was successful. However, due to loss of CPT during the conjugation process the method used is not optimal for this CPT liposome formulation, and further studies are needed to find a more suitable preparation method or a more stable immunoliposome formulation.

ABBREVATIONS

AF594	Alexa Fluor 594
CPT	Camptothecin
DB	4-(Dodecyloxy)-benzoic acid
DDAB	Dimethyldioctadecylammonium
DNA	Deoxyribonucleic acid
DOTAP	1,2-Di-oleyl-3-trimethylammonium-propane
DPH	1,6-Diphenyl-1,3,5-hexatriene
DPPG	L-α-Dipalmitoylphosphatidylglycerol
DSPE	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine
EPC	Egg phosphatidylcholine
EPR	Enhanced permeability and retention effect
FBS	Fetal bovine serum
HSA	Human serum albumin
BSA	Bovine serum albumin
LUV	Large unilamellar vesicle
MAL	Maleimide
MLV	Multilamellar vesicle
MPS	Mononuclear phagocytic system
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
OLV	Oligolamellar vesicle
PC	Phosphatidylcholine
PEG	Polyethylene glycol
SUV	Small unilamellar vesicle
Topo-I	Topoisomerase I
ULV	Unilamellar vesicle

1. INTRODUCTION

The development of high-throughput screening methods and combinatorial drug design during the 1990s and the move towards a more target-based approach to drug discovery have resulted in the identification of drug candidates with increased lipophilicity, high molecular weight and limited solubility [1, 2]. This situation, in combination with the decline in productivity in drug discovery and increased interest in the repositioning and reformulation of already marketed drugs by the pharmaceutical industry [3] have increased the interest in advanced drug delivery systems in recent years.

Liposomal drug delivery has proven to be a versatile delivery system for a range of compounds applied in the field of medicine, such as drugs, immunomodulators and imaging agents, and it has received an increasing amount of attention over the last two decades. As drug vehicles, liposomes have the advantage of being biocompatible and able to incorporate both lipophilic and hydrophilic compounds in their structure. Additionally, the surface properties of liposomes can be tailored according to circumstances by changing the composition of the liposome membrane. Modifications of the surface chemistry of liposomes have led to the development of sterically stabilized long-circulating liposomes and immunoliposomes capable to delivery drugs by active targeting [4].

1.1 Camptothecin

1.1.1 Discovery and Early Clinical Trials

In the 1950s researchers discovered that extracts from the tree *Camptotheca acuminate* exhibited high antitumoral activity, initiating the search for the active compound or compounds [5]. As a result, the alkaloid Camptothecin (CPT) was isolated from the stem wood and characterized by Wall and Wani in 1966 [6].

The antitumoral effect exhibited by isolated CPT in animal studies was very promising and led to early clinical trials. Due to the poor water solubility of CPT, the clinical trials were conducted using the more water soluble sodium salt of the drug. The results from the clinical trials showed that CPT had poor efficacy and only a small group of patient responded to treatment. In addition, the side effects proved to be severe. Consequently the interest in CPT as a potential anticancer agent subsided, and it was not until several years later that a

comparison between CPT and its sodium salt showed that the sodium salt only retained 10-20 % the activity compared to native CPT [5, 7].

1.1.2 Mechanism of Action

Renewed interest in CPT as an anticancer agent came in the 1980s, when it was shown that its cellular target was the nuclear enzyme topoisomerase I (Topo-I) [8]. Topo-I belongs to the topoisomerase enzymes that control the topological state of the deoxyribonucleic acid (DNA) during replication and transcription. The enzyme relaxes supercoiled DNA before replication and transcription by causing a single strand break, which allows the broken strand to rotate around the DNA and uncoil it. After the supercoiling of the DNA has been removed, Topo-I religates the single strand break [9]. CPT acts by stabilizing the transient cleavage complex that is formed between Topo-I and DNA right before the uncoiling begins. The effect of CPT's binding and stabilization of the complex is reversible and CPT does not cause any DNA damage in itself, the damage is done by Topo-I. The binding of CPT leads to retardation of the process of DNA religation and Topo-I ultimately collide with the replication forks, leading to DNA damage and cell death (Figure 1.1) [9, 10].
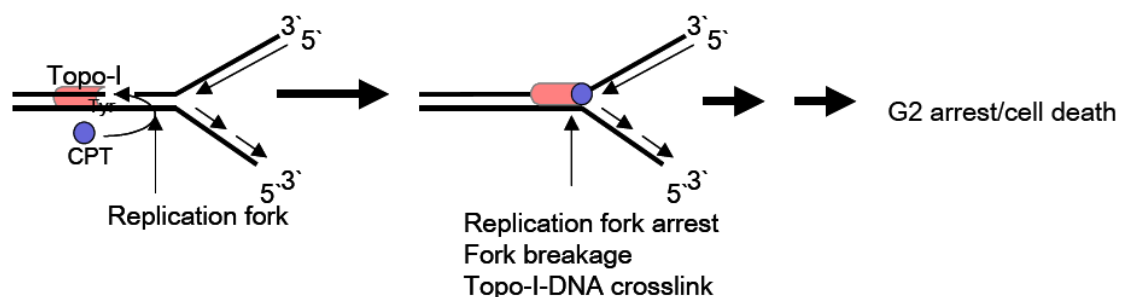
Figure 1.1: CPT binds to Topo-I and arrest the replication fork which ultimately leads to cell death (reprinted with permission [11]).

As the effect of CPT is related to the process of DNA replication and transcription, the cells most sensitive to CPT are cells in the S-phase of the cell cycle. Cancer cells achieve a state of chronic proliferation by bypassing the systems that regulates and control the entry and progression of the cell cycle [12]. The high frequency of proliferation in cancer cells forces

them into the S-phase of the cell cycle more often than normal cells, which in turn leads to an overexpression of Topo-I and a higher sensitivity to the action of CPT.

1.1.3 Stability and Toxicity

Due to the hydrolytic reactivity of CPT's lactone ring, CPT exists in a pH dependent equilibrium between a poorly water soluble lactone form and a water soluble carboxylate form (Figure 1.2). The equilibrium favors the active lactone form at pH values below 6, and the inactive carboxylate form at pH 7 and above [6, 13].

Lactone form   Carboxylate form
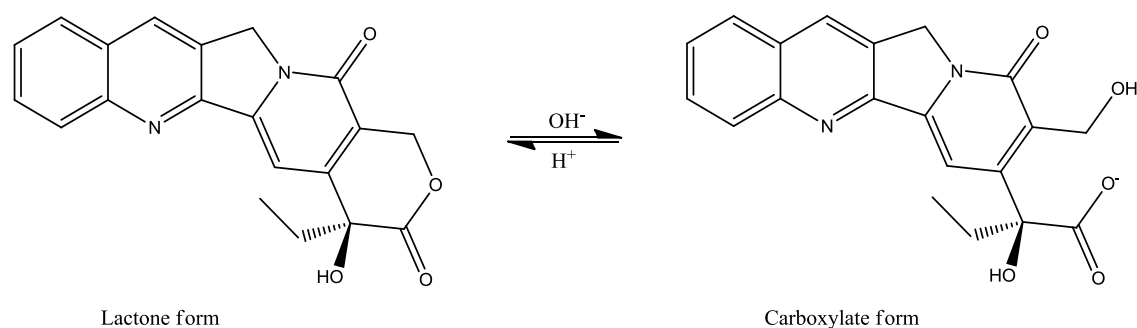
Figure 1.2: The pH dependent equilibrium between CPT's lactone and carboxylate form.

The presence of human serum albumin (HSA) also influences the CPT equilibrium. CPT's carboxylate form show a 150 to 200-fold greater affinity to HSA than the active lactone form, and the equilibrium will therefore favor the formation of the carboxylate form in the presence of HSA in accordance with Le Chatelier's principle [14-16]. This equilibrium affects the bioavailability of the drug since HSA-bound CPT is no longer available for cellular uptake and cannot reach its target. In contrast, certain blood constituents, including high-density lipoproteins, low-density lipoproteins and the membrane components of erythrocytes have demonstrated a stabilizing effect on the lactone form by extending its half-life in blood [17].

CPT derivatives have been developed to counter the solubility and stability problems inherent to the lactone form. Chemical modification of the quinoline ring in the CPT structure has yielded several compounds with improved stability or solubility compared to the parent compound. 9-Aminocamptothecin, irinotecan, topotecan and lurtotecan are a few of the most promising compounds achieved by this type of modification, all of them are water soluble, but due to the short half-life of 9-aminocamptothecin and the lack of response from treatment

with lurtotecan in clinical trials, the only compounds that have reached the market are topotecan and irinotecan (Figure 1.3) [18]. In addition to modification of the quinoline ring, researchers have also investigated ways to further stabilize the lactone ring by means of chemical modifications, producing homocamptothecins with increased stability, but varying clinical results [18]. So although irinotecan and topotecan are water soluble and demonstrates an increased stability in the presence of HSA compared to CPT, the hydrolytic reactivity of the lactone ring still remains a challenge for further development and realization of CPT and its derivatives' full potential in a clinical setting [14].
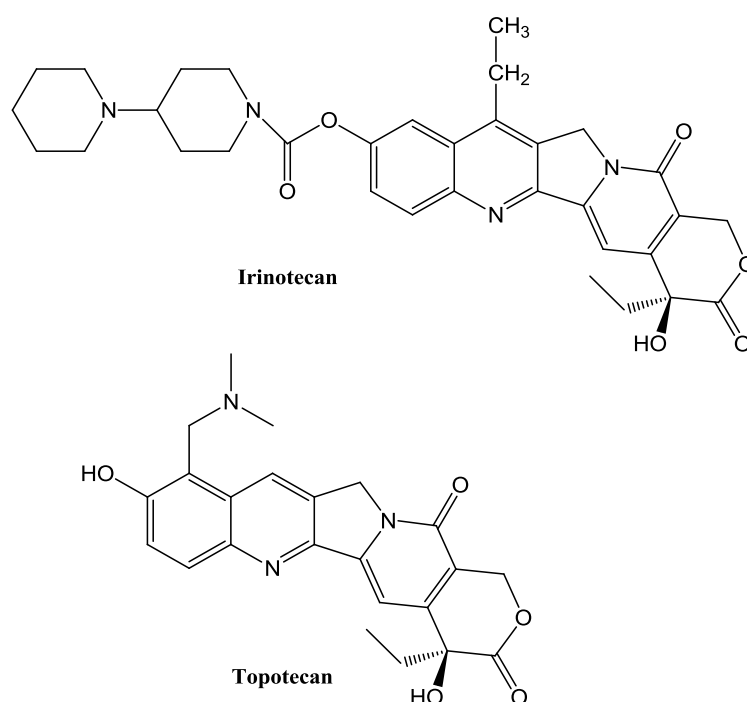
Figure 1.3: Chemical structure of the two CPT derivatives commercially available on market [18].

A possible alternative to chemical modification and stabilization of the lactone ring could be the use of a drug delivery system. Burk *et al.* showed that CPT was stable in its lactone form when it was incorporated into the lipid bilayers of liposomes [19]. The CPT lactone form is located in the acyl chain region of the lipid bilayers, protected from the aqueous phase and the fate of hydrolysis (Figure 1.4) [20, 21]. The use of liposomes as a drug vehicle for water soluble CPT derivatives have also shown promising results, as the lipid bilayers act as a barrier to the outside environment and the pH of the aqueous core can be adjusted to favor the lactone form [21].
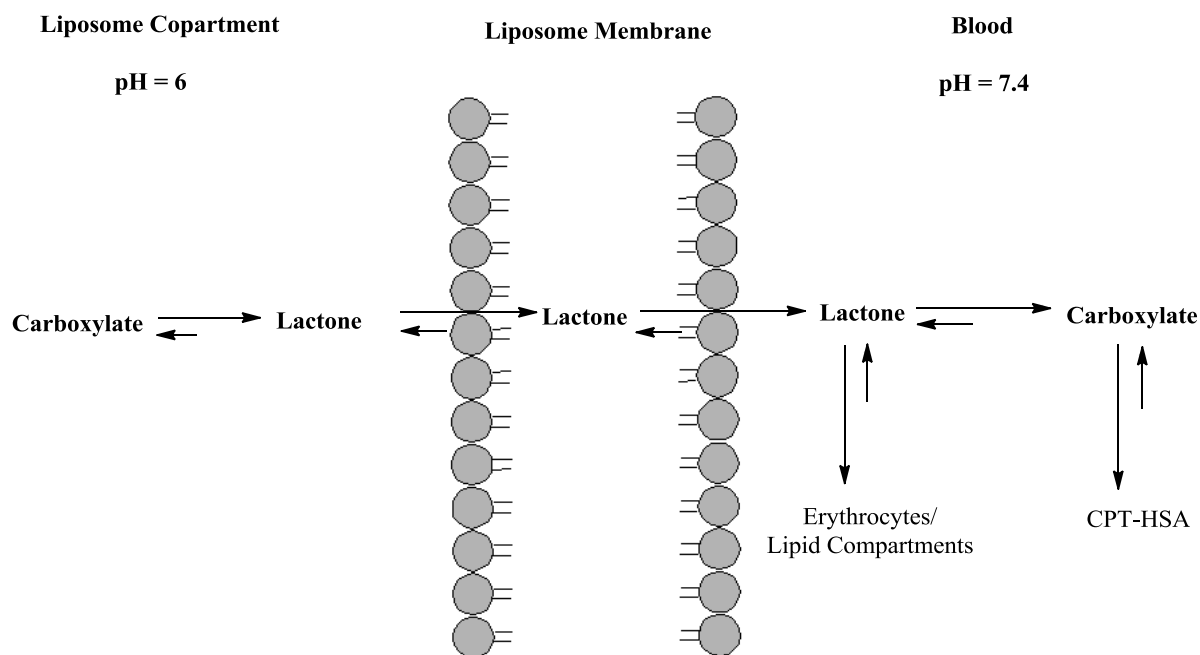
Figure 1.4: Schematic illustration of the equilibrium state of CPT's lactone and carboxylate form in different environments and the stabilization of the lipophilic lactone form in the liposome membrane (modified version of the illustration by Naderkhani [22] with permission).

1.2 Liposomes as Drug Vehicles

Liposomes are spherical vesicles with membranes composed of lipids. The lipids in the membrane are arranged in a bilayer that separates the liposome's aqueous core from the external environment. Liposomes self-assemble when lipids are dispersed in aqueous media and the process can be encouraged by applying mechanical force [23, 24]. Both hydrophilic and lipophilic drugs can be incorporated into the liposome structure, where hydrophilic drugs will be trapped in the aqueous core and lipophilic drugs will be incorporated into the lipid bilayers of the membrane (Figure 1.5). The liposomes' ability to accommodate both lipophilic and hydrophilic drugs combined with their biocompatibility and biodegradability makes them suitable drug vehicles for several different types of drugs [25].
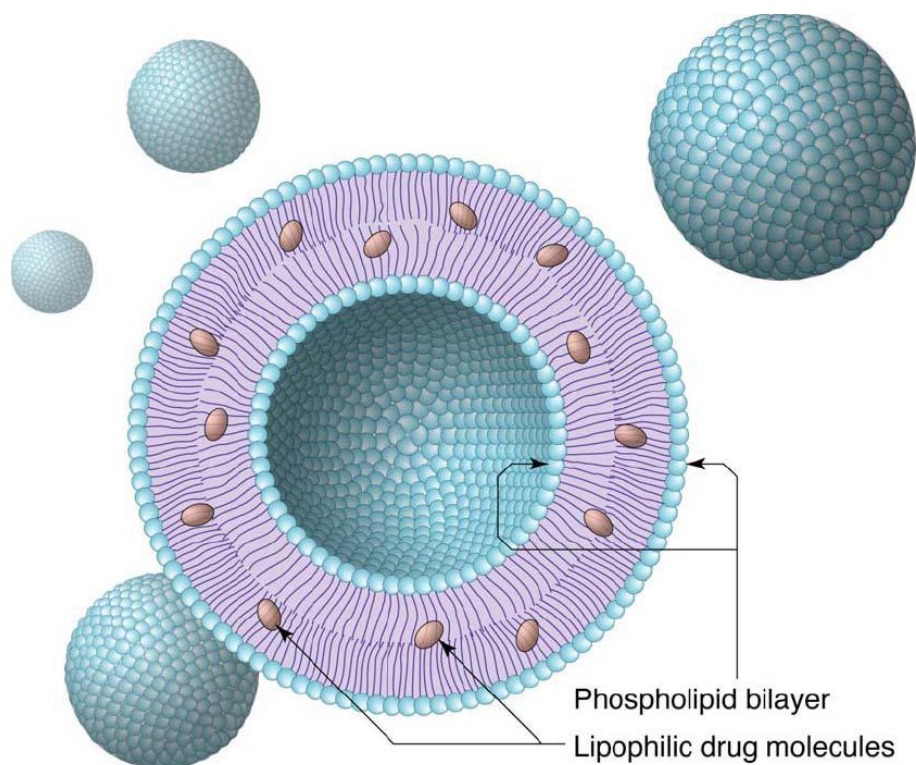
Figure 1.5: Liposomes with lipophilic drug molecules incorporated into the lipid bilayer (reprinted with permission [11]).

The preparation of liposomes as drug vehicles are often performed by the film hydration method and can usually be divided into different processes, mixing of the lipid components dissolved in organic solvents, evaporation of the organic solvents, hydration of the lipid film, and size reduction. The lipids are usually dissolved in an organic solvent before the solvent is removed from the mixture (e.g. by solvent evaporation or freeze-drying), resulting in dried lipids that can be hydrated to form liposomes [26]. Hydrophilic drugs can be incorporated into the liposome core by adding them to the aqueous solution used for hydration, while lipophilic drugs can be incorporated into the membrane by mixing them with the lipid components prior to solvent evaporation [23]. The size and lamellarity of the liposomes formed after hydration can be controlled by the application of a mechanical force to the dispersion, for example by exposure to sonication or simple handshaking [26].

1.2.2 Lamellarity, Size and Surface Charge

Liposomes which consist of only one lipid bilayer separating the core from the rest of the aqueous environment are classified as unilamellar vesicles (ULVs) (Figure 1.6). Oligolamellar vesicles (OLVs) (Figure 1.6) and multilamellar vesicles (MLVs) are liposomes that have more than one lipid bilayer separating the inner core from the aqueous environment. The structures can furthermore be classified according to their size, where unilamellar vesicles can be defined as small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs), depending on their size range. Liposome can have a size of approximately 20 nm to several μm [23, 24].

Liposome size is also an important factor in relation to the circulation time of liposomes in the blood stream. The uptake and clearance of liposomes from the blood stream by the mononuclear phagocytic system (MPS) is size dependent. The blood components responsible for the recognition and opsonization of foreign particles recognize and bind to the large liposomes faster than smaller liposomes, leading to shorter circulation time for liposomes larger than 200 nm. Liposomes between 70-200 nm have a larger surface area and thus manage to evade binding and recognition for a longer period of time, while smaller liposomes with a diameter below 70 nm are extraverted in the fenestrated blood vessels of the spleen [23, 27]

Other factors that are of importance to the liposome clearance from the blood stream are the membrane fluidity and surface charge of the liposome. The incorporation of lipid components that reduce membrane fluidity has demonstrated a positive effect on the circulation time by reducing the binding of blood components, while liposome formulations with cationic and anionic lipids have shown increased opsonization and clearance from the blood [23].

ULV   OLV
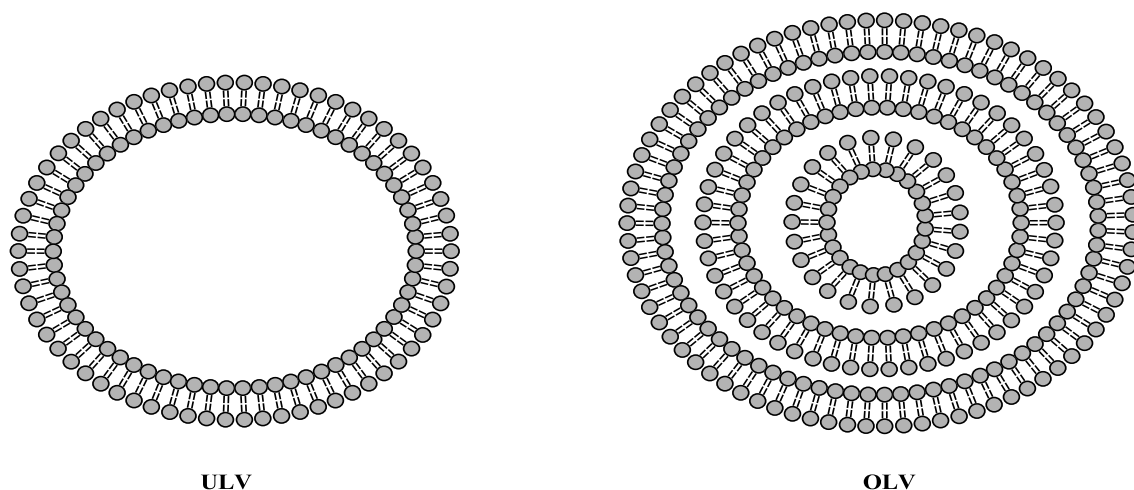
Figure 1.6 Illustrations of an ULV and an OLV.

1.2.3 Lipid Components and Composition

Liposomes can be composed both by natural and artificial lipids. Biological membranes, such as cell membranes have a high proportion of phospholipids in their compositions. There are two different types of phospholipids; sphingolipids and phosphodiglycerides [24]. The primary lipid used in liposomal drug carriers is phosphatidylcholine (PC) (Figure 1.7), which can be both extracted from natural sources and produced synthetically [23]. PC lipids consist of a lipophilic tail group and a zwitterionic hydrophilic head group. The amphiphilic nature of PC and other lipids results in spontaneously association in aqueous environment to form lipid bilayers. The tail groups form an inner lipophilic environment, while the head groups protrude into the aqueous environment.
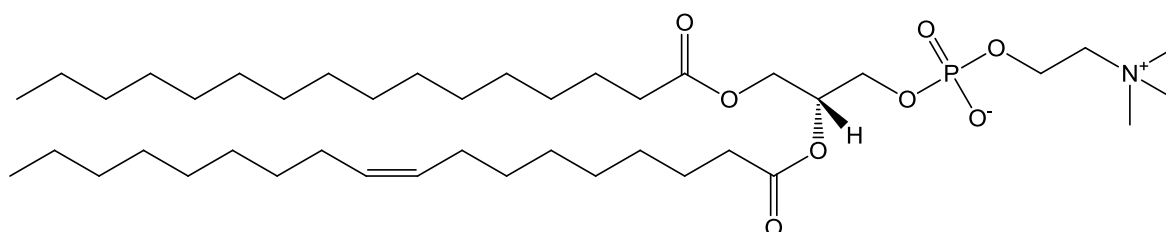
Figure 1.7: Chemical structure of the predominantly phospholipid in egg PC [28].

The fluidity or mobility of the lipid bilayer is determined by the lipid transition temperature, which is the temperature when the lipid bilayer goes through a phase transition from the more organized and rigid "gel" state to the "fluid" state. The "fluid" state is the most flexible and permeable state of the lipid membrane, leading to leakiness in the membrane structure due to the increased lateral mobility of the lipids. The factors that influence the transition temperature of the given lipid bilayer, is the chain length of the tail group and the degree of saturation of the acyl chains. Consequently, the transition temperature in the lipid mixture can be manipulated by the addition of different types of lipids and chemical compounds, such as 4-(Dodecyloxy)-benzoic acid (Figure 1.8), leading to increased stability, enhanced retention and incorporation of the liposome formulation. The addition of cholesterol to liposome compositions in the fluid state has demonstrated a reduction in membrane fluidity by reducing the lateral mobility of the lipids in the bilayer. This also prevents the insertion of blood components from the MPS when circulating in the blood, thereby increasing circulation time [23].

The addition of certain types of lipids or fatty acids to the liposome formulation can also be used to modify the surface charge of the liposomes. For example by adding cationic lipids such as 1,2-di-oleyl-3 trimethyl-ammonium-propane (DOTAP) (Figure 1.9) and dimethyldioctadecylammonium (DDAB) (Figure 1.10) to the composition of the liposome membrane, both the surface charge and transition temperature of the liposome membrane is affected.
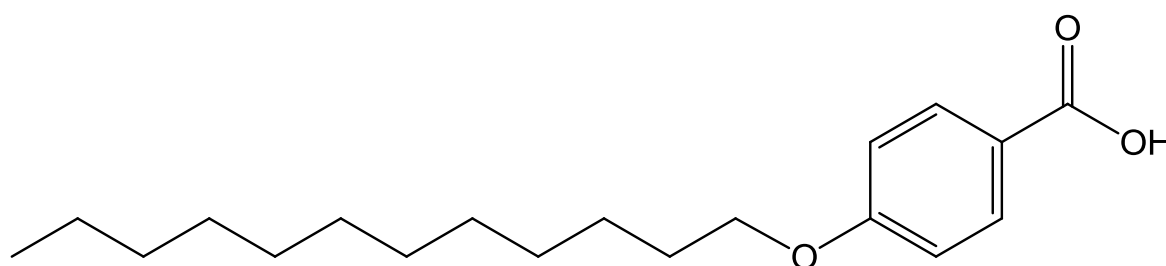
Figure 1.8: Chemical structure of DB [29].
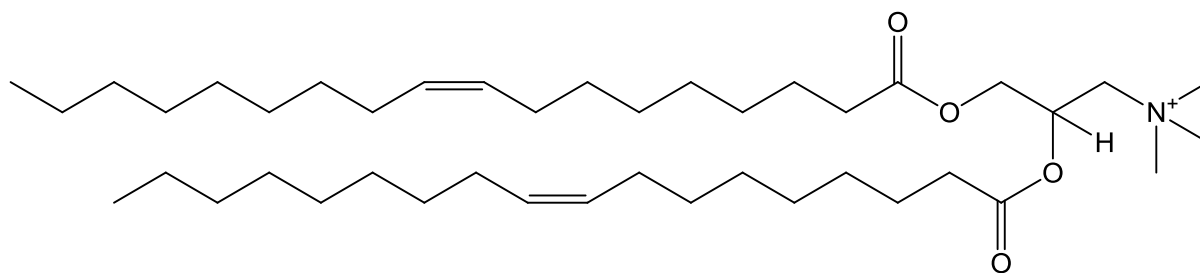
Figure 1.9: Chemical structure of DOTAP [30].
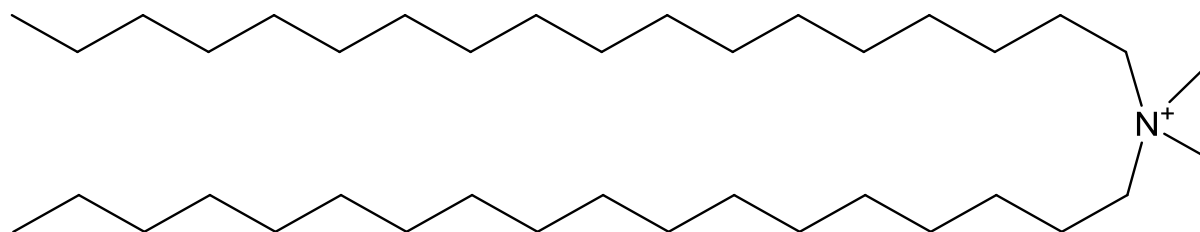
Figure 1.10: Chemical structure of DDAB [31].

Decorating the liposome surface with polyethylene glycol (PEG) (Figure 1.11) has been shown to extend blood-circulation time by "hiding" or "stealthing" the liposome from the immune system and reducing mononuclear phagocyte system uptake. The reason for the stealthing effect of PEG is that the PEG-molecules inhibit or delay the binding of opsonins to the liposome surface [32]. The PEG molecule can be inserted into the lipid membrane by using a lipid anchor, in this thesis, DSPE-PEG, where a PEG molecule have been conjugated with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine was used (Figure 1.12).
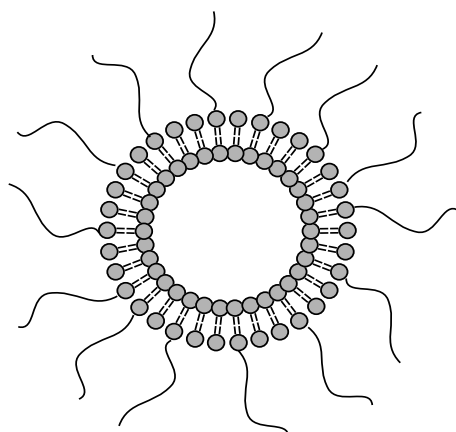
Figure 1.11: Illustration of a stealth liposome decorated with PEG.
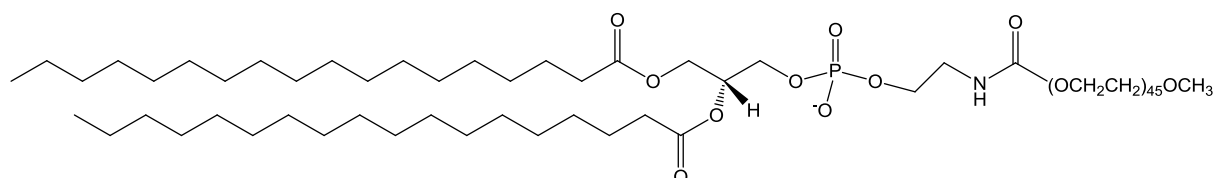
Figure 1.12: Chemical structure of DSPE-PEG 2000 [33].

1.3. Liposomes as Drug Vehicles for Anticancer Agents

Tumors have different vasculature compared to healthy tissues, because the increased angiogenesis in tumors leads to a dysfunctional basement membrane in the blood vessels. The blood vessels of the tumor therefore becomes more "leaky" than in healthy tissues, allowing relatively large particles ranging from $10 - 200$ nm in size to leave the blood vessels and accumulate in the interstitial space [34]. The leaky blood vessels combined with a non-functional lymphatic drainage system in tumors, results in enhanced accumulation of particles in tumors. Liposomes have demonstrated the ability to accumulate in tumors due to this enhanced permeability and retention effect (EPR) (Figure 1.13), thereby leading to an increased delivery of drug to the tumor tissue compared to healthy tissue [35].

The utilization of EPR combined with the biocompatibility of liposomes and their ability to entrap and retain drugs, have led to the use of liposomes as drug vehicles for anticancer agents, such as anthracyclines. One formulation that is available clinically is a PEGylated liposome formulation of doxorubicin. The formulation demonstrates a good efficacy towards tumors and a marked reduction in side effects compared to free doxorubicin [36, 37]. The

liposomes limits the amount of free drug in blood able to cause side effects, and the PEGylation prolongs the circulation time of the liposomes, thereby increasing the chance of drug accumulation by EPR and enhancing the therapeutically efficacy of the treatment.

A combination of the passive targeting achieved with PEGylated liposomes through EPR and the principle of active targeting can hopefully further enhance the therapeutic efficacy of anticancer agents and reduce the associated side effects.
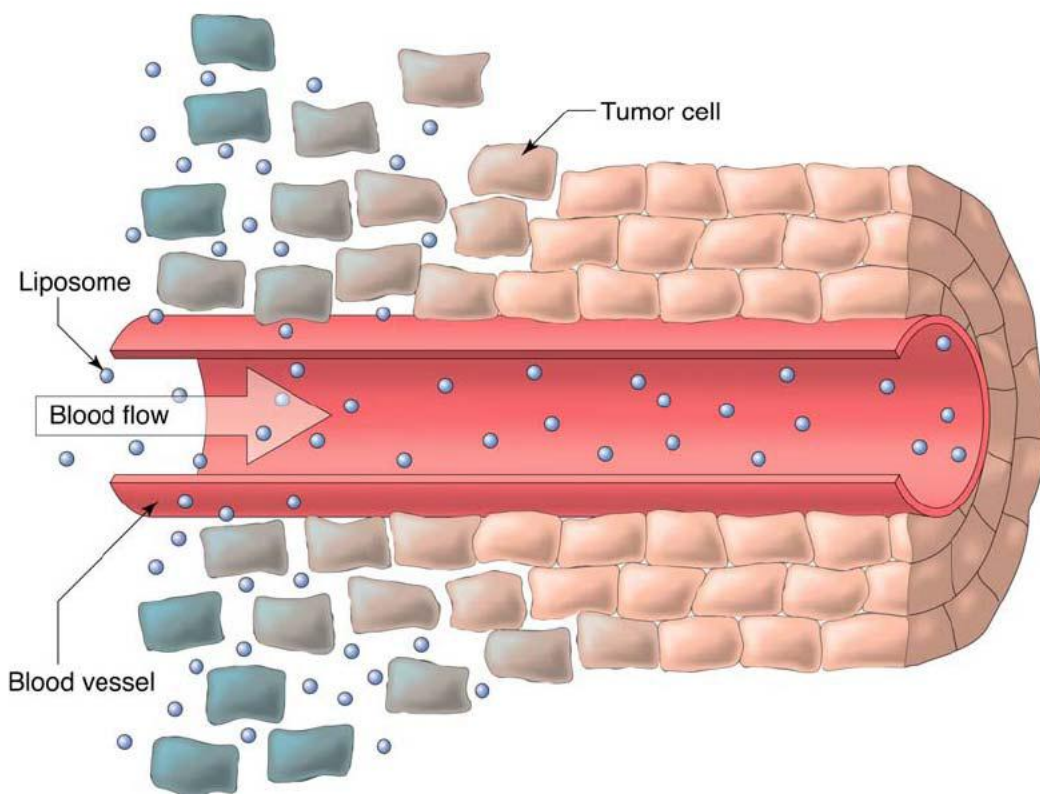
Figure 1.13: Accumulation of liposomes in tumor tissues due to EPR (reprinted with permission [11]).

Active targeting can be achieved by the addition of a ligand to the liposome surface that recognizes and binds to the target tissue. The ligand used for targeting can vary according to the characteristics of the target tissue. Liposomes where the ligands used for targeting is a type of antibody is called immunoliposomes (Figure 1.14). By using a type of antibody specific to an antigen found on the surface on cancer cells the immunoliposomes are able to recognize and bind to both malignant cells in the systemic circulation and to the tumor tissue made accessible through extravasation from the blood vessels by EPR [38].
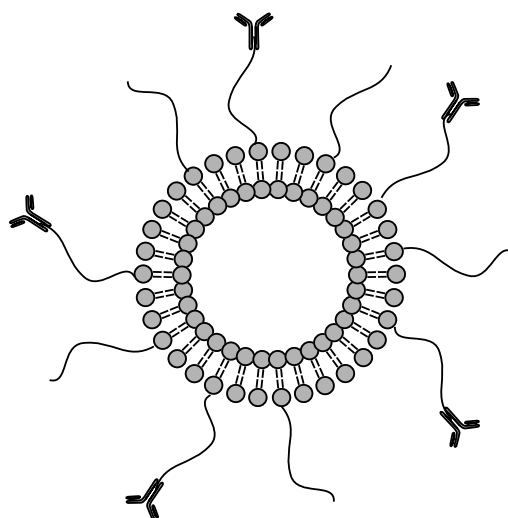
Figure 1.14: Illustration of a PEGylated immunoliposome, where the antibodies are conjugated with the PEG molecules on the liposome surface.

1.3.1 CPT-loaded Liposomes

Previous studies performed on liposomes loaded with CPT have shown promising results according to incorporation using a composition of EPC and the cationic lipid DOTAP [39]. This formulation has also been tested *in vivo* in a mouse tumor model. The results showed that the liposomes accumulated very fast in the liver, probably due to the positive surface charge, and it also seemed like some of the drugs were prematurely released [40]. These results indicated a need for investigation of PEGylated liposome formulations, which was supported by the findings, reported by Naderkhani, that a certain degree of PEGylation is necessary to avoid fusing of CPT loaded liposomes [22].

2. AIM OF STUDY

The aim of this study was to determine the incorporation ability of CPT for various PEGylated liposome formulations, and to test the CPT retention in these formulations in different physiological media with the aim of prolonging the circulation time and improving the ability to delivery CPT through adequate incorporation and retention of the drug.

We also wanted to observe the effect of incorporating different cationic lipids into the liposome membrane and see if the incorporation of the neutral artificial lipid DB could have a positive effect on the amount of CPT incorporated into the liposome membrane, similar to the effect reported by Maitani [41].

Subsequently, the targetability of the most promising formulation towards the EGFR-receptor expressed on specific cancer cells was to be tested.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Assay Kits

Table 3.1: Assay kits.

Assay Kit	Manufacturer/Supplier
Bio-Rad Protein Assay	Bio-Rad Laboratories, USA
Phospholipides enzymatique PAP 150	bioMérieux sa, France

3.1.2 Chemicals

Table 3.2: Chemicals.

Chemical	Purity	Quality	Manufacturer/Supplier
Acetic acid	100 %	Glacial, for analysis	Merck, Germany
Acetonitrile	≥ 99.9 %	For HPLC	Sigma-Aldrich Chemie GmbH, Germany
Albumin	≥ 96 %	From bovine serum	Sigma-Aldrich Chemie GmbH, Germany
Albumin, Fluorescein isothiocyanate Conjugate	-	From bovine serum	Sigma-Aldrich Chemie GmbH, Germany
Antibody, EGFR (EGFR.1)	-	Mouse monoclonal IgG_{2b}	Santa Cruz Biotechnology Inc., USA
(S)-(+)-Camptothecin	> 95.0 %	For laboratory use only	TCI Europe, Belgium
Chloroform	≥ 99.8 %	For HPLC	Merck, Germany
1,6-Diphenyl-1,3,5-hexatriene	98 %	-	Sigma-Aldrich Chemie GmbH, Germany
di-Potassium hydrogen phosphate	Min. 99.0 %	For analysis	Merck, Germany
di-Sodium hydrogen phosphate-dihydrate		For analysis	Merck, Germany

Dithiothreitol	≥ 99 %	-	Sigma-Aldrich Chemie GmbH, Germany
Methanol	Min. 99.9 %	For HPLC	Sigma-Aldrich Chemie GmbH, Germany
Ortho-Phosphoric acid 85 %	-	For analysis	Merck, Germany
Sodium chloride	≥ 95 %	For analysis	Sigma-Aldrich Chemie GmbH, Germany
Sodium hydroxide	Min. 98 %	For analysis	Riedel-de Häen, Germany
Potassium dihydrogen phosphate	> 99.5 %	For analysis	Merck, Germany
Triethylamine	≥ 99.5 %	For HPLC	Sigma-Aldrich Chemie GmbH, Germany
Triton® X-100	97 %	-	Sigma-Aldrich Chemie GmbH, Germany

3.1.3 Equipment

Table 3.3: Equipment.

Equipment	Type	Manufacturer/Supplier
Analytical Scale	Sartorius BP211D with printer	Sartorius AG GmbH, Germany
Bath sonicator	Branson 1510	Branson Ultrasonics, USA
Centrifuge	Biofuge Stratos with swing bucket rotor (#3047)	Heraeus Instruments, UK
Centrifuge tubes	Polycarbonate tubes, thick-wall	Beckman Instruments, USA
Filter	0.22 µm cellulose acetate filter	Sartorius AG GmbH, Germany
Filter	0.22 µm non-sterile syringe filters	Pall Life Sciences, USA
Fluorescence spectrophotometer	Perklin Elmer LS 55 Fluorescence Spectrometer	Perklin Elmer, UK
Gel column	PD-10 Desalting column with Sephadex G-25 medium, pre-packed	GE Healthcare Bio-Sciences AB, Sweden
HPLC instrument 1	Waters 2690 Separation module Waters 474 Scanning Fluorescence Detector Symmetry C18-columm (3,9x150 mm)	Waters, USA
HPLC instrument 2	Waters 2795 Separations module Waters 2475 Multi λ fluorescence detector Symmetry C18-columm (3,9x150 mm)	Waters, USA
Microcentrifuge	Biofuge pico	Heraeus Instruments, UK
Microplate multi-detection reader	POLARstar Galaxy with fluorescence filters	BMG LABTECH GmbH, Germany
Microtitre plates	NUNC MicroWell 96UPS, Unsterile	NUNC A/S, Denmark
Microtitre plate reader	Spectra Max 190 Microplate	Molecular devices, USA

	Spectrophotometer	
Microtubes	Plastibrand microtubes 1,5 mL	BRAND GmbH + CO KG, Germany
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
Rotary evaporator	Büchi Rotavapor R-124 with Büchi Vac V-500 vacuum pump system with Büchi Vacuum Controller B-721 and Büchi Waterbath B-480	Büchi, Switzerland
Scale	Sartorius LP620S	Sartorius AG GmbH, Germany
Spin column	Nanosep 3K Omega 3000 MWCO	Pall Corp., USA
Spin column	Vivaspin 6 300, 000 MWCO	Sartorius Stedim Biotech GmbH, Germany
Ultracentrifuge	Beckman L8-M	Beckman, USA
Ultraviolet crosslinker	UV 1000 Ultaviolet crosslinker	Hoefer Scientific Instruments
Vortex	MS2 Minishaker IKA	IKA Works GmbH & Co, Germany
Zetasizer Malvern	Nano series	Malvern Instruments, UK
Zetasizer capillary cells	Folded capillary cells	Malvern instruments, UK

3.1.4 Lipids

Table 3.4: Lipids used in the liposome compositions.

Chemical	Abbreviation	Charge	Manufacturer/Supplier
1,2-Di-oleyl-3-trimethylammonium-propane	DOTAP	Positive	Avanti Polar, USA
4-(Dodecyloxy)-benzoic acid	DB	Neutral	Sigma-Aldrich Chemie GmbH, Germany
Dimethyldioctadecylammonium	DDAB	Positive	Avanti Polar, USA
Egg phosphatidylcholine	EPC	Zwitterionic	Lipoid GmbH, Germany
Monomethoxy polyethylene glycol-maleimide	DSPE-PEG-MAL	Neutral	Creative PEGWorks, USA
N-(Carbonyl-methoxypolyethylenglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine	DSPE –PEG	Neutral	Lipoid GmbH, Germany

3.1.5 Solutions

Isotonic Phosphate Buffered Saline (PBS) 0.025 M

I. Potassium dihydrogen phosphate	3.40 g
II. di-Potassium hydrogen phosphate	4.35 g
III. Sodium chloride	16.00 g
IV. Distilled water	ad 2000 mL

I-III are dissolved in IV, and the pH of the solution is either adjusted to 6.0 or 7.4 with sodium hydroxide depending on if the buffered solution is going to be used for liposome preparation or retention studies. The pH is monitored using a pH-meter (Metrohm Ltd, Switzerland). The solution is then filtered through a 0.22 μm filter (cellulose acetate filters, Sartorius AG GmbH, Germany).

Bovine serum albumin

For parts of the retention studies isotonic phosphate buffered saline 0.025 M, pH 7.4 with bovine serum albumin (BSA) was used. This was made by the addition of 1.00 g BSA per liter PBS.

Phosphate Buffer 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 of III, before II is added to the solution. The rest of III is added up to 1000.0 ml, before the solution is filtered through a 0.22 μm filter (cellulose acetate filter, Sartorius AG GmbH, Germany).

Triton-Solution 5% (w/w)

I. Triton X-100   5.0 g

II. PB 0.025 M pH 3.0   ad 100 g

I is dissolved in II.

Mobile Phases for HPLC-analysis

Mobile Phase A (75 %TEAA/25 % acetonitrile)

I. Acetonitrile   250 mL

II. Filtered TEAA buffer   750 mL

I and II are mixed together, and the pH of the mixture is then adjusted to 5.99.

Mobile Phase B (65 %TEAA/35 % acetonitrile)

I. Acetonitrile   350 mL

II. Filtered TEAA buffer   650 mL

I and II are mixed together, and the pH of the mixture is adjusted to 6.32.

Mobile Phase C (95 % acetonitrile/ 5 % water)

I. Distilled water   50 mL

II. Acetonitrile   950 mL

I and II are mixed together.

3.2 Methods

3.2.1 Preparation of Liposomes

A 2 mg/mL stock solution of CPT in chloroform:methanol (4:1 volume ratio) was prepared. The 25 mg/mL stock solutions of DOTAP and DDAB in chloroform were used as received from the manufacturer. For all other lipids except for DB as described below, stock solutions were prepared at 100 mg/mL. The volume of each stock solution required to produce the desired formulation (Table 3.5) was then taken from the prepared stock solutions and mixed together in a round bottom flask. For liposome formulations containing DB, the required amount of DB was weighed out using an analytical scale (Sartorius AG GmbH, Germany) and added to the round bottom flask containing the remaining dissolved lipid components for the formulation. CPT stock solution was added to reach a final concentration of 1 mg/130 μmoles lipid.

Table 3.5: Lipid composition of the different liposome formulations given in mol %.

Lipid Components / Liposome Formulation	EPC	DOTAP	DB	DDAB	DSPE-PEG	DSPE-PEG-MAL
EPCPEG	99 %	-	-	-	1 %	-
DTPEG	79 %	20 %	-	-	1 %	-
DB	75 %	-	25 %	-	-	-
DBPEG	74 %	-	25 %	-	1 %	-
DBDTPEG	54 %	20 %	25 %	-	1 %	-
DDABPEG	74 %	-	-	25 %	1 %	-
EPCPEGMAL	99 %	-	-	-	0.5 %	0.5 %
DTPEGMAL	78 %	20 %	-	-	1 %	1 %

The organic solvents in the solution of CPT and lipids were removed using a rotary evaporator with a vacuum pump system (Büchi, Switzerland). The solvents were removed from the solution at a pressure of 200 mbar for 45 minutes, in a water bath at 50 °C. The lipid film was then removed from the water bath and the pressure reduced to 50 mbar for 3 hours.

The resulting dry lipid films were rehydrated with PBS pH 6 at room temperature. The lipid concentration of the resulting liposome dispersion was 130 μmol/mL, which corresponds to 100 mg/mL for 100 % EPC. A vortex (IKA Works GmbH & Co, Germany) was used to ensure that the films had been completely rehydrated and that all the lipids were detached from the glass of the round bottom flask.

3.2.2 Size Reduction

Direct probe sonication was used to reduce the size and the lamellarity of the liposomes in the dispersions after rehydration. The liposome dispersions were sonicated in round bottom flasks on ice using a probe sonicator with a 19 mm needle probe tip (Sonics and Materials, USA) and 40 % output. Sonication cycles were set to 2 minutes, with 10 minutes cooling of the probe and liposome dispersions between each cycle. The liposome formulations without DB and DDAB were sonicated with the number of cycles described in the method established by Naderkhani [22]. For liposomes composition with DB or DDAB the sufficient number of sonication cycles needed to reach the appropriate size had to be established during the study by confirming the liposome size with PCS-analysis. After sonication and size determination, the liposome dispersions were stored in the refrigerator overnight at 4 °C to equilibrate.

3.2.4 Size Determination

Size determination of the liposomes was performed using a Submicron Particle Sizer, model 370 (Nicomp, USA). The instrument uses photon correlation spectroscopy (PCS) to determine the size distribution of the liposomes. For the analysis and sample preparation the method and parameters described earlier [42] was used with a few modifications.

Parameters used:

- 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: 2
- Run time: 15 minutes

3.2.3 Ultracentrifugation

After the liposome dispersions had been allowed to equilibrate overnight, they were ultracentrifugated in 3 ml thick wall polycarbonate centrifuge tubes with a Optima L8-M ultracentrifuge and a SW60Ti rotor (Beckman, USA) at a speed of 32 000 rpm (100 000 g), for 30 minutes at 10 °C. Liposome formulations containing DDAB were ultracentrifugated at 25 °C due to a temperature related change in the viscosity of the formulations that made the separation process during ultracentrifugation at 10 °C inadequate.

The ultracentrifugation separated CPT loaded liposomes from liposome aggregates, any titanium particles from the sonication probe and CPT crystals, giving a pellet and a supernatant.

Subsequently the supernatants were separated from the pellets and used in further size determination, incorporation and retention studies.

3.2.5 Conjugation of BSA to Liposomes

In search for a suitable method for the attachment of antibodies to the liposome surface, BSA was used as a substitute for the antibodies. Both BSA labeled with fluorescein isothiocyanate (FITC) and unlabeled BSA were used in the method development.

3.2.5.1 Labeling of BSA with Alexa Fluor 594

The unlabeled BSA was labeled with Alexa Fluor 594 (AF594) by incubating 50 µL of 2 mg/mL BSA stock solution with 30 µL 1 mg/mL stock solution of amine reactive AF594-carboxylic acid succinimidyl ester for 3 hours at room temperature.

After incubation the excess dye was separated from the proteins using a pre-packed PD-10 desalting column (GE Healthcare Bio-Sciences AB, Sweden). The column was first equilibrated with 20 mL PBS pH 7.4. The volume of BSA + AF594 was raised to 1 mL by addition of 920 µL PBS pH 7.4, before the sample was added the column. After the protein sample had entered the column completely, 2 mL PBS pH 7.4 was added bringing the colored

BSA to the bottom of the column. The collected 3 mL of eluent was then discarded before 1 mL PBS pH 7.4 was added to the column and the colored BSA was collected as the eluted protein. The presence of BSA in the eluent was confirmed using a protein assay (Bio-Rad Laboratories, USA).

3.2.5.2 Conjugation of BSA

Conjugation of BSA-AF594 to Liposomes Containing DSPE-PEG-MAL (as described in Table 3.5)

Half of the proteins eluted above were incubated for 1 hour with 100 mM dithiothreitol (DTT), and the proteins were separated from the excess DTT using the same column methods as above.

Both the DTT treated and the non-treated proteins were then incubated for 1 hour at room temperature with liposomes formulations containing 0.5 % or 1 % DSPE-PEG-MAL, expected to react with thiol groups on the proteins to create stable bonds between the liposomes and the proteins (Figure 3.1)[43].
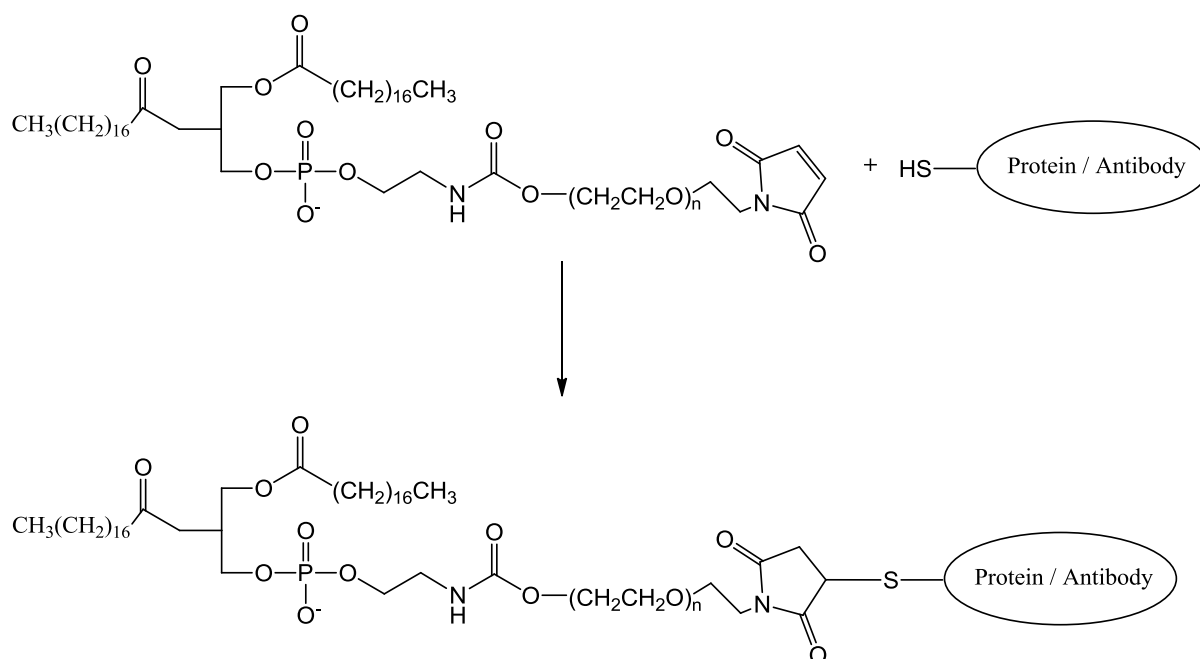
Figure 3.1: Chemical reaction between the protein/antibody and DSPE-PEG-MAL [43].

Conjugation of BSA-FITC to Liposomes Containing DSPE-PEG-MAL

BSA-FITC was incubated with liposome formulations containing 0.5 % or 1 % DSPE-PEG-MAL, and 0.5 % or 1 % DSPE-PEG for 1 hour at room temperature as mentioned above.

3.2.5.3 Purification of BSA Conjugated Liposomes

The liposomes were separated from unreacted BSA using a Vivaspin 6 (Sartorius Stedim Biotech GmbH, Germany) spin column with a molecular weight cut off at 300 000 Da and a Biofuge Stratos centrifuge with a #3047 swing bucket rotor (Heraeus Instruments, UK). Initially, the spin column was centrifuged at 10 000 g, but due to high loss of lipids the speed was later reduced to 1900 g. When the sample had been filtrated through the membrane, the column was washed three times with 500 µL PBS pH 7.4 before the retained liposomes with BSA were collected from the membrane.

3.2.5.4 Confirmation of BSA Conjugated Liposomes

The presence of BSA in the liposome samples was confirmed by measuring the fluorescence intensity. The fluorescence of the liposomes conjugated with BSA-AF594 was measured using a Perklin Elmer LS 55 fluorescence spectrometer (Perklin Elmer, UK) with the excitation slit set to 2.5 nm, emission slit set to 5 nm, excitation wave of 590 nm, emission wave of 617 nm and integration time set to 1 sec. Liposomes conjugated with BSA-FITC were analyzed using a POLARstar Galaxy microplate multi-detection reader (BMG LABTECH, Germany) with a 485 nm excitation filter and a 538 nm emission filter.

3.2.5.5 Quantification of Phosphatidylcholine in BSA Conjugated Liposomes

The presence of phosphatidylcholine was confirmed and quantified using the Phospholipides enzymatique PAP 150 kit (bioMérieux sa, France) according to the method described in section 3.3.9, Quantification of Lipid Content, below.

3.2.5.6 Quantification of CPT in BSA Conjugated Liposomes

Quantification of the amount of CPT incorporated into the liposomes conjugated with BSA was performed according to the method described in section 3.2.8, Separation and Quantification of Camptothecin, below.

3.2.6 Conjugation of EGFR Antibodies to Liposomes

Using the method developed for the labeling and conjugation of BSA as a starting point, 300 µL of the 200 µg/mL EGFR stock solution were mixed with 30 µL of the 1mg/mL AF594 stock solution and conjugated for 1 hour at room temperature. The conjugated EGFR-AF594 were then purified on a Nanosep 3K Omega (Pall Corp., USA) at 5000 g with a Biofuge pico microcentrifuge (Heraeus Instruments, UK), before it was mixed with 50 µL DTPEG-MAL and conjugated at room temperature for 2 hours. Finally the EGFR conjugated liposomes were separated from the conjugation mixture on the Vivaspin 6 (Sartorius Stedim Biotech, Germany) spin column and washed three times with 80 µL of PBS pH 7.4.

3.2.7 Retention Studies

The different liposome formulations ability to retain incorporated CPT was determined using Slide-A-Lyzer dialysis cassettes. A sample volume of 100 µL of the liposome suspension was used in each cassette.

The cassettes were placed in 300 mL PBS pH 7.4, 300 mL PBS pH 7.4 + BSA, 50 mL fetal bovine serum (FBS) + 50 mL PBS pH 7.4 or 100 mL human erythrocytes, and incubated at 37 °C for 24 hours.

10 µL samples were drawn from the cassettes at 0, 5 and 24 hours.

3.2.8 Separation and Quantification of Camptothecin

To determine the amount of CPT in the liposomes, reversed phase high performance liquid chromatography (HPLC) was used. The method is based on the work of Warner and Burke [44] with some modifications. Liposome samples were diluted 1:100 with 5 % (w/w) triton solution, which cause disruption in the liposome structure due to the presence of the nonionic surfactant. This releases the incorporated CPT from the liposomes and results in a more

accurate quantification of CPT. After sample preparation, the lactone and carboxylate form were separated on a C-18 column using gradient elution and a gradual increase in the pH and acetonitrile content of the mobile phases. The amount of CPT was subsequently quantified based on a standard curve created from CPT standards of the lactone and carboxylate form with known concentrations in the range of 0.5 μM to 5.0 μM.

Overview of the method for HPLC instrument 1:

Table 3.6: Compositions of the different mobile phases that were used.

Mobile Phase	Acetonitrile	TEAA buffer	Distilled water	pH
A	25 %	75 %	-	5.99
B	35 %	65 %	-	6.32
C	95 %	-	5 %	-

- 474 scanning fluorescence detector
- Detection Wavelengths: Excitation λ=360 nm, Emission λ=440
- Column: Waters Symmetri C18-column (3.9x150 mm)
- Injection volume: 10 μL
- Flow rate: 1.0 ml/min
- Run time: 15 min
- Column temperature: 30°C
- Sample temperature: 25°C

Overview of method for HPLC instrument 2:

The mobile phases used with the second HPLC instrument were the same as for the first HPLC instrument. The following configurations and parameters used:

- 2475 scanning fluorescence detector
- Detection Wavelengths: Excitation λ=360 nm, Emission λ=440
- Column: Waters Symmetri C18-column (3.9x150 mm)

- Injection volume: 10-20 μL

- Flow rate: 1.0 ml/min

- Run time: 11 min

- Column temperature: 30°C

- Sample temperature: 25°C

3.3.9 Quantification of Lipid Content

The amount of phosphatidylcholine in the samples was determined by the use of enzyme assays. Assays were performed using the Phospholipides enzymatique PAP 150 kit (bioMérieux sa, France).

The enzyme kit catalyzes the hydroxylation reaction between phospholipids and water by the help of the enzyme phospholipase D. Choline oxidase then oxidizes the product of the reaction, choline, yielding betaine and hydrogen peroxide. Hydrogen peroxide reacts with phenol and 4-aminoantipyrine, a reaction that is catalyzed by the enzyme peroxidase. The reaction yields the dye quinoneimine and enables the amount of phospholipids to be determined by measuring the absorbance of treated samples (Figure 3.2).

$$\text{Phospholipids} + \text{H}_2\text{O} \xrightarrow{\text{Phospholipase D}} \text{Choline} + \text{phosphaditic acid, lysophosphaditic acid, N-acylsphingosyl phosphate}$$

$$\text{Choline} + 2\,\text{O}_2 \xrightarrow{\text{Choline oxidase}} \text{Betaine} + 2\,\text{H}_2\text{O}_2$$

$$2\,\text{H}_2\text{O}_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4\,\text{H}_2\text{O}$$
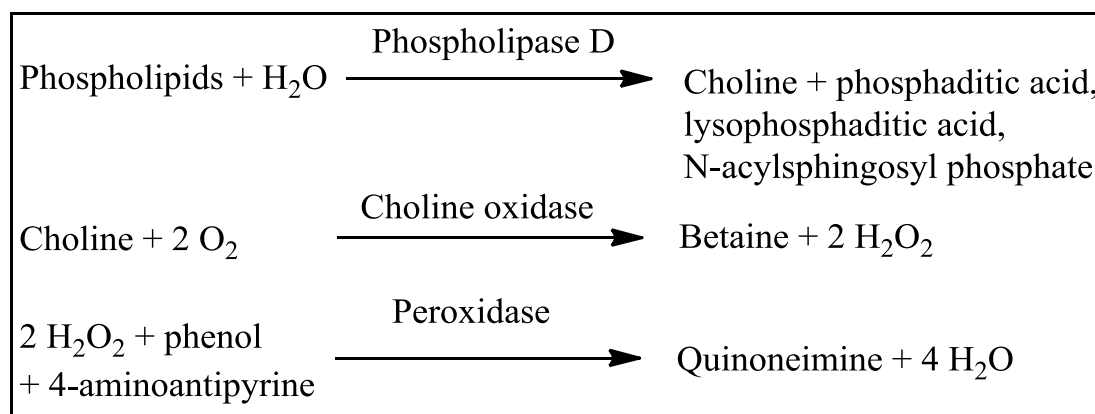
Figure 3.2: Reaction cascade that leads to the formation of the red dye in the enzyme kit (Instructions, Phospholipides enzymatique PAP 150 enzyme kit, bioMérieux sa, France).

The enzyme assays were performed in triplicates using microtiter plates (NUNC A/S, Denmark) and a suitable microtitre plate reader (Molecular devices, USA). The wells on the microtiter plates were filled with 200 μL reagent from the enzyme kit and 50 μL of the

liposome samples. When the reagent and the samples had been thoroughly mixed, the microtiter plates were incubated at 37 °C for 10 minutes before the absorbance of the assays was measured at 505 nm wavelength as specified in the instructions included with the Phospholipides enzymatique PAP 150 enzyme kit (bioMérieux sa, France).

Absorbance results for the different liposome samples were then used to calculate the recovery of phospholipids in the supernatant after ultracentrifugation (Equation 3.1) and adjust results from the incorporation studies. The supernatant phospholipid content was compared to the phospholipid content of the liposome dispersion before ultracentrifugation.

$$Phospholipid\ recovery\ = \frac{Mean\ absorbance\ supernatant}{Mean\ absorbance\ liposome\ dispersion}$$

Equation 3.1: Equation used to calculate the phospholipid recovery after ultracentrifugation.

For the quantification of the lipid content in the conjugated liposomes a standard curve was created using choline standards with a concentration of 0.25, 0.5 and 0.7 µg/µL made from the choline standard in the enzyme kit with a concentration of 3.1 g/L (4 mmol/L). The standard curve was incubated at 37 °C for 10 minutes with the rest of the liposomes samples before the absorbance was measured at 505 nm wavelength, as described above.

3.2.10 Determination of Zeta Potential

The zeta potential of liposomes is used as an estimation of the surface charge, and since the surface charge is an important liposome characteristic that among other things affects stability, it is an essential parameter to measure. The potential was measured using a Zetasizer Nano ZS (Malvern, UK).

The Zetasizer capillary cell was cleaned with 96 % ethanol and distilled water using a 1 mL syringe before analysis as recommended by the manufacturer. The liposome samples were diluted 1:10 with PBS pH 6.0 and the zeta potential measured for 10 cycles with a voltage of 4 mV.

3.2.11 Determination of Fluorescence Anisotropy

Fluorescence anisotropy is used to assess the fluidity of the liposome membrane, by measuring the degree of rotational freedom of the flouresence probe in the lipid bilayer by polarized light.

1 mM of the liposome samples were mixed with 2µM 1,6-Diphenyl-1,3,5-hexatriene (DPH) in a 1:1 ratio to reach a final volume of 4 mL. The mixture was then allowed to equilibrate in the dark, for 24 hours at room temperature. After equilibration the fluorescence anisotropy of the mixture was measured at 20 ℃ and 37 ℃ using a Perklin Elmer LS 55 fluorescence spectrometer (Perklin Elmer, UK) with the excitation slit set to 5 nm, emission slit set to 2.5 nm, excitation wave of 364 nm, emission wave of 432 nm and integration time set to 1 sec.

3.2.12 Statistical Methods

To test if the changes in the lipid composition gave significant changes in the incorporation or retention of CPT, student's t-tests for comparison of two means was performed. A significance level of $p < 0.05$ was always used. The hypotheses determined the choice of a one or two sided t-test.

4. RESULTS

4.1 PEGylated Liposomes

PEGylated liposomes have the ability to prevent the fusing of liposomes due to steric hindrance by the polymer molecule, and to prolong the circulation time in the blood vessels by evading the binding of opsonins. The prolonged circulation time of liposomes in the blood increase the chance of drug accumulation by the EPR. PEGylated liposome formulations are therefore very interesting in drug delivery of anticancer agents.

4.1.1 Particle Size of PEGylated Liposomes

The results from the PCS-analysis performed on the 5 different PEGylated formulations, probe sonicated according to Table 4.1, showed that all the formulations achieved a mean particle size < 200 nm (Figure 4.1). Furthermore, they also showed an increase in mean diameter with the addition of DOTAP, DB and DDAB to the formulation. A similar increase was seen in the number of sonication cycles needed to reach the appropriate liposome size < 200 nm for formulations containing DB and DDAB (Table 4.1).

The liposome formulation of DB without PEG was probe sonicated and analyzed to be used for comparison in relation to the incorporation studies of CPT.

Table 4.1: Number of sonication cycles for the different liposome formulations.

| Formulation	Number of Cycles (1 Cycle = 2 minutes)
DB	3
DBDTPEG	4
DBPEG	4
DDABPEG	3
DTPEG	2
EPCPEG	2
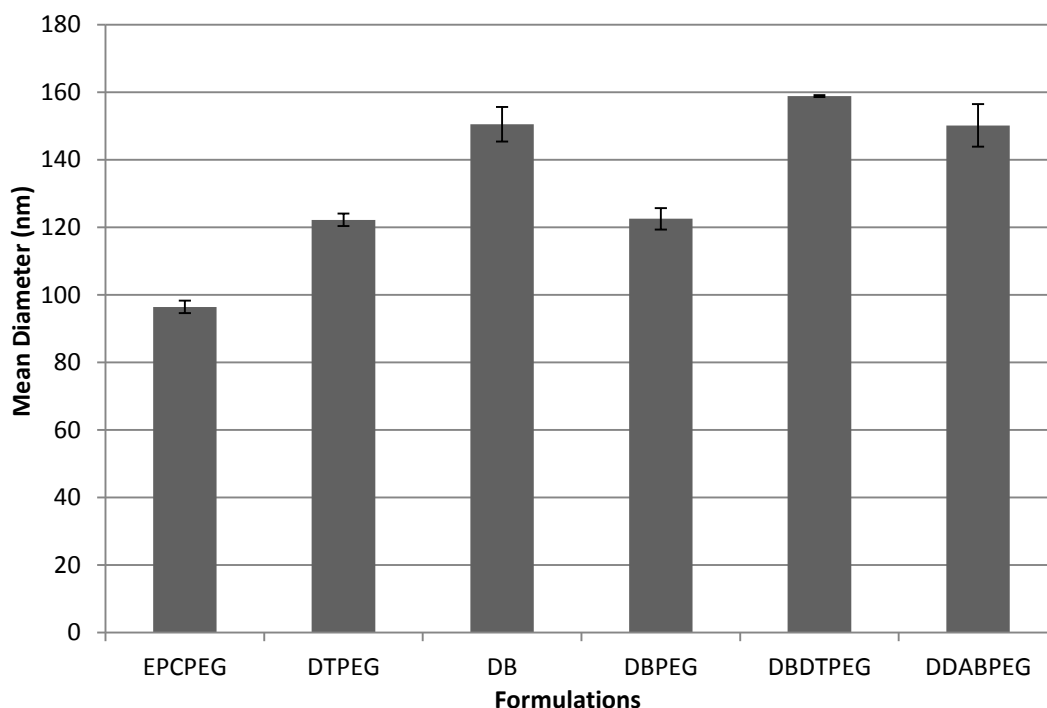
Figure 4.1: Mean particle size of the non-PEGylated DB formulation and the PEGylated formulations (n = 3).

4.1.2 Incorporation of CPT in PEGylated Liposomes

The incorporation capacity of CPT for the different PEGylated liposomes investigated in this study was determined. The results are summarized in Figure 4.2 together with incorporation data for non-PEGylated versions of EPCPEG, DTPEG and DBPEG. Based on the data presented, the inclusion of additional lipid components in the membrane of EPCPEG has a positive effect on the incorporation of CPT, with the exception of DBPEG. Amongst the four formulations with increased incorporation ability, DTPEG showed the highest incorporation of CPT. Incorporation of CPT in the DB formulation without PEG was determined for the comparison with DBPEG.
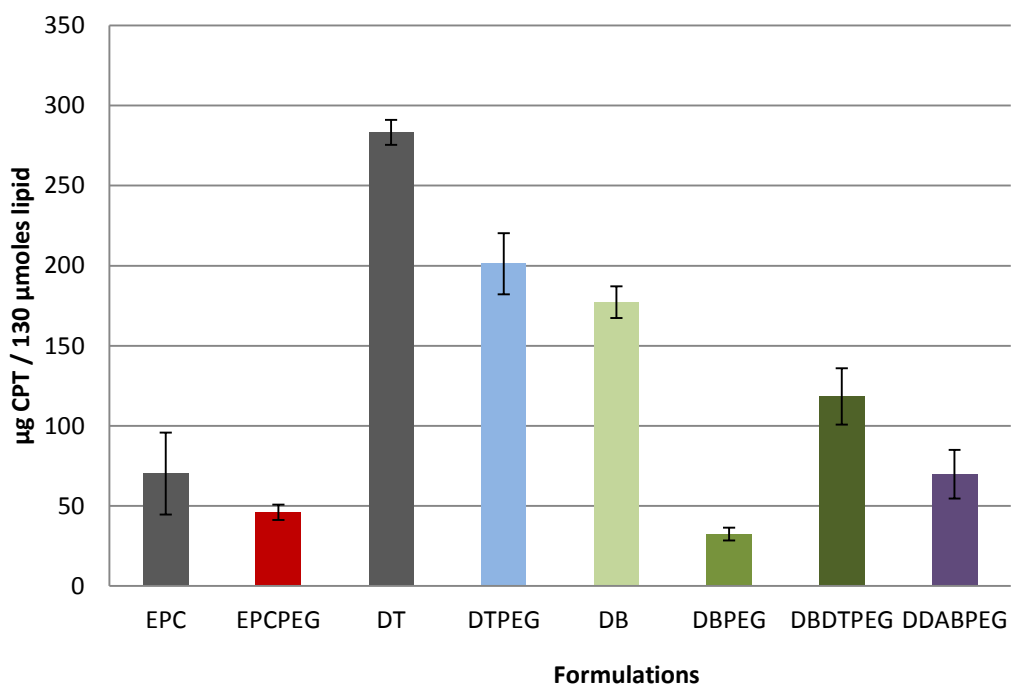
Figure 4.2: Incorporation of CPT in non-PEGylated and PEGylated versions of the different liposome formulations (n = 3). The results colored in grey are the incorporation data of the 100 % EPC formulation and the 20 % DOTAP formulation, from Naderkhani, 2011[22].

4.1.3 Retention Studies of PEGylated Liposomes

The drug retention ability of a potential drug vehicle is as important as the ability to incorporation a drug, since poor retention could translate to rapid loss of the incorporated drug before the drug vehicle even has reached its target inside the body. Consequently, the retention ability of the PEGylated liposomes also had to be investigated in different media to find the most promising formulation.

4.1.3.1 Retention Studies in PBS pH 7.4

To investigate how the different formulations were able to retain CPT over time, liposomes were first incubated at 37°C in PBS pH 7.4. Data from the retention studies is presented in Figure 4.3. Retention ability seems to increase by the incorporation of DOTAP and DB in the lipid membrane, and the two formulations with the best retention ability in PBS pH 7.4 is DTPEG and DBPEG.
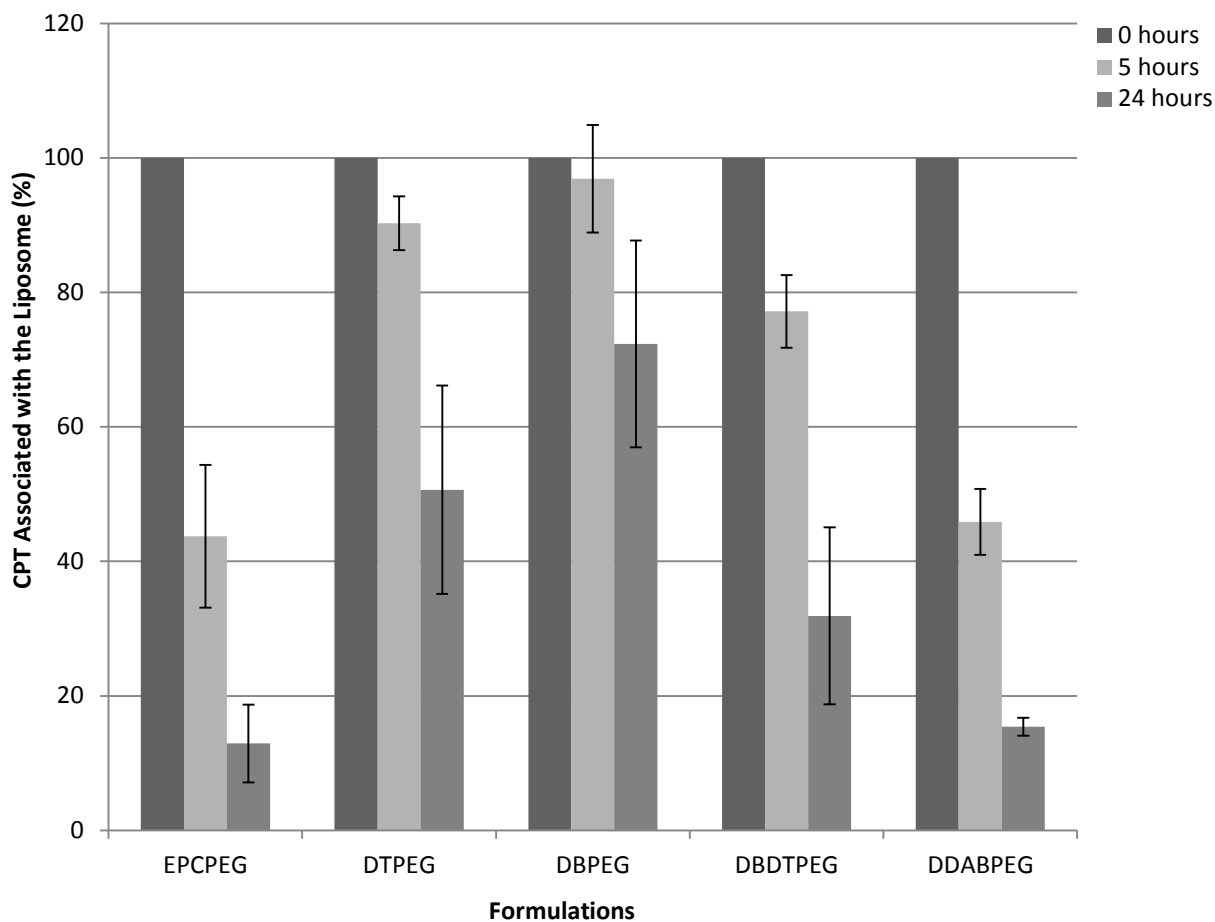
Figure 4.3: Retention of CPT in the different formulations after 0, 5 and 24 hours in PBS pH 7.4 at 37 °C (n = 3).

4.1.3.2 Retention Studies in PBS pH 7.4 with BSA

The influence of BSA on the retention ability of CPT for the different formulation was then investigated by incubating the liposome formulations in PBS pH 7.4 with 1 mg/mL BSA at 37 °C for 24 hours. Results from the studies, presented in Figure 4.4, were similar to the results from the retention studies in PBS pH 7.4 without BSA. The exceptions are the retention of DDABPEG which increased significantly at 5 hours, and the retention of EPCPEG which increased significantly at 5 and 24 hours.
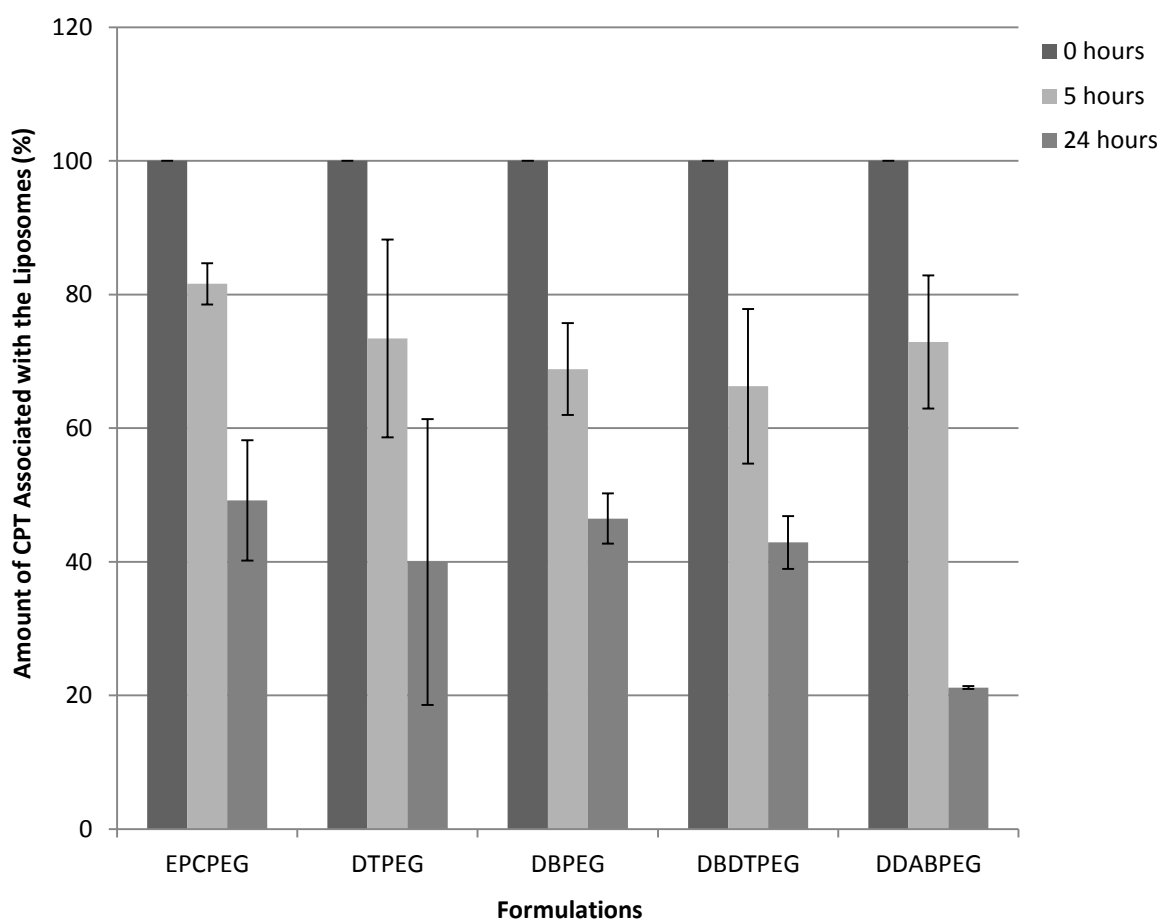
Figure 4.4: Retention of CPT in the different formulations after 0, 5 and 24 hours in PBS pH 7.4 + BSA at 37 °C (n = 3).

4.1.3.3 Retention Studies in PBS pH 7.4 and FBS

The three most promising formulations from the incorporation study and retention studies in PBS pH 7.4 and PBS pH 7.4 with BSA, DTPEG, DDABPEG and DBDTPEG, was examined further in a mixture of PBS pH 7.4 and FBS. The EPCPEG formulation was included as a control in this study. As can be seen in Figure 4.5, the retention of the DDABPEG formulation increased significantly in the presence of FBS. For the DTPEG formulation and the DBDTPEG formulation, retention was unchanged or slightly decreased in the presence of FBS compared to the result in BSA.
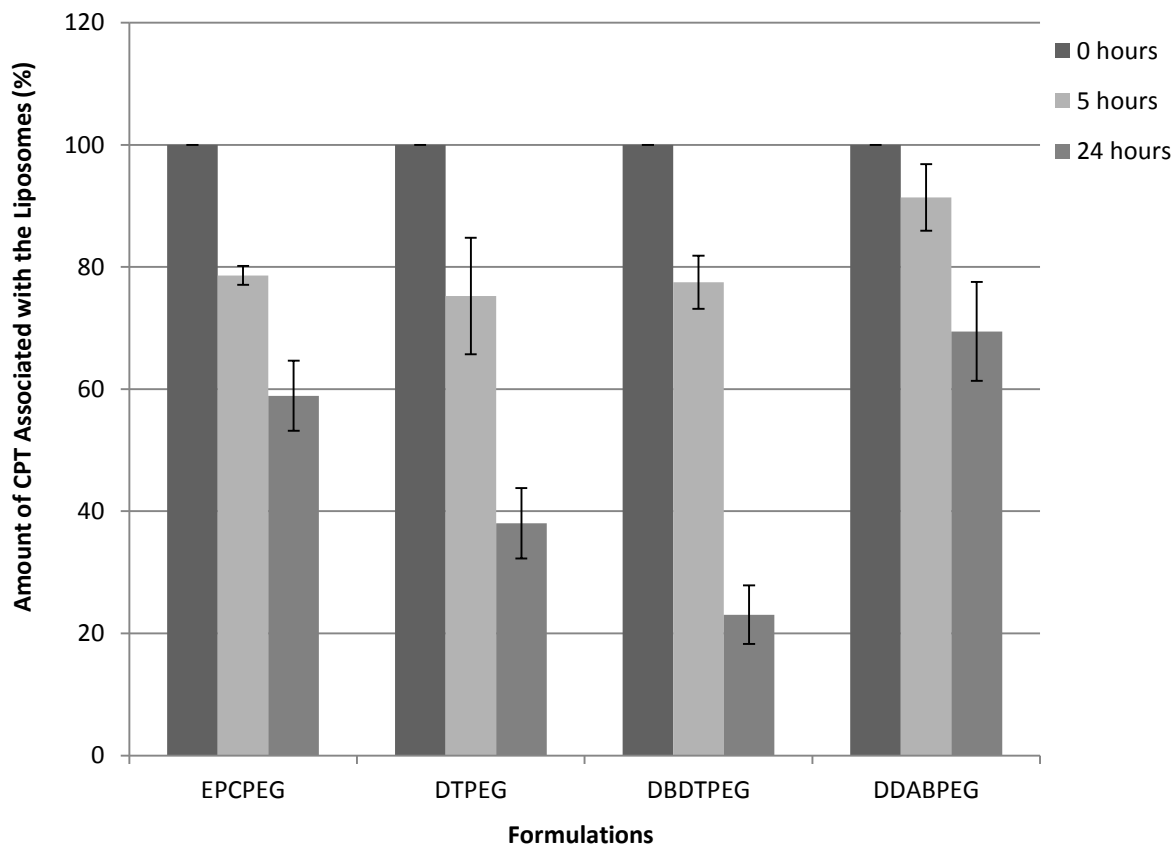
Figure 4.5: Retention of CPT in the different formulations after 0, 5 and 24 hours in PBS pH 7.4 + FBS (1:1) at 37 °C (n = 3).

4.1.3.4 Retention Studies in Erythrocytes

Based on results from the incorporation study and the retention studies in different media, the DTPEG formulation was determined to be the most promising of the five formulations investigated, and was therefore chosen as the formulation for a retention study in human erythrocytes. The results are shown in Figure 4.6, and indicate only a slight decrease in the retention after 5 hours incubation with erythrocytes compared with the results from the previous retention studies.
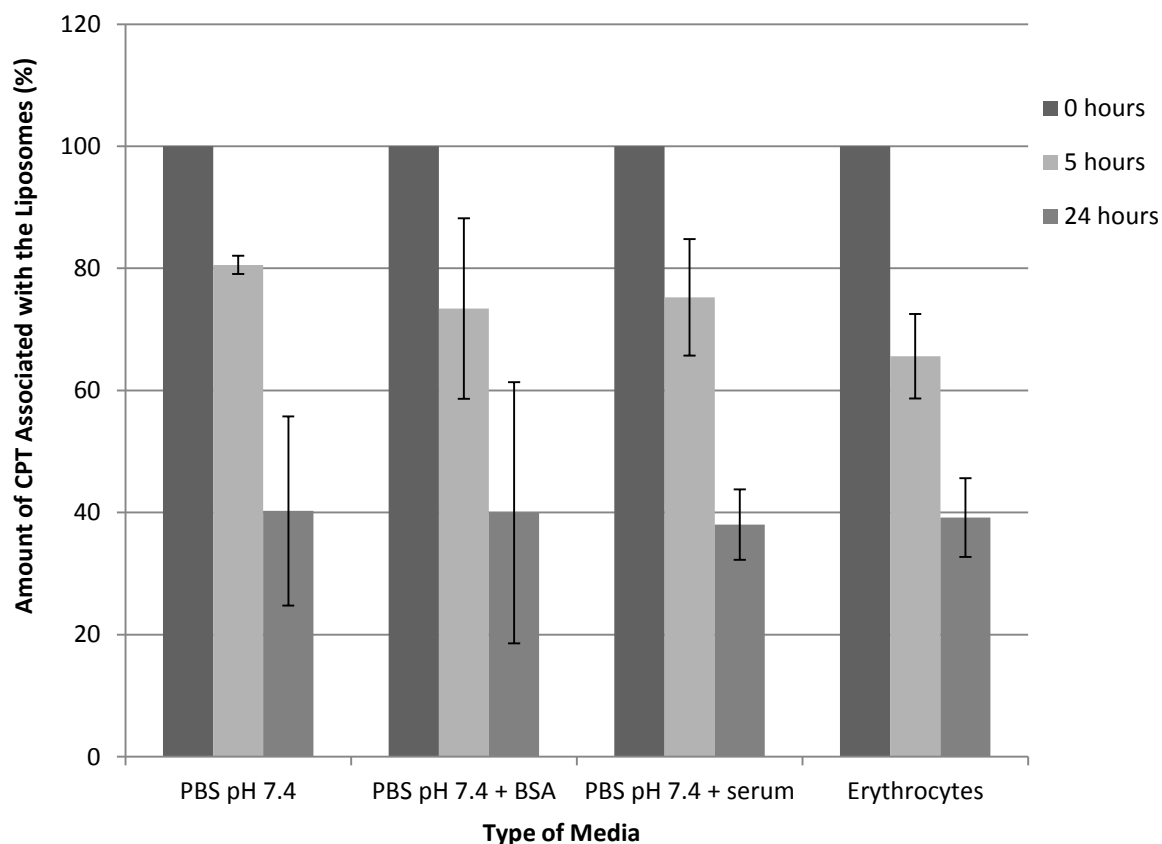
Figure 4.6: Retention of CPT in DTPEG after 0, 5 and 24 hours in PBS pH 7.4, PBS pH 7.4 + BSA, PBS pH 7.4 + FBS and erythrocytes at 37 °C (n = 3).

4.1.4 Zeta Potential of PEGylated Liposomes

The zeta potential of the different formulations was measured to find out if the incorporation of CPT in the PEGylated liposome could be influenced by the surface charge of the liposomes. Figure 4.7, show that the formulations with the cationic lipids (DOTAP and DDAB) have a significantly higher zeta potential compared to the formulations with non-charged lipids such as EPC and DB.
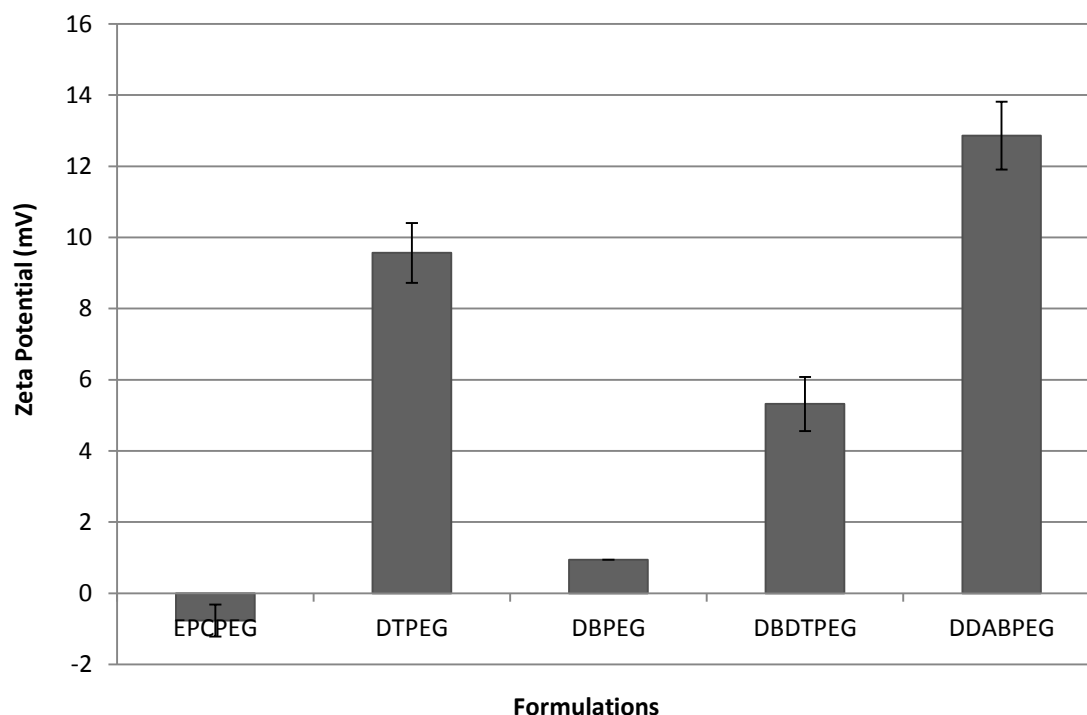
Figure 4.7: Zeta potential measured at pH 6.0 for the different formulations (n = 3).

4.1.5 Fluorescence Anisotropy of PEGylated Liposomes

The fluorescence depolarization anisotropy of the liposome formulations with DPH were measured to find out if there was any correlation between membrane fluidity and the incorporation and retention ability of CPT for the different formulations. DPH was used as the membrane probe in the measurements, because of its inherent fluorescent property and ability to equilibrate in the liposome membrane. The measured anisotropy depends on the DPH molecules degree of rotational freedom in the membrane structure as an indication of the membrane fluidity. A high degree of rotational freedom for DPH in the liposome membrane would translate to a low value of anisotropy because less of the polarized light would be conserved, and a low degree of rotational freedom would accordingly result in a high value of the measured anisotropy. The values presented in Figure 4.8 shows that the measured anisotropy of the different liposome formulation at 20˚C is approximately 0.24 for all the formulation except DDABPEG, which demonstrate a significant increase in anisotropy. However, the measured anisotropy values at 37˚C are approximately 0.2 for all formulations.
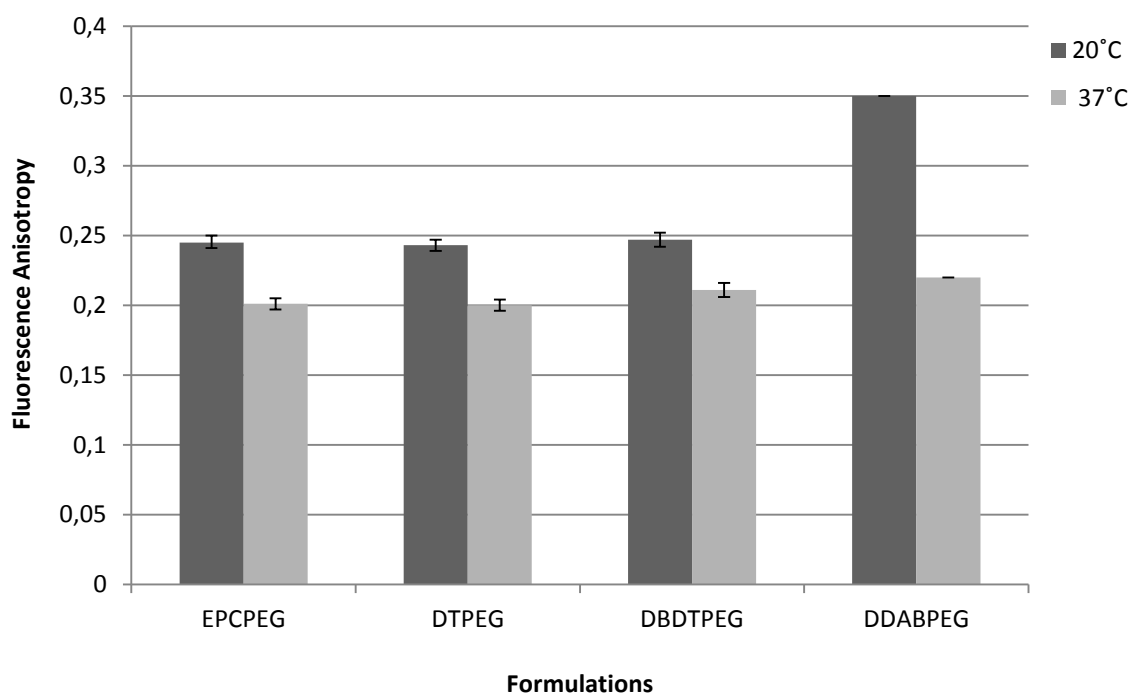
Figure 4.8: Measured fluorescence anisotropy for EPCPEG, DTPEG, DBDTPEG and DDABPEG at 20 ˚C and 37 ˚C.

4.2 Preparation of Immunoliposomes

Based on the results from the incorporation study and the different retention studies, it was determined that the DTPEG formulation was the most promising liposome formulation of the five formulations investigated. The DTPEG formulation was therefore chosen as the best candidate for the preparation of a PEGylated liposome conjugated with EGFR antibodies. Maleimide terminated DSPE-PEG (DSPE-PEG-MAL) was added to the liposome formulation so that the liposomes could conjugate in the presence of BSA/EGFR antibodies.

The conjugation method was first conducted with BSA as a test, before the liposomes were conjugated with the EGFR antibodies.

4.2.1 Particle Size of Liposome with Maleimide Terminated PEG

The mean particle size of DTPEGMAL and DTPEGMAL-BSA as determined by PCS-analysis after probe sonication for 2×2 minutes, is presented below in Figure 4.9 together with the EPCPEGMAL formulation for comparison. As seen in the figure, the mean size of

DTPEGMAL is slightly increased compared to the size of EPCPEG, while the conjugation of BSA to the liposome produced no significant change in the size measured by the PCS.
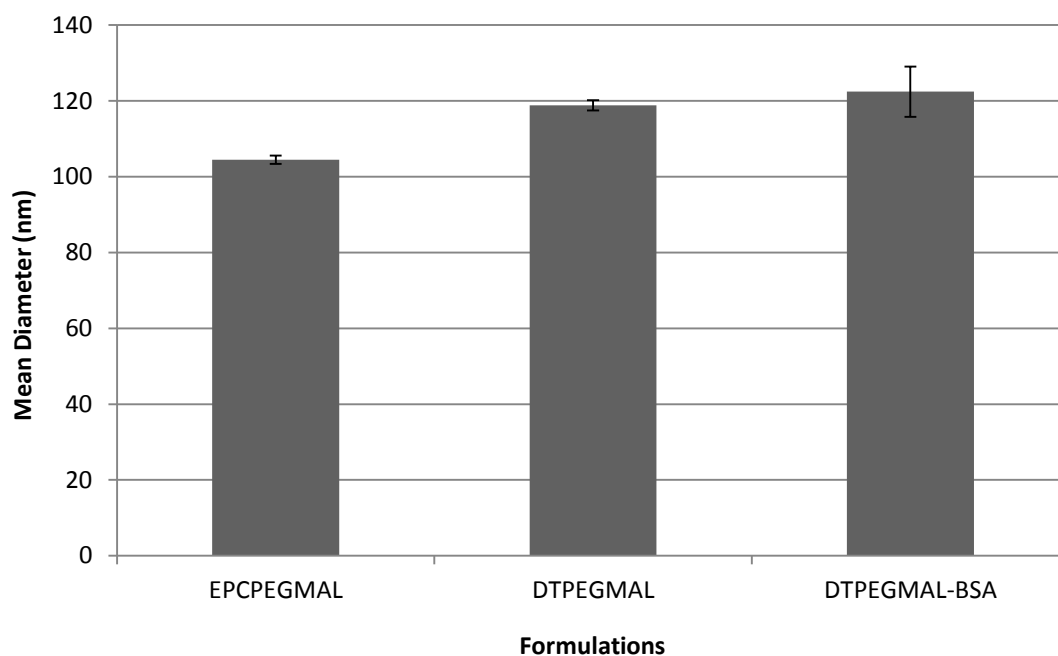

Figure 4.9: Mean particle size of the liposomes formulation with DSPE-PEG-MAL as determined by PCS-analysis (n = 3). The liposome size of the DTPEGMAL-EGFR formulation was not determined due to the small sample volume.

4.2.2 Incorporation of CPT in Liposomes conjugated with BSA and EGFR

The incorporation of CPT in the liposome formulation conjugated with BSA and EGFR was determined using the same method as for the other liposome formulation used earlier in this study. The results are presented in Figure 4.10 and show that the incorporation of CPT in the conjugated liposome formulations is significantly lower than the incorporation of CPT in the control.
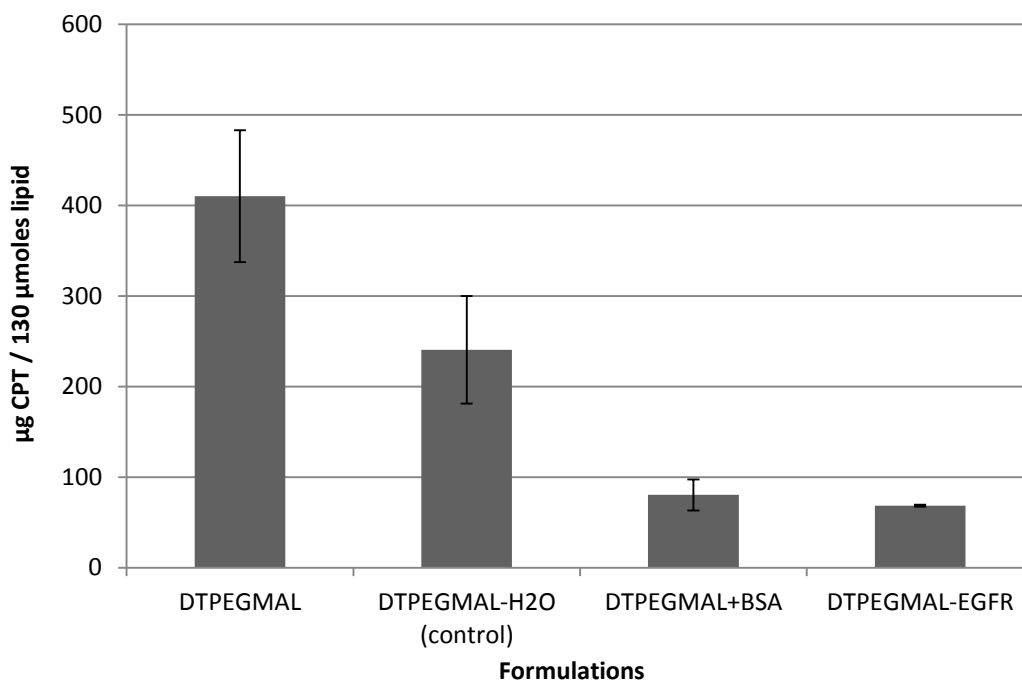
Figure 4.10: Incorporation of CPT in DTPEGMAL, DTPEGMAL-BSA and DTPEGMAL-EGFR together with control (n = 3 for all formulations except DTPEGMAL-EGFR, where n = 1).

4.2.3 Retention of Liposome Conjugated with BSA

The retention of CPT for both DTPEGMAL and DTPEGMAL-BSA was observed to see if the addition of DSPE-PEG-MAL to the formulation had an influence on the retention ability of the formulation. DTPEGMAL was observed in both PBS pH 7.4 and in PBS pH 7.4 + FBS, while DTPEGMAL-BSA only was observed in PBS pH 7.4.

Retention for DTPEGMAL is almost the same in PBS pH 7.4 + FBS as in PBS pH 7.4, except for the increase after 24 hours observed in PBS pH 7.4 + FBS, as can be seen in Figure 4.11.

DTPEGMAL-BSA demonstrated no apparent difference in retention ability compared to the control and the retention ability observed is very similar to DTPEGMAL in PBS pH 7.4, except for a marked decrease in the retention after 24 hours (Figure 4.12).
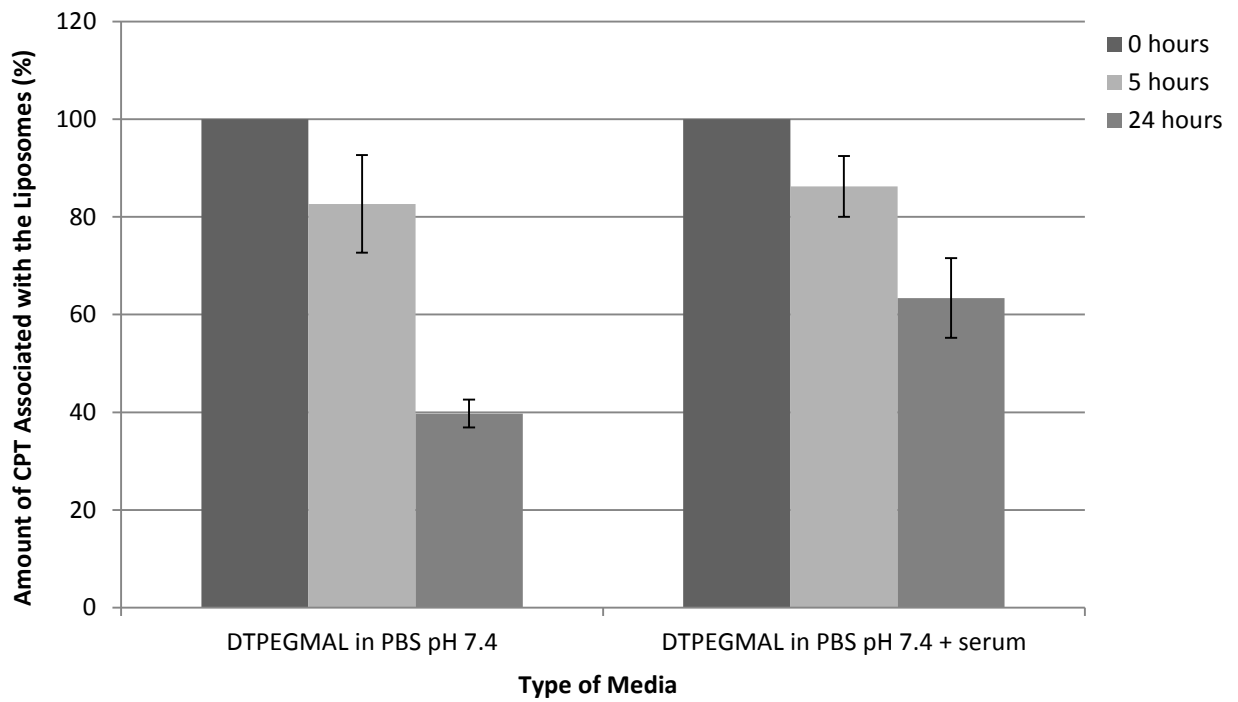
Figure 4.11: Retention of CPT in DTPEGMAL after 0, 5 and 24 hours in PBS pH 7.4 and PBS pH 7.4 + FBS at 37 °C (n = 3).
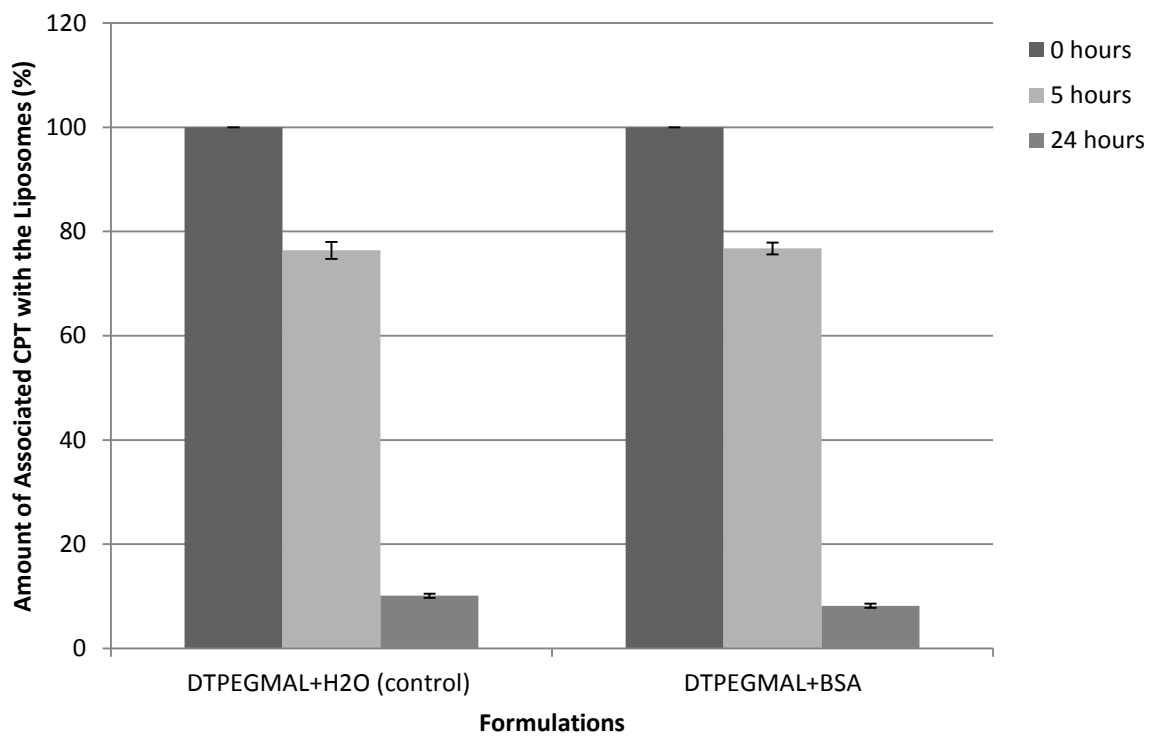

Figure 4.12: Retention of CPT in DTPEGMAL-BSA after 0, 5 and 24 hours in PBS pH 7.4 at 37 °C (n = 3).

4.2.4 Zeta potential of Liposomes Conjugated with BSA

The zeta potential of the DTPEGMAL formulation was measured before and after conjugation with BSA at pH 6.0 to see if there could be observed any change in the surface charge of the liposome after conjugation. Figure 4.13 summarize the measurements and show that the surface charge of DTPEGMAL is significantly lower after the conjugation with BSA.
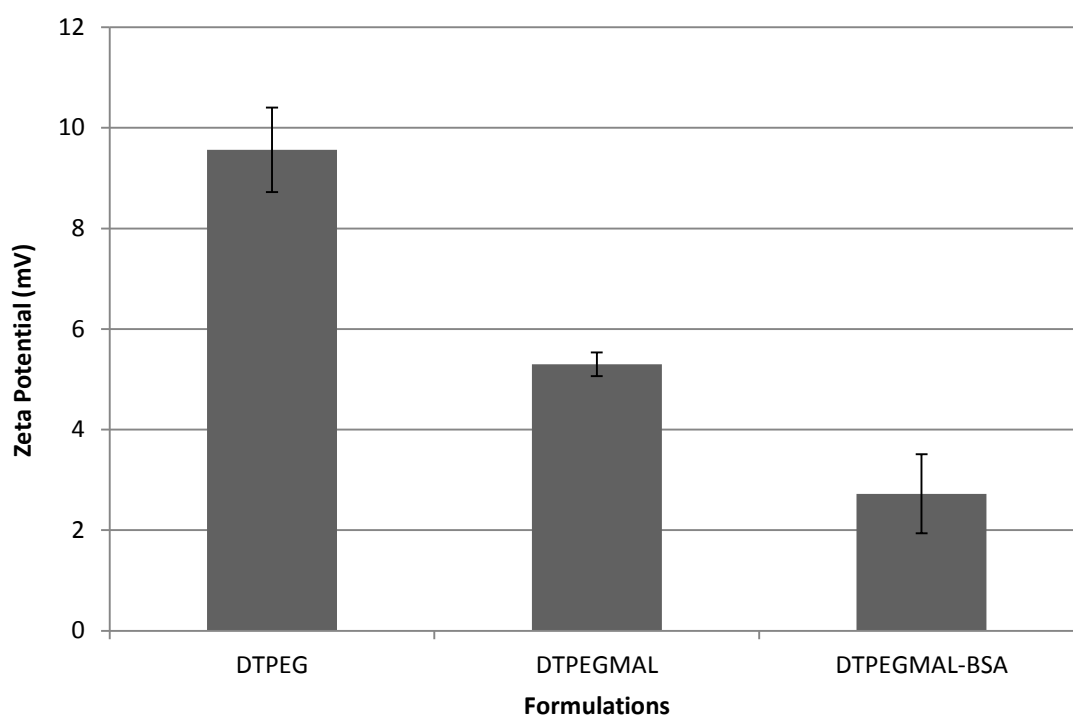
Figure 4.13: Zeta potential of DTPEGMAL and DTPEGMAL-BSA compared to the zeta potential of DTPEG (n = 3).

4.2.5 Fluorescence Determination of BSA

Fluorescence spectroscopy was used to confirm the presence of BSA labeled with FITC on the liposomes after conjugation and purification. The results presented in Figure 4.14, show that fluorescence intensity of DTPEGMAL-BSA is significantly higher than for the control.
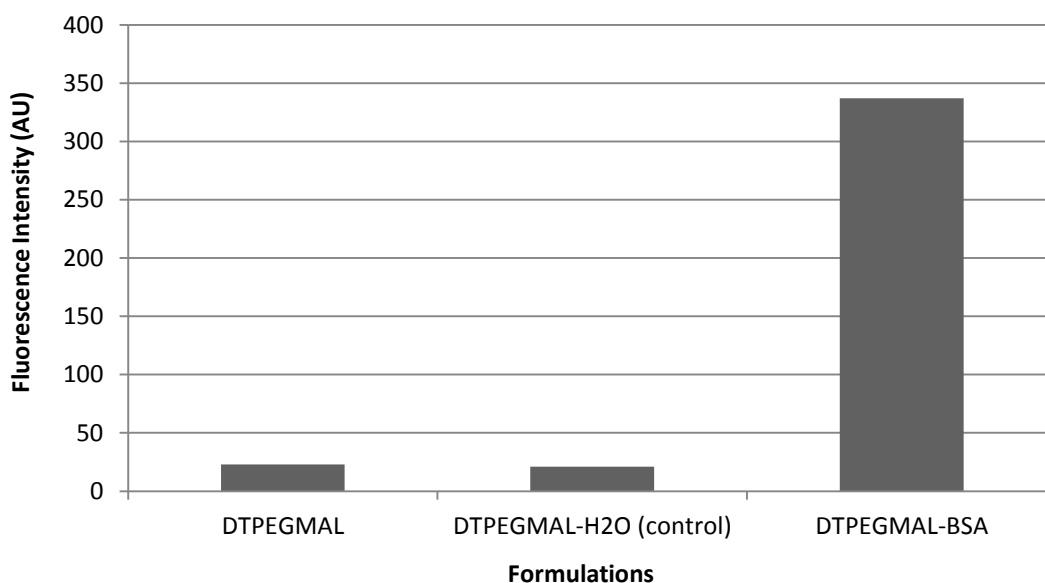
Figure 4.14: Measured fluorescence intensity for DTPEGMAL and DTPEGMAL-BSA with control (n =3).

4.2.6 Fluorescence Determination of EGFR Antibodies

The presence of EGFR antibodies on the surface of the conjugated liposomes were confirmed by measuring the fluorescence intensity of the EGFR antibodies that were prelabeled with AF594. Due to differences in both dyes and instruments used, the measured value cannot be directly compared to the results acquired from the fluorescence determination of BSA, but the result in Figure 4.15 confirm the presence AF594 labeled EGFR antibodies.
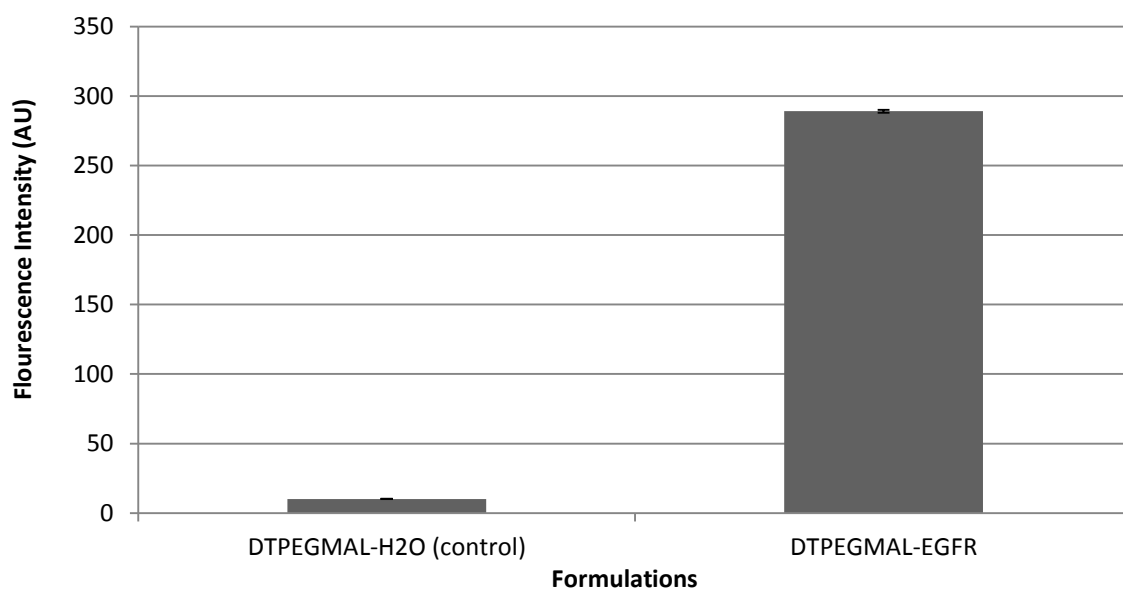
Figure 4.15: Measured fluorescence intensity for DTPEGMAL and DTPEGMAL-EGFR with control (n = 3).

5. DISCUSSION

Effect of Incorporation of DSPE-PEG in the Lipid Bilayer

In one of the previous studies involving liposome formulations of CPT, a decrease in the amount of CPT incorporated in PEGylated liposomes in comparison to non-PEGylated liposomes was observed [22]. By comparing the results from our studies with the results for the non-PEGylated versions of the different liposome formulations (see Figure 4.2), we can clearly see that the inclusion of DSPE-PEG in the liposome membrane have a negative influence on the incorporation capacity of CPT. A small still non-significant decrease in drug incorporation has also been observed for other lipophilic anticancer drugs i.e. paclitaxel upon addition of PEG [45].

The cause of the reduction in the amount of incorporated CPT in PEGylated liposomes can either be attributed to the nature of the PEG molecule protruding on the surface or its lipid anchor, DSPE, which becomes incorporated into the liposome membrane with the addition of PEG to the liposome formulations. The addition of the anchor part containing the saturated fatty acid chains steroyl could lead to a less fluid bilayer which again has been reported to decrease the incorporation of CPT in several studies [39, 46].

Effect of Incorporation of Positively Charged Lipids in the Lipid Bilayer

For the incorporation of CPT in PEGylated liposomes our study showed that the DTPEG formulation, with 20 % DOTAP and 1 % PEG had the highest incorporation of all the liposome formulations that were investigated. DOTAPs stabilizing effect in liposomes have also been observed both for CPT in another study as well as for other poorly soluble anticancer drugs e.g. paclitaxel [47, 48]. In addition to improve the incorporation capacity of lipophilic anticancer drugs, cationic liposome has recently shown to selectively target the tumor vasculature [49]. We therefore wanted to test if other cationic lipids also had this positive effect on the incorporation of CPT, and DDAB was chosen for that purpose. The formulation with DDAB demonstrated lower incorporation of CPT compared to the formulation with DOTAP, indicating that the positive charge of DOTAP's head group is not the only factor that influences the effect DOTAP exert on the liposome membrane. The zeta potential measured for DDABPEG was slightly higher than what was measured for DTPEG (see Figure 4.7). However, DDABPEG showed a higher anisotropy value than DTPEG at 20

°C indicating a more rigid bilayer, which earlier has shown to decrease the CPT incorporation [46, 49]. The reason for the lower incorporation observed in DDABPEG compared to DTPEG could be attributed to the rigidity of the bilayer. However, DDAB does not have a head group like DOTAP, which could also be affecting incorporation.

Effect of Incorporation of DB in the Lipid Bilayer

According to studies by Maitani, the addition of DB to the liposome formulation resulted in an increase in the incorporation and retention of CPT in the liposomes. The reason for this is attributed to the stabilizing of CPT in the bilayer due to π-π interactions between the phenyl group and CPT [41]. When DB was added to the formulation a more than two fold increase in incorporation compared to the EPC formulation was observed. However, when 1 % PEG was added the positive effect from the presence of DB seem to be diminished. The increase in incorporation when DOTAP is added to the formulation with DB is most probably due to the positive effect DOTAP alone has shown on incorporation.

The results from our study do not demonstrate a simple correlation between the surface charge of the different liposome formulations and the incorporation of CPT. The zeta potential seem to influence the incorporation capacity of the different formulations to certain degree, but is clearly just one of multiple factors affecting the incorporation of CPT in liposomes.

Retention of PEGylated Liposomes

The retention ability of all the formulations, except for ECPEG and DDABPEG, remained the same or decreased slightly in PBS pH 7.4 + albumin compared to PBS pH 7.4. If we compare the results from the studies performed in PBS pH 7.4 + BSA with the results from PBS pH 7.4 + FBS, a slight decrease in the retention of DTPEG and a more prominent decrease in the retention of DBDTPEG can be observed. The decrease in retention for these two formulations could be caused by interaction between the liposomes and the components present in FBS.

The anisotropy values measured at 37 °C showed no significant difference between formulations. This is in agreement with the fact that the retentions of CPT in the different formulations neither show any significant differences. Subsequently the summarized retention

results for the DTPEG did not show any significant change in retention ability in the different media, meaning that the formulation is as stable in presence of blood components as in PBS.

All the tested PEGylated formulations show similar CPT-retention properties. However, since DTPEG has an incorporation capacity of CPT that is about twice as high compared to the formulations showing the second most promising incorporation capacity and more than four times higher than the EPCPEG formulation, this is clearly the most promising formulation for further development. We therefore wanted to modify the DTPEG formulation into immunoliposomes that potentially could target receptors found on the surface of the tumor cells.

CPT Incorporation in Functionalized and Conjugated Liposomes

1 % DSPE-PEG-MAL was added to DTPEG to functionalize the formulation. DSPE-PEG-MAL's terminal maleimide groups enable conjugation to proteins through chemical coupling between the maleimide group on the liposomes and the thiol groups on proteins [43, 50]. The functionalized DTPEGMAL formulation demonstrated a marked increase in incorporation of CPT compared to DTPEG. We hypothesize that the increase in incorporation is due to a transient interaction between CPT and the terminal maleimide group on DSPE-PEG-MAL. This is supported by the fact that after the formulation went through the washing steps during the conjugation procedure (DTPEGMAL-H$_2$O (control)) an incorporation capacity similar to DTPEG was observed.

The incorporation results for DTPEGMAL-BSA and DTPEGMAL-EGFR indicate that the amount of incorporated CPT decreases during the conjugation and purification steps in manufacturing process of conjugated liposomes. Incorporated CPT is most likely lost due to leakage of CPT during the purification process and this leakage seems to somehow be connected to the presence of proteins on the surface since we do not see the same decrease in amount of CPT found in the control. Results from studies performed by Yokouchi and colleagues on the effects of adsorption of BSA on liposomal membrane characteristics, have indicated a possible hydrophobic interaction between the liposome membranes of negative and neutral liposome compositions and BSA [51]. The interaction seems to disrupt the

membrane structure and increase the permeability of the bilayer. A similar interaction has also been described for DPPG liposomes by Tsunoda and colleagues [52].

The zeta potential of the DTPEGMAL formulation at pH 6.0 was significantly lower than the zeta potential of DTPEG. This reduction in surface charge could be due to the addition of 1 % DSPE-PEG-MAL to the formulation, leading to increased neutralization of the positive surface charge. After the conjugation the potential was half of the measured value beforehand, thereby indicating the attachment of BSA, since the isoelectric point of BSA in water is 4.7 and the protein therefore confer a negative net charge at pH 6 [53]. It should be noted that a lowering in zeta potential has also shown to have a negative effect on the incorporation of CPT for some formulations.

Confirmation of Conjugated Liposomes

Already from the results from the measurements of the zeta potential there were indications that the BSA was attached to the surface of the liposomes. Moreover, the presence of both BSA and EGFR on the surface of the liposomes was confirmed by fluorescence spectroscopy of the different liposome samples. The about 300 times higher fluorescence intensity observed for the labeled formulations confirmed the successful preparation of a PEGylated liposome formulation conjugated with BSA or EGFR which gives immunoliposomes potentially targeting the EGFR-receptor expressed on colorectal cancer cells.

CPT Retention of Functionalized and Conjugated Liposomes

Retention studies performed on the DTPEGMAL formulation in PBS pH 7.4 and PBS pH 7.4 + FBS show similar results to the retention of the DTPEG formulation. The similarities in retention ability indicate that the addition of DSPE-PEG-MAL to the formulation does not influence the retention properties of the liposome significantly. However, the results from the retention study of DTPEGMAL-BSA and the control in PBS pH 7.4 demonstrate pronounced decrease in retention after 24 hours. The reason for the pronounced decrease might be due to changes in the liposome membrane, but it is difficult to identify the exact reason without doing further investigations.

The method described here successfully conjugated BSA and EGFR antibodies to DOTAP containing liposomes, however, the conjugation procedure resulted in a significant loss of the incorporated drug. In order to identify a targeting method capable of retaining liposomal potency, we also attempted a post insertion method involving the formation of DSPE-PEG-MAL/DSPE-PEG micelles. In this method BSA/EGFR was first conjugated with the DSPE-PEG-MAL/DSPE-PEG and subsequently inserted into the liposome membrane through coincubation with the liposome dispersion [54]. However, a greater loss of CPT using this method was observed. In its current state, the conjugation method does not produce immunoliposomes containing enough CPT to warrant *in vivo* testing in research animals as discussed in "Prospect Regarding the CPT Liposome Formulations for Cancer Therapy" below.

Prospect Regarding the CPT Liposome Formulations for Cancer Therapy

Free CPT has been injected into rats and mice in research settings at around 2mg/kg [55]. For a mouse of 20 g, the injected volume of DTPEG and immunoliposomes, taking the dilution from the conjugation into account, would be about 0.2 and 2 mL, respectively. The recommended injection volume in such research animals (about 0.3 mL) is therefore exceeded for the immunoliposomes while the DTPEG could be appropriate for such study. It is even possible to increase the dose/volume given to partly compensate for the loss of drug from the formulation over time and still be below both the volume and dose limit.

6. CONCLUSIONS

In this study we have investigated the incorporation capacity and retention ability of PEGylated liposome formulations loaded with CPT. We demonstrated that the PEGylation of CPT-loaded liposome formulations cause a slight decrease in the incorporation capacity of CPT, while simultaneously contributing to a more stable retention of the drug in the presence of components derived from blood

PEGylated formulations with DDAB (DDABPEG) and DB (DBPEG) demonstrated lower incorporation ability and were slightly more unstable in regard to retention of CPT compared to the PEGylated formulation with DOTAP (DTPEG). DTPEG showed the highest incorporation of CPT, which was about 2 times higher than the formulation showing the second highest incorporation (DBDTPEG) and more than 4 times higher than the PEGylated control liposomes EPCPEG. DTPEG also showed the most stable CPT retention ability in different media including medium containing erythrocytes. This formulation could also possible be suitable for further *in vivo* studies.

Regarding the targetability of the most promising liposome formulation, DTPEG, we were able to confirm successful conjugation of both BSA proteins and EGFR antibodies to the liposomes. However, our results indicate that the method developed and used for the preparation of immunoliposomes needs to be optimized further if it is to be used for the current CPT liposome formulation since leakage of drug during the process seems to be a problem.

7. FUTURE PERSPECTIVES

The liposome formulation DTPEG could possibly be appropriate for *in vivo* studies. It is even possible to increase the dose/volume given to partly compensate for the loss of drug from the formulation over time and still be below both the volume and dose limit. One approach to further optimize the formulation could be to increase the lipid concentration of the formulation and in that way increase the concentration of CPT. It has also been shown that increased lipid concentration results in higher retention of CPT upon incubation in PBS so this could possible also improve the formulation [40].

The conjugation method needs to be optimized if it is so to be used for this formulation. Possible approaches for optimization includes shortening the conjugation time, and improving post-conjugation purification of the immunoliposomes. In the purification step, free, unbound EGFR antibody is separated from the immunoliposomes. The purification or washing step is considered the governing factor affecting the CPT loss, and could be improved by switching from a spin column based washing to separating antibodies from immunoliposomes on desalting columns. The volumes used in these experiments are small, and with our current efforts, we have not been able to retain enough liposome volume using desalting columns, however, specialized smaller desalting columns has the potential to address this issue.

All formulation described in this thesis are also currently being tested in our lab in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) based cytotoxicity assays using two human cell lines, MRC-5 and HT-29. The first cell line is a non-cancerous cell line derived from human fetal lung fibroblasts, while the second line is derived from human colon adenocarcinoma. The *in vitro* IC_{50} of the different liposomal formulations are being investigated and compared to free CPT. Comparisons will also be done between cell lines to determine different effects on cancerous versus non-cancerous cells.

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