Faculty of Health Science
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# Topical liposomes treated by probe-sonication: Study on process and composition using statistical experimental design and multivariate evaluation

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Master thesis in Pharmacy, September 2015

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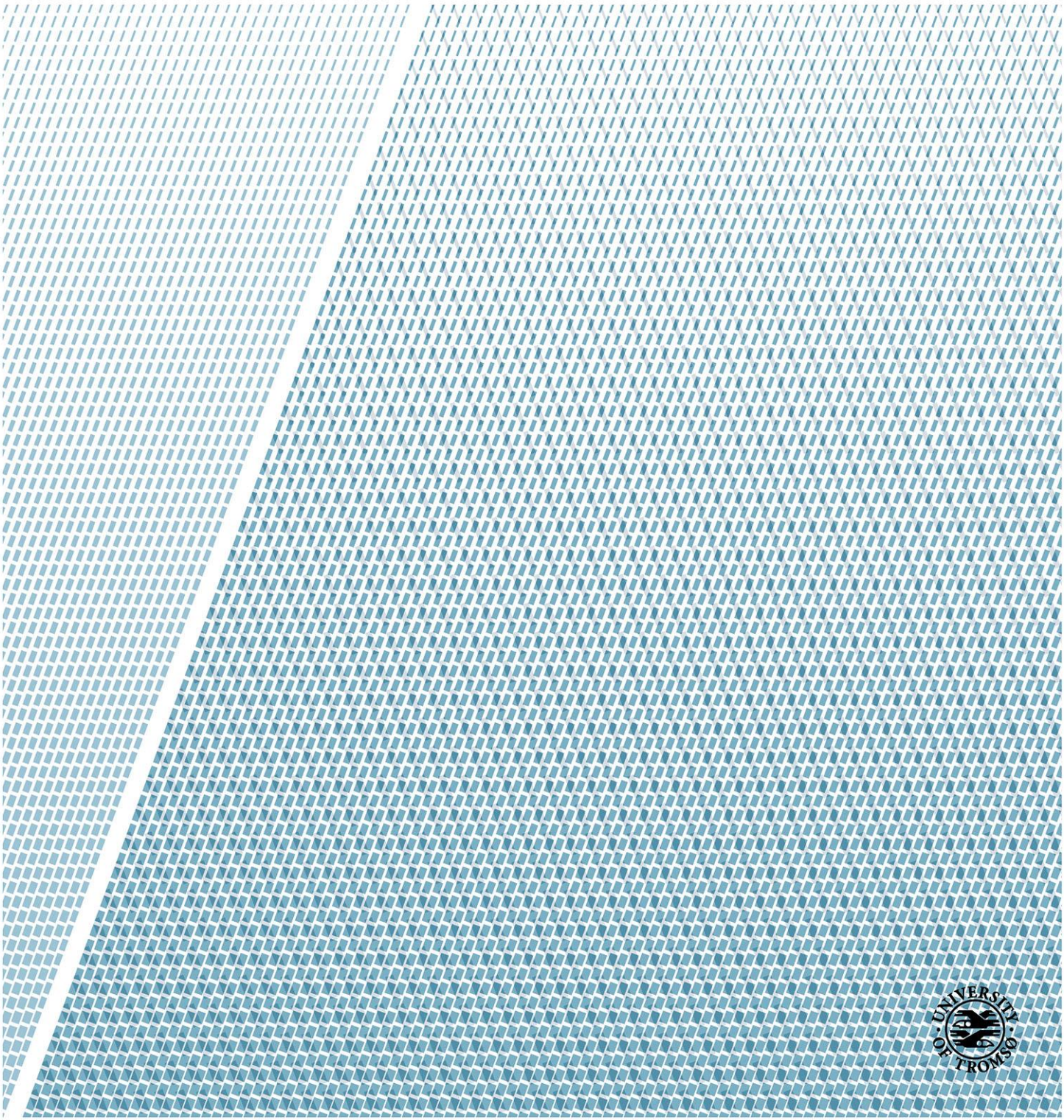

MASTER THESIS FOR DEGREE MASTER OF PHARMACY

# TOPICAL LIPOSOMES TREATED BY PROBE-SONICATION: STUDY ON PROCESS AND COMPOSITION USING STATISTICAL EXPREMENTAL DESIGN AND MULTIVARIATE EVALUATION

**By**

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**SEPTEMBER 2015**

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**TABLE OF CONTENT**

# List of Figures

# List of Tables

## Abstract

A size distribution between 200 and 300 nm and a high drug entrapment is desirable for liposomes intended for topical application. In an early phase of drug development, small batch sizes are also wanted. Dual Asymmetric Centrifugation (DAC) meets these requirement and was our method of choice. But, after successful production of a few DAC-liposome batches with drug entrapment of chloramphenicol (CAM) > 50%, vesicle size 200-300 nm, batch size approx. 500 µl, high lipid content of 40-50% (w/v), the DAC-machine got broken. Thus, probe-sonication was replacing DAC, with the following alterations: (1) The sample volume was increase from 500 µl to between 2 and 8 ml (2) Lipid concentration was reduced from semisolid VPGs to liquid dispersions; 10, 20 and 30 mg/ml lipid conc. in sonicated samples. The efficiency of the DAC method was significantly improved when adding propylene glycol (PG), reducing the needed processing time from 50 min to 2 min and 55 seconds. We therefore wanted to investigate (3) the effect of PG-concentration on the sonication method (levels of between 50 and 200 µl PG was added). Finally, (4) number of 2-minutes sonication cycles was judged critical, and between 2 and 6 cycles were tested. Empty liposomes were produced from Lipoid S-100 and variables investigated using multifactorial design on two levels (+1/-1), plus center points. The design matrix, given by the Unscramble 9.8 software, gave 19 experiments in a ($2^4$) full factorial design. The liposome size distribution varied from 55.8 to 408 nm, except one experiment giving liposome sizes of 876 nm. The ANOVA analysis suggest that within the levels of the variables investigated, sample volume is the most important variable affecting the vesicle size (p-value 0.0145), and number of sonication cycles (p-value 0.0692). Of the 19 experiments, four experiments had the aimed vesicle size of 200-300 nm, and were repeated with CAM added. CAM entrapped in sonicated liposomes was between 23 and 31%, and lower that for the DAC-liposomes (around 50%). An increase in vesicle size was observed when adding CAM for three out of four formulations (mean diameter 769, 837 and 834 nm and 67 nm). Both DAC and probe-sonication are suitable for making liposomes with the aimed vesicle size of 200-300 nm, and with acceptable incorporation efficiency of CAM. For CAM-liposomes, sonication conditions applied in this experimental matrix is too gentle and liposome size bigger than aimed for. Effect of PG on liposome size from sonication could not be demonstrated with the applied PG concentrations.

**Keywords:** Drug delivery system; liposome; probe sonication; multivariate analysis; statistical experimental design, Dual asymmetric centrifugation, Photon correlation spectroscopy

## Sammendrag

En liposom-størrelsesfordeling mellom 200-300nm og en høy legemiddelinkorporering karakteristika som er ønskelige for liposomer beregnet for topikal administrering. I de tidligste legemiddelutviklingsfasene er små batchstørrelser også ønskelig. Dual Asymmetric Centrifugation (DAC) innfrir disse kravene, og var metoden som ble valgt for studien. Etter en vellykket produksjon av et noen DAC-liposom batcher (legemiddelinkorporering av kloramfenikol (CAM) > 50%, vesikkelstørrelse på 200-300nm, batchstørrelse på ca 500 µl, høyt lipidinnhold på rundt 40 til 50% (w/v), ble DAC-apparatet ødelagt. En ny lipidstørrelsesreduksjonsmetode, nemlig «probe sonication». Følgende endringer ble ansett som nødvendig ved overgang fra DAC til «probe sonication» ble valg istede for DAC, med følgende endringer: (1) Prøvevolume ble økt fra 500 µL til mellom 2 og 8 mL. (2) Lipidkonsentrasjonen ble redusert fra vesikulære fosfolipid geler (VPGs) i DAC til flytende dispersjoner med mellom 10-30 mg/ml lipidkonsentrasjon i probe-sonikeringen. Siden effektiviteten av DAC-metoden var vesentlig forbedret ved tilsetning av propylenglykol (PG), og medførte en drastisk reduksjon av den nødvendige sentrifugeringstid fra 50 minutter til 2 minutter og 55 sekunder, ønsket vi å undersøke (3) virkning av PG-konsentrasjonen på sonikeringsmetoden (nivåer på mellom 50 og 200uL PG til liposomdispersjon). Avslutningsvis (4) antall sonikeringsrunder som varte i 2 minutter ble ansett som kritisk for den oppnådde vesikkelstørrelsen. Mellom 2 til 6 sykluser ble utprøvd. Tomme liposomer ble fremstilt fra Lipoid S-100 og variabler som ble undersøkt ved hjelp av multifaktorielt-design på to nivå (+1/-1), i nivåene som ble beskrevet ovenfor, i tillegg til midtpunktene. Den resulterende designmatrisen som angitt av Unscramble 9.8 software, ga 19 eksperimenter i $2^4$ full faktoriell design. Liposomstørrelsesfordelingen varierte fra 55,8 til 408nm, og med et forsøk som ga en liposomstørrelse på 876nm. ANOVA analyse tydet på at det innenfor nivåene av de målte variablene er prøvevolumet den viktigste faktorene som påvirker størrelsen(p-verdi 0,0145), og antallet sonikeringssykluser(p-verdi: 0,0692). Fire av forsøkene hadde det tilsiktede vesikkelstørrelsen på 200 til 300nm, og var gjentatt med CAM. CAM inkorporert i sonikerte liposomer var mellom 23 og 31%, og var lavere enn for DAC-liposomene (omtrent 50%). Dette kan forklares med den høyere lipidkonsentrasjonen i DAC prøvene. Det ble observert en signifikant økning i vesikkelstørrelsen når man legger til CAM i 3 av fire formuleringer (gjennomsnittsdiameter 769, 837 og 834 nm, og 67nm). Inkoporeringen av CAM inn i det ytre bilipidlaget og en mer rigid membranstruktur kan forklare den økte vesikkelstørrelsen. Både DAC og probe-sonikeringen har vist seg som egnede metoder i

fremstillingen av liposomer med den ønskede vesikkelstørrelsen på 200-300nm, og med en akseptabel inkorpoeringseffektivitet av CAM. For CAM-liposomer, er sonikeringsbetingelser som ble brukt i dette forsøksmatrisen for skånesomme og liposomstørrelse er større enn den ønsket størrelsen. Effekten av PG på liposom-størrelse fra sonikeringen kunne ikke påvises med de brukte PG-konsentrasjonene.

**Nøkkelord:** Drug delivery system; liposome; probe sonication; multivariate analysis; statistical experimental design, Dual asymmetric centrifugation, Photon correlation spectroscopy

## List of Abbreviation

CAM Chloramphenicol

DLS Dynamic light scattering

EE % Entrapment efficiency

GB Glass beads

HPLC High performance liquid chromatography

LUV Large unilamellar vesicles

MLV Multilamellar vesicles

MVV Multivesicular vesicles

PA Phosphoric acid

PC Phosphatidylcholine

PCS Photon correlation spectroscopy

PE Phosphatidylethanolamine

PG Propylene glycol

PI Phophatidylinositol

PL Phospholipid

PS Phosphatidylserine

RR % Drug Recovery

SD Standard deviation

SUV Small unilamellar vesicles

ULV Unilamellar vesicles

UV Ultraviolet

## 1. Introduction

Liposomes, or lipid vesicles, are formed when membrane-forming lipids, such as phospholipids, are dispersed in aqueous media (Vemuri and Rhodes, 1995). Liposomes are proven suitable for drug incorporation and sustained drug delivery for systemic application and intravenous administration, but also for topical use. When administrated topically, liposome composition and physiochemical properties will decide the fate of the drug and the liposomal carrier (Taylor et al., 2005).

It has earlier been concluded that for deposition of drug onto the strata of the skin, and for minimal systemic drug uptake and side-effects, the optimal liposome size is approximately 300 nm in diameter (du Plessis et al., 1994). At the same time, as much as possible of the drug should be inside the liposomes to achieve the wanted sustained release effect.

In lab scale liposome production, screening different formulations, it is desirable to use production methods that are suitable for making small batch sizes (taking the high expenses of the raw material into account), easy to apply, fast, gentle, non-expensive, and easy to control. However, conventional methods usually contain several (time consuming) steps. In this study, we wanted to take a closer look at the final, and critical step in the production of liposomes, namely the size reducing step. Probe sonication is applied to reduce the size of the liposomes formed after hydration of lipids, most often hydrated from a dried lipid film, referred to as “dry film dispersion method” (Hurler et al., 2013).

### 1.1. Liposomes as drug delivery system

Liposomes are spherical vesicles that consist of an aqueous core surrounded by phospholipid (PL) bilayers (as shown in Figure 1). They are spontaneously formed when phospholipids are brought in contact with an aqueous medium. The size of the liposomes may range from tens of nanometers to tens of microns in diameter (Papahadjopoulos and Kimelberg, 1974).

Liposomes have the ability to function as drug carrier for both hydrophilic and lipophilic drugs. The hydrophilic drugs can be incorporated into the aqueous core, while the lipophilic drug can be incorporated in the phospholipids membrane (Torchilin, 2012).

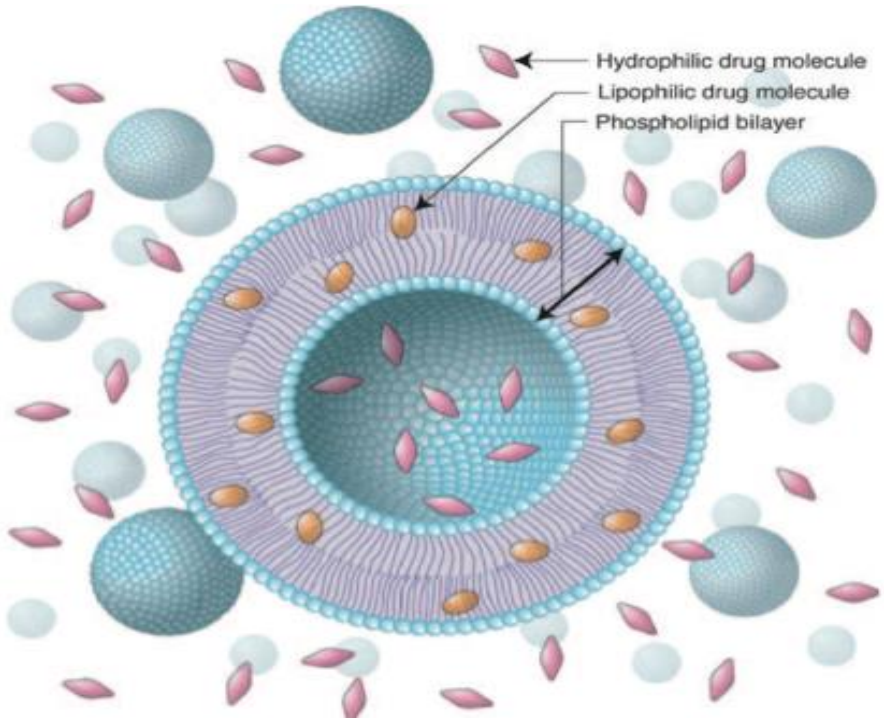


**Figure 1: Liposomes with lipophilic drug incorporated into the lipid bilayer and hydrophilic drug incorporated in the aqueous core (Figure used with permission) (Hupfeld et al., 2006).**

## 1.2. Liposomes in Topical Drug Delivery

The properties of liposomes as drug carrier system, depends on several factors; (1) the physiochemical properties of their membranes, (2) the nature of their components, (3) liposome size, (4) surface charge and (5) lipid organization (Elsayed et al., 2007). In topical application, liposomes are usually entrapped into a vehicle to achieve suitable viscosity and application properties. The size of the liposomes will influence the properties of topical liposomal dosage form (Skalko et al., 1998).

The most important obstacle for topical drug administration is the low diffusion rate of drugs through the stratum corneum. Several methods have been estimated to increase the permeation rate of drugs temporarily. One of the most promising approaches is application of drug in vesicles based on formulations (Bouwstra and Honeywell-Nguyen, 2002). The optimal liposomes size for topical administration is expected to be approximately 300 nm (du Plessis et al., 1994).

Several properties make liposomes attractive as drug delivery system, such as (Torchilin, 2005):

- Biocompatibility and non-toxicity.
- Ability to entrap both hydrophilic and hydrophobic pharmaceutical agents.
- Capability to reduce toxicity and increased stability of entrapped drug.

## 1.3. Phospholipids in Liposomes Products

Phospholipids (PL) are obtained from two major sources, soy beans and egg yolk (Brandl, 2001). When phospholipids are dispersed in aqueous medium, they have a strong tendency to form membranes. This is because their polar heads prefer to interact with the aqueous medium, whereas their long aliphatic chains encourage interaction with one another. Hydrophobic interactions, the Van der Waal forces keep the long hydrocarbon tail together when lipid bilayer is formed (Papahadjopoulos and Kimelberg, 1974).

Phosphatidycholine (PC), has the chemical structure drawn in Figure 2, is the most commonly used phospholipid in preparation of liposomes. It can be obtained from both natural and synthetic sources. PC consists of a polar group which is represented by the quaternary ammonium moiety choline, that is linked to a glycerol back bone by a phosphoric ester, the

other two hydroxyl groups of the glycerol backbone are esterified with fatty acids of different chain length and degree of saturation (number of double bonds) (Brandl, 2001).

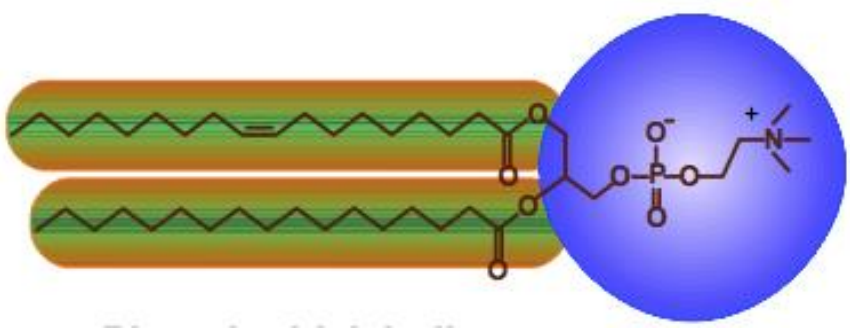

**Figure 2: Chemical structure of phosphatidylcholine (Brandl, 2001).**

The hydrocarbon chain length and the degree of saturation of the acyl chains influence the transition temperature ($T_m$), where the membrane transforms from a rigid, "gel state" to a liquid crystalline state. The fluid phase is the more flexible and permeable phase of the lipid membrane, and more suitable for transition of water over the lipid bilayer. The fluidity or mobility of the lipid layer is determined by whether and to which degree non-lipid drugs may be encapsulated into or adsorbed onto the membrane (Brandl, 2001).

**Table 1:** Overview of the most common phospholipids

| Name of phospholipid | The esterified group | Abbreviation | Net charge at pH 7.0 |
|---|---|---|---|
| Phosphatidic acid | -H | PA | Negative |
| Phosphatidylcholine | $-CH_2CH_2N^+(CH_3)_3$ | PC | Zwitterionic |
| phosphatidylethanolamine | $-CH_2CH_2NH_3^+$ | PE | Zwitterionic |
| Phosphatidylglycerol | $-CH_2CHOHCH_2OH$ | PG | Negative |
| Phosphatidylinositol | $-HC_6H_5(OH)_5$ | PI | Negative |
| Phosphatidylserine | $-CH_2CHNH_3{+}COO^-$ | PS | Negative |

Table 1 is shown the most common PLs, their most common used abbreviations, and the esterifies group that characterizes the different PLs, and also give them the net charge important for inducing electrostatic repulsion and stabilization of liposome dispersions (Ogihara et al., 2010).

PC is consisting of a phosphoric choline head group, a centerpiece of glycerin and a tail with two different fatty acid (R´= fatty acid), as illustrated in figure 1 (Hasengschwandtner, 2005).

## 1.4. Classification and Size Reduction of Liposomes

### 1.4.1. Liposomes Classification

Liposomes can be classified based on the preparation method used, the lipid composition, size and lamellarity (New, 1990, Samad et al., 2007).

According to the method of preparation liposomes may vary in their size and lamellarity (New, 1990), respectively.

- Unilamellar vesicles (ULV) – 25-100 nm in diameter.
- Small unilamellar vesicles (SUV) – they are single bilayer vesicles, around 25-50 nm in size. These are most common to use in intravenous/parenteral drug delivery due to their homogeneous in size range.
- Large unilamellar vesicls (LUV) – these are large single bilayer vesicles of more than 100 nm in diameter. They can entrap a high amount of hydrophilic drug due to their large aqueous compartment compared with SUVs.
- Multilamellar vesicles (MLV) – they consist of a large number concentric lamellar, due to their large lamellarity bilayers single bilayer vesicles, of 100 nm to several micrometer in diameter.
- Multivesicular vesicles (MVV) – more than 1000 nm in diameter.

Figure 3 gives a visual explanation on how we see the different liposomes classes, explained by words in the text above.

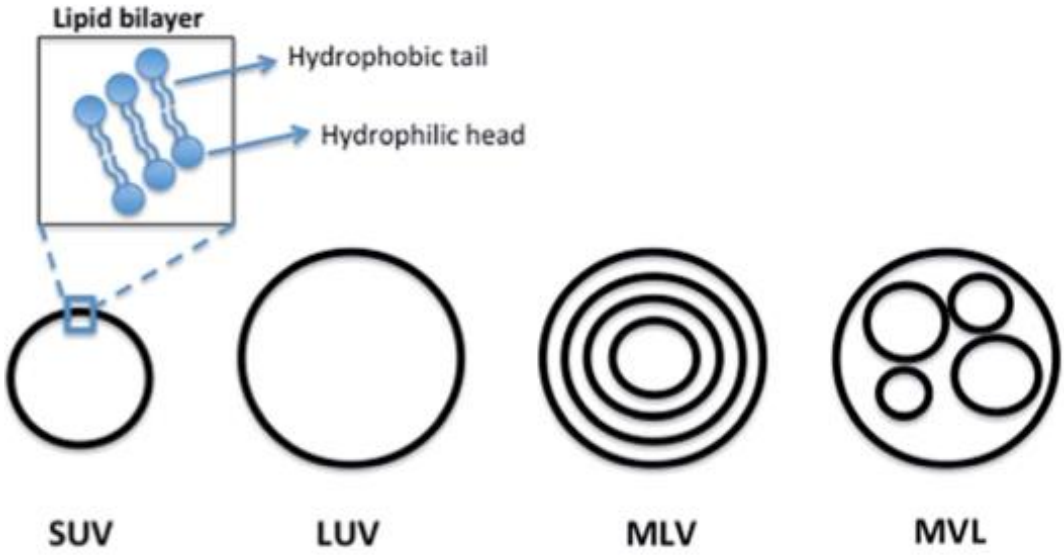


**Figure 3: Schematic representation of small and large unilamellar vesicles (SUV/LUV), multilamellar vesicle (MLV) and multivesicular vesicles (Salim et al., 2014).**

### 1.4.2. Sonication

Sonication is a simple method for size reduction in preparation of liposomes (Woodbury et al., 2006). There are two types of sonication methods; probe sonication and bath ultrasonic disintegrator (New, 1990). When high-intensity ultrasound is applied to aqueous dispersion of polar lipids it may lead to formation of small lipid vesicles (Richardson et al., 2007). The probe tip sonicator delivers high energy to lipid dispersion that may overheat the lipid dispersion and causes degradation (New, 1990). Spontaneous generation and collapse of small cavities shows when the sound waves are spreading through aqueous dispersion polar lipid with frequencies between 16 and several hundred kHz (Taylor et al., 2005).

Sonication is, due to its speed and simplicity, one of the most commonly used methods to reduce the original vesicle size to ensure the desire size, lamellarity and homogeneity properties of liposomes (Woodbury et al., 2006).

Advantages:

- Ability to produce smaller liposomes (Müller et al., 2004).
- Can be performed directly on hydrated MLVs (Taylor et al., 2005).

- Effective for reduction of large MLVs to more homogenous dispersion of SUVs (New, 1990) (Barenholz et al., 1977).

However, some disadvantages exist for the method, accordingly;

- High-energy input during sonication lead to high risk of oxidation of the phospholipids (Müller et al., 2004).
- Direct contact between sample and sonication probe cause possible metal impurities in the sample.
- Poor reproducibility (New, 1990).
- Require constant cooling (New, 1990).

Size distribution and lamellarity of liposome after sonication have been investigated in several studies (Müller et al., 2004). Frequency and power are the most important parameters of ultrasound, but the ultrasound characteristics also described to be directly dependent on power input and duration of sonication (Silva et al., 2010). When comparing the effect of ultrasound of different frequencies the faster reduction of mean size vesicle is achieved at the lower frequency (Yamaguchi et al., 2009).

### 1.4.3. Liposomes Size Reduction by Dual Asymmetric Centrifugation (DAC)

Dual asymmetric centrifugation (DAC) is method that can be used for homogenization and reduction of particle size for liposomes as previous reported by Massing and his group (Massing et al., 2008). This method is a specific kind of centrifugation method. The major difference of DAC from normal centrifugation is that the vial is turned around its own centre when normal centrifugation is processed. According to this the backward rotation of the sample lead to an overlaid agitation of the inward and outward movement of the sample (Figure 4). Due to adhesion between the sample material and the rotating vial, the centrifugation around its own centre pushes the sample in the opposite direction. DAC is performed in a sealed vial, a design that makes it suitable for sterile production of liposomes or also suitable to entrap toxic/radioactive compounds. Glass beads are used to increase the homogenization effect of the liposomes dispersion (Massing et al., 2008).

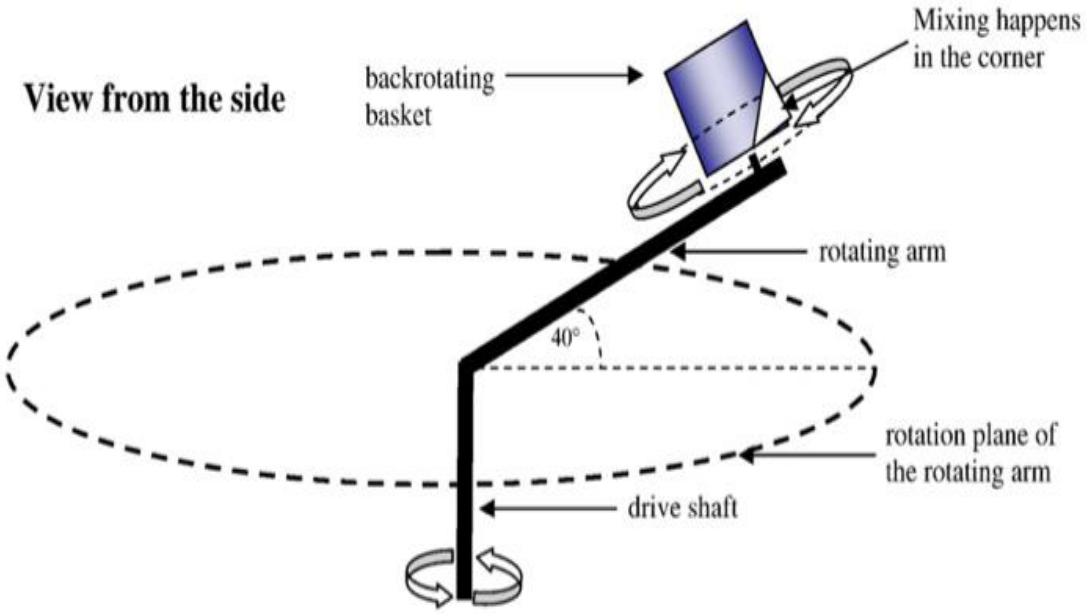


**Figure 4: Schematic picture of the principle of dual asymmetric centrifuging (Massing et al., 2008).**

The advantages of using DAC for homogenization liposomes dispersion are;

- DAC is suitable for producing small batches of VPGs and subsequently, liposome dispersion in a standard injection vial under sterile conditions.
- DAC is simple and gentle with respect to sensitive substances (Tenambergen et al., 2013).
- Reproducible method (Massing et al., 2008).
- High entrapment efficiency for hydrophilic drugs due to the same amount of water outside and inside of the liposomes (Massing et al., 2008).

Limitations might be that only viscous liposome formulations are suitable to be size reduced by DAC, as the homogenization effect depends on the sample to adhere to the rotating vial surface. Vesicular phospholipid gels (VPGs) are very concentrated liposomal dispersion, where the liposomes form a three dimensional network, and have a viscous consistence (Massing et al., 2008). VPGs therefore are the preferred liposomal intermediate applied in the DAC liposome processing method.

## 1.5. Size Analysis of Liposomes

Several techniques are used for determination of liposomes size and size distribution, such as photo correlation spectroscopy (PCS), electron microscopy, size exclusion chromatography (SEC), analytic ultracentrifugation, sedimentation field flow fractionation and small angle X-ray diffraction and scattering (Grabielle-Madelmont et al., 2003, Hupfeld et al., 2006). In this project, PCS was the method of choice.

### 1.5.1. Photon Correlation Spectroscopy (PCS)

Photon correlation spectroscopy (PCS), also called dynamic light scattering (DLS), is used for analysis of mean vesicles diameter of small-suspended vesicles, such as liposome vesicles. PCS measures the scattered light intensity at a 90° angle. The time dependent fluctuations in the intensity of scattered light, that results from vesicles (liposomes) random Brownian motion due to collisions between suspended vesicles and solvent molecules. The PCS instrument focuses laser light on sample, and it registers the movement from vesicles to solution. The analysis is based on time dependence of fluctuation. The small vesicles diffuse and move much faster than the large vesicles. Therefore; the rate of fluctuations of scattered light intensity vary accordingly (Torchilin and Weissig, 2003). PCS is a simple and rapid method to determine the particle size and size distribution of liposomes (Goldburg, 1999).

## 1.6. Chloramphenicol as Model Drug

Chloramphenicol (CAM), originally called Chloromycetin, was the first antimicrobial drug obtained from Streptyomyces species (Ehrlich et al., 1947). We applied CAM as model antimicrobial drug in this study. CAM is a neutral drug, since it contains both nitrogen and nonionic chlorine. It is stable at room temperature when stored in airtight container (Ehrlich et al., 1947). It degrades in solution due to exposure to light over longer time-period (Anderson et al., 2012). CAM has a molecular weight of 323.1 g/mol.

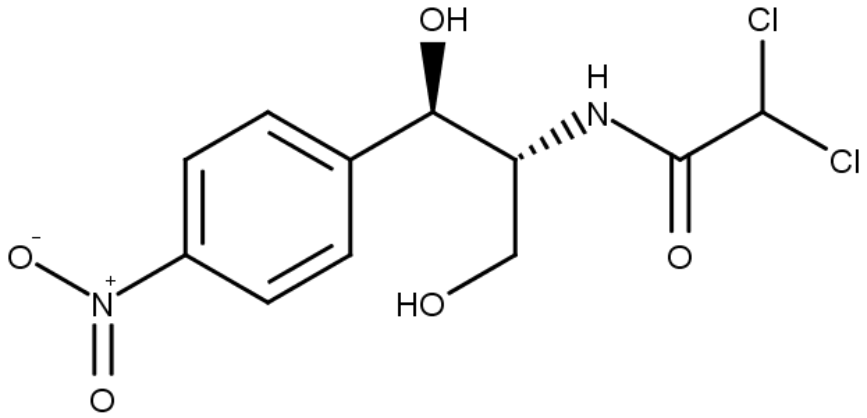


**Figure 5: Structure of chloramphenicol.**

This antimicrobial agent inhibits protein synthesis by binding to the large ribosomes subunits (50S) in microorganisms. CAM has a broad spectrum activity against both Gram positive and Gram negative bacteria, rickettsias and chlamydia (Anderson et al., 2012). CAM has effect on an array of bacteria (Helms and Quan, 2006). Although the applicability of CAM in the treatment of wound remain to be confirmed through broader clinical evaluation, preliminary results by Heal (Heal et al., 2009), indicate that a single administration of CAM ointment to suturated wounds after minor surgery procedure resulted in relative reduction in infection rate of about 40 %.

CAM was selected as a model antimicrobial drug for this study, as its low aqueous solubility represents pharmaceutical challenge when applied in topical dosage form (Anderson et al., 2012).

### 1.7. Experimental design and multivariate analysis

In fractional factorial deigns all combination factors are used to see if a change in variable could make a variation on the response or has an effect on the response. This design often gives much information as possible about the main effect of the design variables with a minimum of experiments. Due to this we were studied the main effect of the four variables and their response were investigated in this study (Esbensen et al., 2002).

By full factorial design it is enable to study the main effect of the individual variables, and all interaction between the combinations of two design variables with 2 or more factors. This study design is often used for extensive study of the effects of few variables, especially variables with more than two levels. In general the form to represent full factorial design n factors with two levels is; $2^n$ experiment. The general formula is $p^n$, where p is denoting the number of the levels and n is denoting the number of factor that is investigated with p levels. This design is permitted the study of the interaction between designs variables based on the confounding pattern of the design (Esbensen et al., 2002).

Multivariate data analysis is based on the statistical experimental principle of the multivariate statistics that involved many variables/factors and responses. It is used to perform the effects of all variables on the responses (Esbensen et al., 2002).
In this thesis we have used factorial design and multivariate analysis for the optimization studies on probe sonication of liposome to get as much information as possible out of minimum numbers of experiments.

## 2. Aim of Study

The main objective of this study was to investigate the robustness and optimize the probe sonication procedure applied for size reduction of liposomes used for topical drug delivery. Experiments were carried out using factorial design and multivariate analysis to investigating the effect of phospholipid concentration, number of sonication cycles, amount of propylene glycol added and total sample volume. Experiments with the most promising results in regard to liposome size from our design were investigated further by the addition of model drug CAM. Liposome size and drug entrapment efficiency was compared with results obtained using alternative protocols for liposome production with DAC.

# 3. Material and Methods

## 3.1. Materials

### 3.1.1. Chemicals

**Table 2: Chemicals**

| Chemicals | Purity/Quality | Manufacturer |
|---|---|---|
| Acetic acid | ≥ 99.8 % | Sigma-Aldrich, St. Louis, USA. |
| Acetonitrile for HPLC | | Sigma –Aldrich, Chemie Gmbh Steinheim, Germany. |
| Chloramphenicol | ≥ 98 % | Sigma-Aldrich, Steinheim, Germany |
| Chloroform | 99.0-99.4 % | Sigma-Aldrich, St. Louis, USA. |
| Disposable Culture tubes | | Kimble Chase, USA |
| Methanol | ≥ 99.9 % | Sigma-Aldrich, St. Louis, USA. |
| Lipoid S 100 (soybean lecithin) | ≥ 94 % phosphatidylcholine | Lipoid GmbH, Ludwigshafen, Germany. |
| Potassium phosphate monobasic | ≥ 99.0 % | Sigma-Aldrich, St. Louis, USA. |
| Propylene glycol | | NMD A/S, Oslo, Norway |
| Sodium chloride | | Sigma-Aldrich, St. Louis, USA. |
| Sodium phosphate dibasic | ≥ 99.5 % | Sigma- Aldrich Darmstadt, Germany |

### 3.1.2. Preparation of Phosphate Buffered Saline

Phosphate buffered saline (PBS) pH 7.4 was composed of 8 g of sodium chloride (NaCl), 0.19 g of potassium dihydrogen phosphate ($KH_2PO_4$) and 2.98 g of disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) were dissolved in 1000 ml of distilled water, and pH was adjusted to be pH 7.4 by using 1M HCl (Pavelic et al., 2005).

The pH is measured using a pH-meter (Metrohm Ltd, Switzerland). Further, the solution was filtered through a 0.22 µm filter (Cellulose acetate filters Sartorius AG GmbH, Germany) before use.

### 3.1.3. HPLC-mobile phase

The mobile phase consist of 55 % Milli-Q water, 45 % MeOH, and 0.1 % acetic acid. The mobile phase was filtered through a 0.2 µm pore size filter prior to analysis (pharmacopeia, August 1, 2015)

### 3.1.4. Instruments

**Table 3: Instruments**

| Equipment | Model/Type | Producer |
|---|---|---|
| Bath sonicator | Branson 5510 | Branson ultrasonics cleaner, Danbury USA |
| Büchi waterbath | Büchi B-480 | Büchi labortechnik, Flawil , Switzerland |
| Büchi vacuum controller | Büchi vacuum controller B-721 | Büchi, Switzerland |
| Dialysis membrane | Visking, Size 1, Inf. Dia.8/32, 6.3mm: 30 M (approx.) | Medicell membrane Ltd., London, UK |
| Dual asymmetric centrifuge | SpeedMixer DAC 150 FVZ | SpeedMixer DAC 150 FVZ, Hauschild GmbH & Co KG, Hamm, Germany. |
| HPLC | Water e2795 Separation module<br>Water 2489 UV/visible Detector<br>Xselect™ column C18- 2.5µm (3.0x75 mm). Software: Empower Pro, Empower3 software, 2010, Water Corporation, Milford USA. | Waters, Milford, USA<br>Waters, Milford, USA<br><br>Waters, Dublin, Ireland. |
| Biocap LAF-bench | Biocap RNA/DNA AC 230 V, 50 Hz, 73 w | Erlab, Val de Reuil, Cedex France |
| Filter | 0.22 µm non-sterile Syringe filters | Pall Life Science, USA |
| Filter | 0.22 µm cellulose acetate filter | Sartorius AG, Göttingen, Germany |
| Milli-Q filtered water | | |
| Milli-Q filter (0,22 $\mu$m) | | Merck Millipore KGaA, France |

| | | |
|---|---|---|
| Distilled water | | |
| Rotary-evaporator | Büchi rotavapor R-124 rotary evaporator with vacuum pump v-500- system | Büchi, Switzerland |
| PCS | Submicron Particle Sizer, model 370. Software: CW 388 Version 1.68, Nicomp, Santa Barbara, USA | Nicomp particle sizing system, Santa Barbara, USA |
| pH meter | 744 pH meter Metrohm | Metrohm Ltd., Switzerland |
| Probe sonicator | Sonics high intensity ultrasonic processor 500 Watt model, Needle probe 13 mm | Sonics and Materials, USA |
| Vortex Mixer | Genie 2$^{TM}$ Bender & Hobein AG | Vortex Genie, Zurich, Switzerland |

## 3.2 Preparation of liposomes

### 3.2.1. Preparation of the lipid films

Lipid films were prepared using two different film hydration methods depending on the final size reduction method applied:

#### *3.2.1.1. Lipid film for sonication*

Briefly, soybean lecithin (Lipoid S 100, from Lipoide GmbH, Ludwigshafen, Germany) was dissolved in 20 ml of methanol and the organic solvent was removed by using a rotary evaporator with a vacuum pump system (Buchi, Switzerland) (Figure 6). When preparing drug containing liposomes CAM was also dissolved together with the lipid (Appendix 2). The solvent was removed from the dispersion at a pressure of 150 mbar for 20 minutes in water bath at 45 ±1°C. The pressure was adjusted to 50 mbar for about 1 hour to remove the traces of solvent and to obtain a dry film.

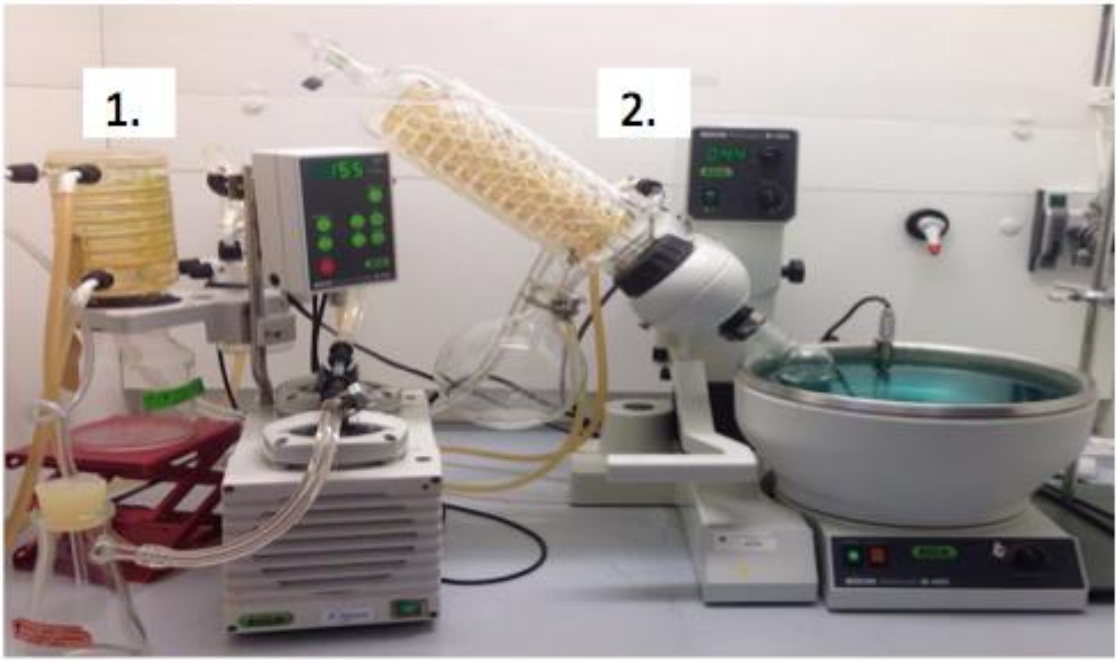


**Figure 6: Instrumentation for drying the lipid film rotary evaporator (2.) with a vacuum pump system (1.)**

#### *3.2.1.2. Lipid film for DAC*

Briefly, when making empty liposomes 200 mg lipoid S 100 was dissolved in 10 ml chloroform/methanol (2:1) in brown injection vial with 30 ml capacity. For drug containing liposomes 20 mg of CAM was added to the lipid solution. The organic solvent was removed by evaporation under a stream of $N_2$-gass for 1 ½ hour.

### 3.2.2. Hydration of lipid films

The dried lipid films were hydrated according to the procedures described under.

#### *3.2.2.1 Hydration of lipid films for sonication*

The dry lipid films were hydrated with different amount of phosphate buffer saline (PBS pH 7.4) and propylene glycol (PG), to form the liposomes dispersion. The lipid dispersion in the round bottom flask was mixed and shaken to ensure that all the lipids were evenly dispersed in the aqueous solvent. The dispersion was kept in a refrigerator at 4 °C overnight before size reduction and further characterization.

#### *3.2.2.2 Hydration of lipid films for DAC*

The lipid film for not containing PG was hydrated with 200 µl phosphate buffers saline (PBS 7.4), and 14 glass beads added and mixed on vortex for 2 minutes. For PG-liposomes 100 µl PG was added together with 200 µl PBS. The dispersion in the vial was kept in a room temperature for 1 hour, and thereafter transferred to the refrigerator (4°C) overnight prior to size reduction and further characterization.

### 3.2.3. Size reduction of liposomes

For size reduction of liposomes, two different methods were applied as described under.

#### *3.2.3.1. Size reduction by probe sonication*

Probe sonication was used to reduce the original size of liposomes and the lamellarity of the liposomes in the dispersion after hydration of the lipid film. The needle dimension of probe sonicator was 13 mm, and it was carefully placed in the centre of the glass vial containing liposomes dispersion, expecting that the position of the probe may influence the ability to minimize the vesicle size (Antimisiaris et al., 2008). The liposome containing glass vial was immersed into a ice-bath for cooling, and the intensity was at 40 % amplitude. Sonication cycles time was standardized to 2 minutes, with 2 minutes resting time on ice-bath between

each cycle, to reduce the risk for lipid degradation. The liposomes dispersion was kept overnight in refrigerator at 4°C before the characterization.

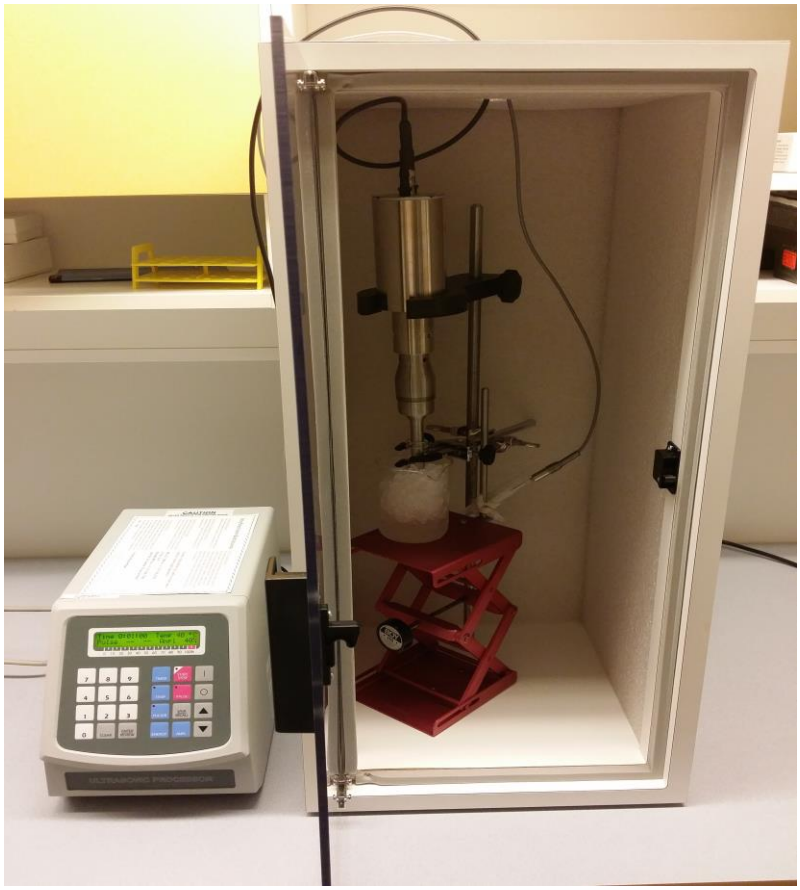

**Figure 7: Picture of the probe sonicator applied in this study.**

#### *3.2.3.2. Size reduction by DAC*

The dispersion was kept at a room temperature before homogenization of liposomes by using a DAC (SpeedMixer DAC 150 FVZ). The liposome dispersion without PG was run for 50 minutes and liposomes dispersion with PG was run for 2 minutes and 55 seconds at 3500 rpm.

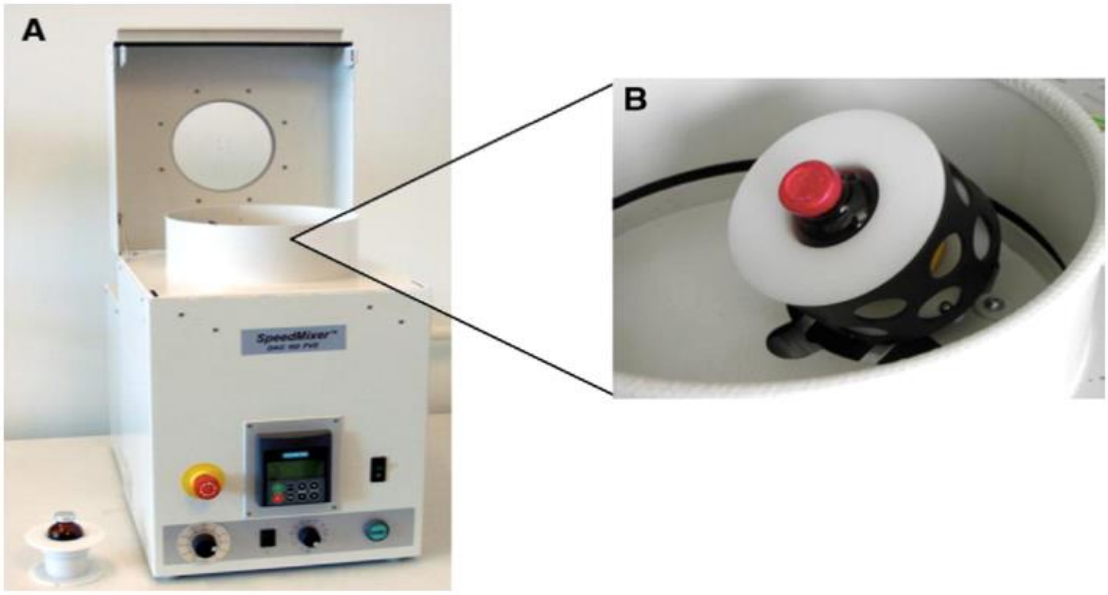


**Figure 8: schematic illustration of Dual asymmetric centrifuge (DAC) (Massing et al., 2008).**

## 3.3. Characterization of Liposomes

### 3.3.1. Drug entrapment and recovery

To determine the entrapment efficiency for CAM, the liposomally unentrapped CAM was separated from the free drug by dialysis. Dialysis was performed in dialysis tubing. The dialysis tube (Size 1, Inf. Dia. 8/32, 6.3 mm: 30 M Medicell, Membrane Ltd., London, UK.) was filled with CAM loaded liposome dispersion of 1 ml, and placed in a glass beaker containing 500 ml of PBS pH 7.4. The dialysis was performed for 4 hours. After dialysis, content of the dialysis bag was diluted 1:50 (V/V) and the dialysis medium (unentrapped drug) was dissolved 1:10 (V/V) in the HPLC-mobile phase.

The efficiency of drug encapsulation and drug loading of liposomes were calculated according to equations below:

**Equation 1:**

$$\text{Entrapment efficiency (EE \%)} = \frac{B}{A} \times 100$$

Where A is the quantified total amount of CAM in the liposome dispersion and in the dialysis medium, and B is amount of CAM in the dialysis bag measured after dialysis

**Equation 2:**

$$\text{Recovery (\%)} = \frac{A}{C} \times 100$$

Where A is the quantified total amount of CAM in the liposome dispersion and in the dialysis medium and C is the calculated amount of CAM that should have been present in the bag based on the weighted mass of CAM put into the formulation.

### 3.3.2. HPLC quantification of chloramphenicol

The standard curve was made from 20 mg/ml stock solution of chloramphenicol (20 mg CAM) in methanol (MeOH), by making standard solutions with concentration of 2.5, 5, 10, 25, 50 and 100 $\mu$g/ml. The standard solutions were prepared by diluting the stock solution by the mobile phase. The flow rate was set to 0.4 ml/min, and UV detector wavelength was 280 nm. The column used was XTerra$^{TM}$ RP C-18, and the temperature of the column was set to 30°C, and the temperature of the samples was set to 30°C during the chromatographic separation. The total run time was 8 min, and the volume injected was 20 µl. All standard solution was injected in triplicate to perform all analyses of the standard solution, respectively.

For quantification of CAM, HPLC was applied using the method described in 4.X.Y. As illustrated in Figure 9, the method gave a linear calibration curve, with a satisfactory linear regression line having the $R^2$-value 0.9999.

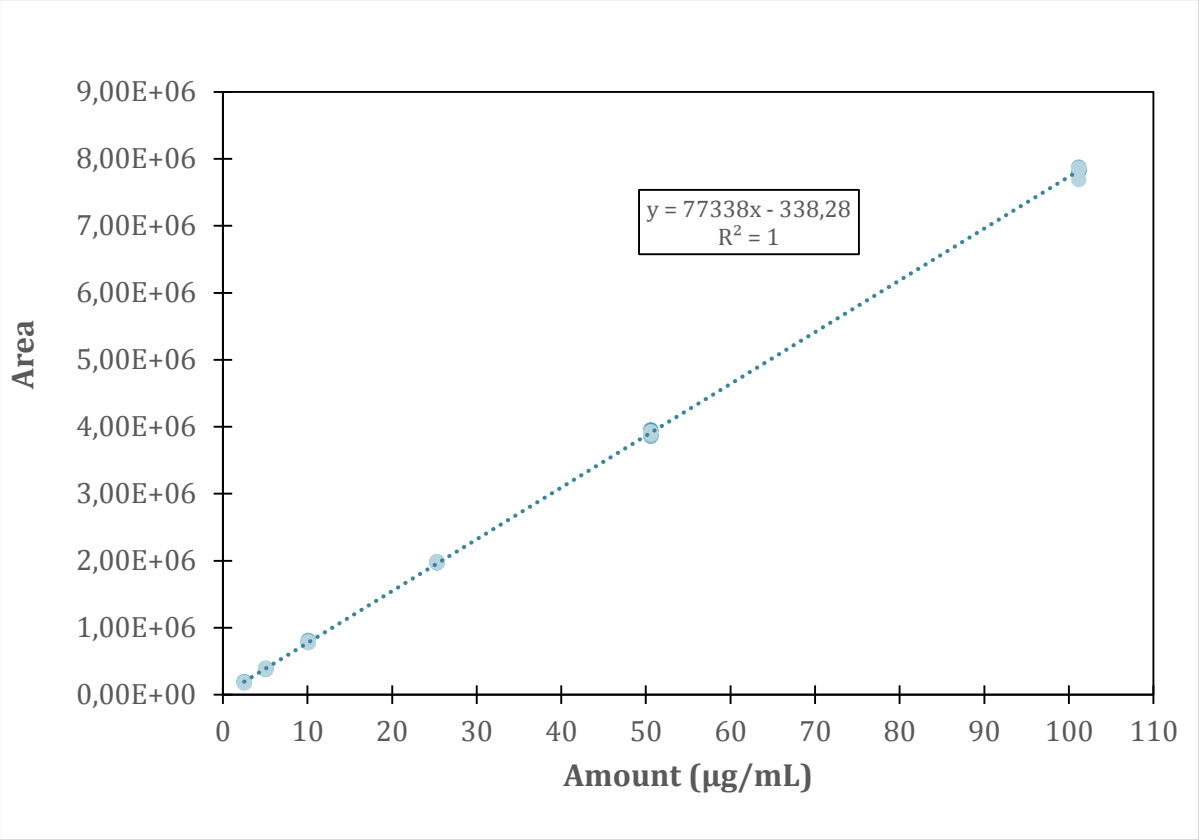


**Figure 9: The HPLC-calibration curve for chloramphenicol.**

### 3.3.3. Liposome size analysis

#### *3.3.3.1. Photon correlation spectroscopy*

The vesicle size and size distribution of sonicated liposomes were determined by Photon correlation spectroscopy (PCS) on NICOMP™ model 370 (Santa Barbara, CA, USA). The vesicles were evaluated using volume weighted results and software was in vesicle mode NICOMP distribution appeared preferable over Gaussian distribution and was used to evaluate the data, as a chi-squared value < 3.0 is preferable for using the Gaussian mode. But as also the preferable value of a fit error of < 1.5 was exceeded for NICOMP. A low value of fit error indicates the accuracy and reproducible of the result, and also the relative stable or settled of the distribution analysis result. A $Chi^2$-value describes the quality of the fit. When a $Chi^2$-value is low the measured value deviate less from the expected frequency within the specific sample. According to obtain the needed lowest $Chi^2$-value the baseline may adjust. Residual value over 10 describes the presence of aggregate particles that are not considered to be in the sample. In accordance to literature, the optimal value of residual may be as close as possible to zero (Frantzen et al., 2003).

In order to avoid contamination from dust particles, the cuvettes (borosilicate glass) to be used for determination were filled with Milli-Q water and sonicated for 10 minutes in ultrasonic bath, and further rinsed with PBS pH 7.4 (0.2 µm pore size syringe filter) before use. The vesicle dispersion was diluted with freshly filtered PBS pH 7.4 until the intensity of 250-350 kHz was achieved to ensure the accuracy and reproducible the analyzed results (Hupfeld et al., 2006).

All samples were analysed three times. The analysis run time was 10 minutes. Vesicles mode was applied, and the temperature and viscosity were defined before each measurement. The temperature was 24±1℃

PCS Parameters used:

- Nicomp distribution /Gaussian distribution
- Channel width: Auto set
- Temperature/ Liquid viscosity: 23 ℃: 0.9325, 24 ℃: 0.9111, 25 ℃: 0.8904
- Liquid index of refraction: 1.333
- Intensity set point: 300 ± 50 kHz
- Laser/ wavelength: Helium neon laser: 632.8 nm

- Scattering angle: 90℃ (fixed angle)
- Toggle solid/vesicle particle: Vesicle particle
- Number of cycle: 3
- Run time: 10 minutes

### 3.4. Experimental design and multivariate evaluation

Four variables were investigated to identify the significance; sonication cycles (2, 4 and 6), total sample volume (2, 5 and 8 ml), lipid concentration (10, 20 and 30 mg/ml) and amount of propylene glycol (50, 125 and 200 µl). Table 7 shows the first 11 experiments of the fractional design that 8 experiments suggested for this study to produce by fraction factorial design $2^{4-1}$ and expanded to $2^4$ full factorial design (table 8 the 19 experiments) (Lundstedt et al., 1998).

By taking into consideration all possible parameters that could influence the liposome size and during incorporation during probe sonication some of them were standardized. Moreover, reduced the number of experiments, statistical experimental design and statistical multivariate analysis of results that were used. For investigation of all variables two different levels (+1/-1) were applied in addition to center points in this study. Fractional factorial design $2^{4-1}$ was applied to produce the firs 11 experiments, and determined which of the parameters that was most significant on vesicle size during probe sonication. Further, it was expanded to a full factorial design $2^4$ to produce the 19 experiments based on the preliminary results for increasing the information of significant/ influential on vesicle size during probe sonication. Hence, the four promising experiments were selected for further investigating the incorporation of CAM in the formulation

### 3.5. Statistical evaluation

The statistical data analyses were performed, and the p value of $\leq 0{,}05$ was considered statistically significant.

ANOVA was used to study the main effects of all design variables in the multifactorial designs, and to evaluate the significance of effects on of the experiments variables on the mean vesicle size distribution of the product of liposomes dispersion.

## 4. Results and Discussion

### 4.1. Manufacturing of liposomes by Dual Asymmetric Centrifugation (DAC)

When starting this project, the initial goal was to establish a new DAC method for producing liposomes for topical application. This method was based on the previous described method by Massing and coworkers (Massing et al., 2008). As you can see in Table 4, we succeeded in producing liposomes with high entrapment > 50% and with the wanted size distribution of between 200-300 nm (Hurler et al., 2012). Since these two formulations have approximately the same size distribution, but totally different homogenization time at 2 minute and 55 seconds with PG, and 50 minutes without PG. It demonstrates that PG makes the size reduction more efficient. It suggested that an interaction of PG with phospholipid bilayer allowing more bilayer flexibility and smaller vesicles as mentioned earlier by Manconi and her group (Manconi et al., 2009). Similar entrapment efficiency was resulted for CAM liposome with PG and CAM liposome without PG even if applying of PG increase the drug solubility in accordance to Bhalekar literature (Bhalekar et al., 2009). Therefore, the results were considered not significant difference in entrapment efficiency between formulation with and without PG. The polydispersity index was reduced with longer mixing time, and this higher in liposomes added PG.

**Table 4: Characteristics of DAC liposomes with and without Propylene glycol (n= 1)**

| Formulations | Mean vesicle size (mean± SD) nm | P.I.* | EE** (% ± SD) |
|---|---|---|---|
| **CAM liposomes** | 282 ± 30.1 | 0.13 | 51.2 ± 2.3 |
| **CAM liposomes with PG** | 230 ± 51 | 0.25 | 49.7 ± 2.8 |

* Polydispersity index ** Entrapment efficiency

The values in Table 5 characteristic for different batches of liposomes without PG and the one given in Table 4, and we here investigated the effect of the storage in refrigerator overnight

on the measured size distribution and polydispersity. The liposome size distribution was found to be similar 1 hour after Speed Mixing and day after liposomes dispersion was kept in refrigerator, but of convenience we decided to the PCS measurements after one night storage. Entrapment efficiency and size distribution result was considered well reproducible when comparing with the same liposome described in Table 4.

**Table 5: Characterization of DAC liposomes 1 hour after Speed Mixing and after storage in refrigerator overnight.**

| **Freshly made liposomes** | | **Liposomes stored at 4℃ over nigh** | | | |
|---|---|---|---|---|---|
| **Mean vesicle size (mean± SD) nm** | P.I.* | Mean vesicle size (mean± SD) nm | P.I.* | EE ** (% ± SD) | RR*** (% ± SD) |
| **263.07 ± 13.66** | 0.083 | 217.70 ± 0.95 | 0.132 | 54.07 ±0.81 | 98.70 ±0.78 |

* Polydispersity index ** Entrapment efficiency *** Drug recovery

After these initial trial productions we unfortunately had to stop using the DAC method as the machine broke down, and we needed to find an alternative method to DAC. We then chose to go for the probe sonication method. Since we were interested to produce the desire mean vesicle size between 200-300 nm, we wanted to learn what factors should be controlled in the process to obtain this liposome size distribution. As tools for this we applied factorial design and multivariate analysis.

### 4.2. Multifactorial design for sonication approach

Based on the results from earlier experience on DAC-manufacturing regarding effect on PG on liposome size, and the need for having a reproducible method for small sample preparation of liposome using sonication, we decided investigate the effect of 4 different factors/design

variables on the vesicle size obtained after sonication (Table 6). To get as reproducible results as possible all other parameters were kept constant, such as sonication probe, amplitude, sample holder, length of sonication cycles (2 min). Table 6 represent the levels of the different variabels used in the factorial design, using the level (+1/-1) and centre points.

**Table 6: Defined variable levels for fractional and full factorial design with center points**

| | Sonication cycles | Sample volume (mL) | Lipid concentration (mg /mL) | Volume of propylene glycol (µL) |
|---|---|---|---|---|
| **High level (+1)** | 6 | 8 | 30 | 200 |
| **0** | 4 | 5 | 20 | 125 |
| **Low level (-1)** | 2 | 2 | 10 | 50 |

### 4.2.1. Fractional factorial design ($2^{4-1}$) and multivariate analysis

We decided to apply fractional factorial design, and using the Unscramble 9.8 software (Camo AS, Norway) a $2^{4-1}$, the design, and the experimental set-up given was as described in Table 7. In the design we had 11 experiments, including the three centre points. In agreement with Lundsted by this design we could study the main effect of many variables (3 to 15) or several responses, but interaction can not be evaluated from this design (Lundstedt et al., 1998).

The 11 experiments from this design was carried out in randomize ordered and as numbered in the second column of Table 7. The table showed also the overview over the different compositions of liposomes that were used under liposomes preparation.

**Table 7: Design matrix for fractional factorial design ($2^{4-1}$) with center points.**

| **Factor** | | A = BCD | B = ACD | C = ABD | D = ABC |
|---|---|---|---|---|---|
| **Experiment (Ordered)** | **Experiment (Randomized)** | **Sonication Cycles** | **Sample volume (mL)** | **Lipid concentration (mg/mL)** | **Volume of propylene glycol (µL)** |
| **1** | **8** | -1 | -1 | -1 | -1 |
| **2** | **6** | +1 | -1 | -1 | -1 |
| **3** | **9** | -1 | +1 | -1 | -1 |
| **4** | **11** | -1 | -1 | +1 | -1 |
| **5** | **10** | +1 | +1 | -1 | -1 |
| **6** | **4** | +1 | -1 | +1 | -1 |
| **7** | **2** | -1 | +1 | +1 | -1 |
| **8** | **1** | +1 | +1 | +1 | -1 |
| **CP 1** | **7** | 0 | 0 | 0 | 0 |
| **CP 2** | **3** | 0 | 0 | 0 | 0 |
| **CP 3** | **5** | 0 | 0 | 0 | 0 |

Figure 10 showed the mean vesicles size that were determined by PCS from all 11 experiments. Of these 11 liposome dispersion; dispersion no 1, 2 and 9 gave the promising mean vesicle size we were interested to investigate in this project, namely between 200-300 nm. For more details on polydispersity and fit error from the PCS measurement see Appendix Table 1.

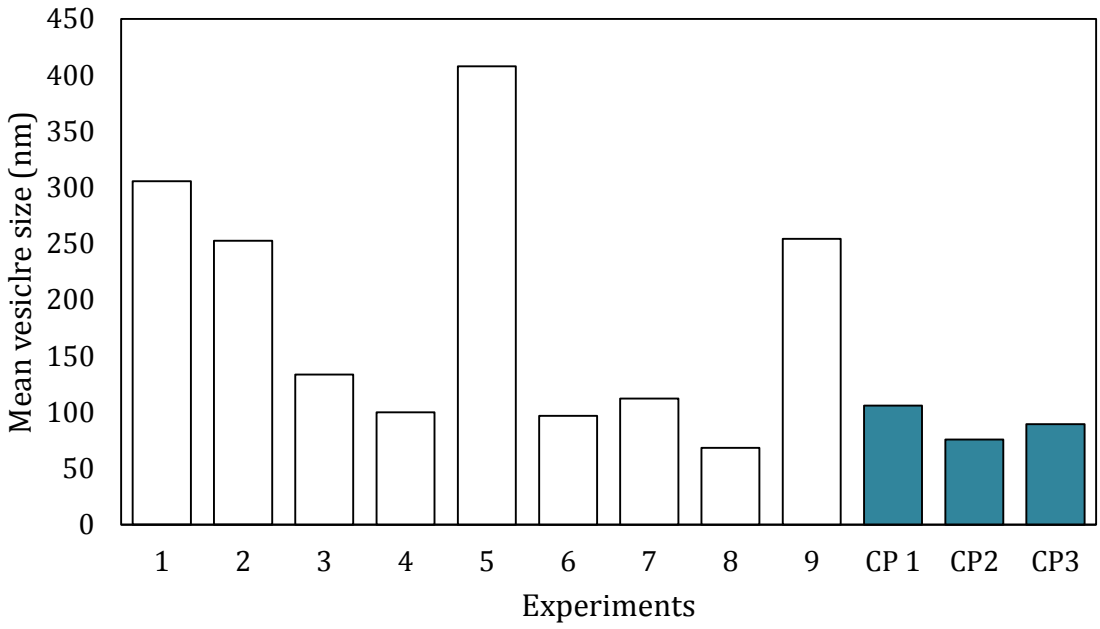


**Figure 10: Vesicle size with only the first 11 experiments.**

#### *4.2.1.1. Standard deviation of probe sonication method*

As it is illustrated in Figure 11 the three center points are produced using the same composition and sonication conditions. Therefore, we would expect that all samples also should have the same vesicle size. But as you can see the actual mean vesicle size measured varied from 105.95 nm for CP1, 75.87 nm for CP2 and 89.38 nm for CP3.

The standard deviation of the center points indicates the reproducibility and reliability of probe sonication method, and tells us that for a more accurate knowledge about the variables and their effect on our response, liposome size, more than one repetition of all exprements would have been given more strength to the results.

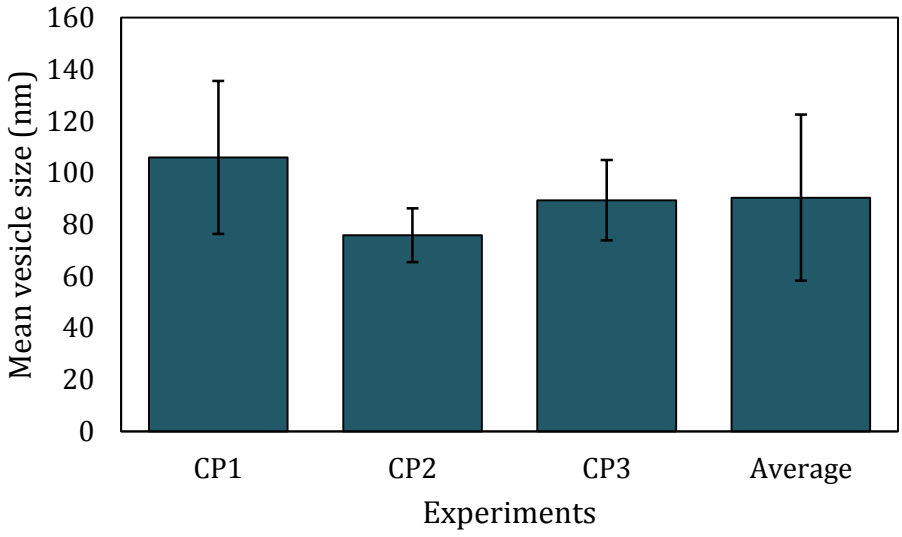


**Figure 11: Vesicle size and P.I. for the three center points.**

#### *4.2.1.2. Statistical evaluation of variables response on vesicle size*

To get more information out of the result we used ANOVA. And the results that were obtained represented in Figure 11.

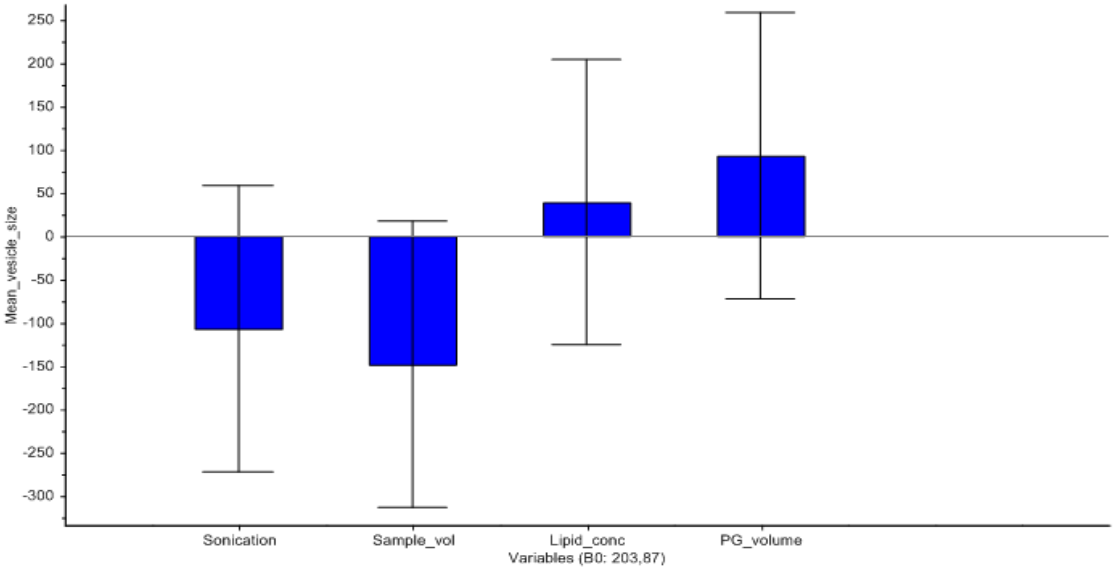


**Figure 12: Regression coefficient and their confidence interval for response mean vesicle size from the experiment variables.**

Figure 12 indicates the regression coefficient and their confidence interval for response mean vesicle size. According to Figure 12, the sample volume has the biggest effect on the vesicle size of the four factors, with a p-value of 0.07 (Appendix 3). The *p*-value of $\leq 0.05$ was considered statically significant. If we reduced the significance percentage criteria to 10 % sonication cycle wanted to be statically significant according to the ANOVA analysis results. Because of the lack of significance from these results we then decided to expand to a full factorial design, to see if this could improve the model and its resolution significance of the influence of the variables on the vesicle size from the sonication.

### 4.2.2. Full-factorial design ($2^4$) and multivariate analysis

When expanding the experimental design in to a full-factorial design, the resulting experimental set-up was as described in Table 8. The new experiments we had to execute are given in light blue. By full factorial design it is possible to study more closely the main effect of the individual variable on the response variables, as well as the effect of the interaction between the variable.

**Table 8: Design matrix for full factorial design ($2^4$) with center points.**

| **Factor** | | A | B | C | D |
|---|---|---|---|---|---|
| **Experiment (Ordered)** | **Experiment (Randomized)** | **Sonication Cycles** | **Sample volume (mL)** | **Lipid concentration (mg/mL)** | **Volume of propylene glycol (µL)** |
| **1** | **11** | -1 | -1 | -1 | -1 |
| **2** | **12** | +1 | -1 | -1 | -1 |
| **3** | **9** | -1 | +1 | -1 | -1 |
| **4** | **10** | -1 | -1 | +1 | -1 |
| **5** | **14** | +1 | +1 | -1 | -1 |
| **6** | **17** | +1 | -1 | +1 | -1 |
| **7** | **19** | -1 | +1 | +1 | -1 |
| **8** | **15** | +1 | +1 | +1 | -1 |
| **9** | **16** | -1 | -1 | -1 | +1 |
| **10** | **8** | +1 | -1 | -1 | +1 |
| **11** | **3** | -1 | +1 | -1 | +1 |
| **12** | **4** | +1 | +1 | -1 | +1 |
| **13** | **1** | -1 | -1 | +1 | +1 |
| **14** | **2** | +1 | -1 | +1 | +1 |
| **15** | **6** | -1 | +1 | +1 | +1 |
| **16** | **7** | +1 | +1 | +1 | +1 |
| **CP 1** | **5** | 0 | 0 | 0 | 0 |
| **CP 2** | **18** | 0 | 0 | 0 | 0 |
| **CP 3** | **13** | 0 | 0 | 0 | 0 |

In Figure 13 all PCS results with mean vesicle size for the total 19 experiments included in full factorial design is given. From these result experiment 10 gave the aimed vesicle size, giving us in total 4 experiments with the wanted liposome size distribution.

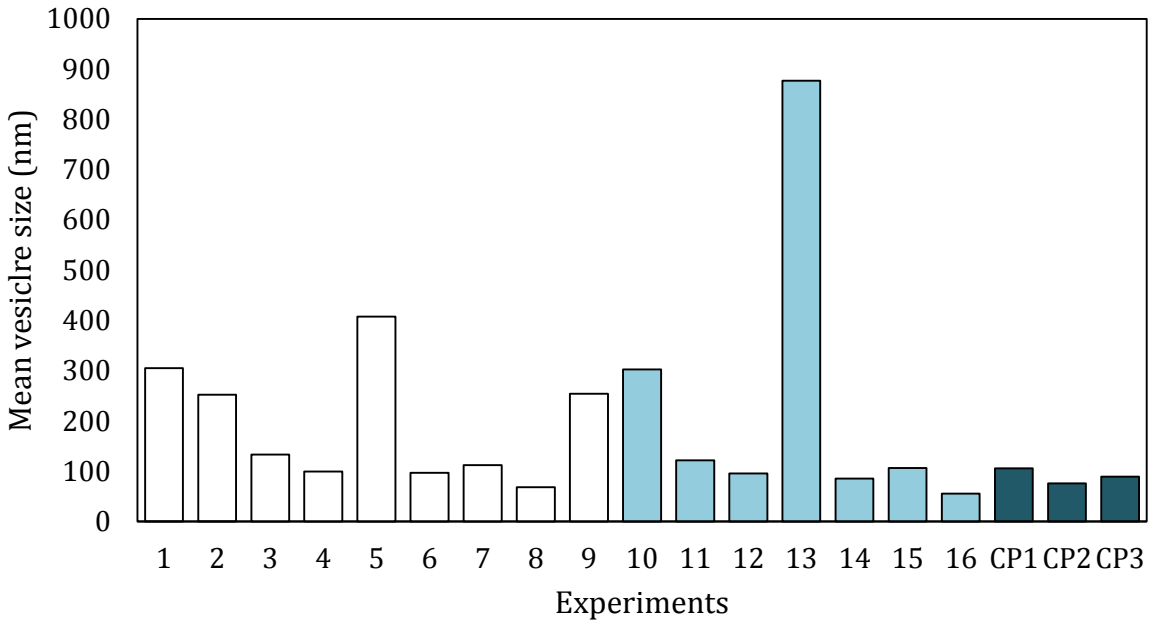


**Figure 13: Vesicle size as measured by PCS for all 19 experiments included in full factorial design $2^4$.**

The advantage of full factorial design is that we may estimate the main effect of all design variables and all interaction effects. Adding these 8 new experiments to the fractional design gave as expected more information regarding the effect of the experiment variables on the final liposome size. According to the ANOVA test (Figure 14), sample volume had a significant effect on the mean vesicle size with the p-value 0.0145. Number of sonication cycles and lipid concentration also were close to significant with the p-value of 0.0692 and 0.7091, respectively. The variable that gave the least effect was the PG volume. This most suddenly can be explained by the relatively low concentration range investigated. This tells as that the lipid: PG ratio should be decreased in more diluted liposome dispersion as compared to vesicular phospholipids gels that we produced by DAC (Table 4). CAM liposomes with PG obtained size distribution after speed mixed in 2 minutes and 55 seconds compared with 50 minutes when without PG.

Also for the three other variables a more pronounced effect might be observed if there was more difference between the +1and the -1 level.

The statistical model showed that sonication cycle and the interaction of sonication cycle and lipid concentration (A+C) considered being significant if the significance percentage criteria

decreased to 10 %. The statistical model that was used in this project would most probably have obtained more significant values with more repetition of the experiments.

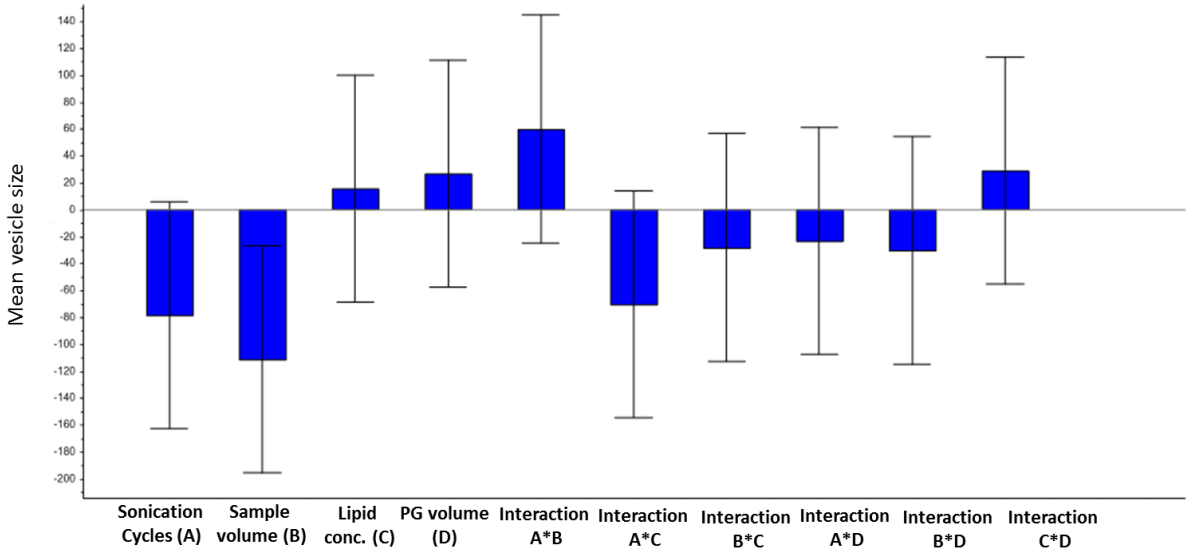


**Figure 14: Regression coefficient and their confidence interval for response mean vesicle size.**

The chosen experiments for further use with CAM were experiment; 1, 2, 9 and 10, since the size we aimed to investigate this drug incorporation should have a size range between 250-350 nm.

### 4.3. Drug loaded sonicated liposomes

The CAM-liposomes was prepared in the same way as the empty liposome. The only difference was that CAM was added to the lipid solution before when making the lipid film. The entrapment efficiency was determined by using dialysis method for separation of the unentrapped drug from liposomes containing drugs (Pavelic et al., 1999). The lipid drug ratio was the same for all formulations (Appendix 2). The mean vesicle size and polydispersity index was determined by PCS method. Characteristic for CAM containing liposomes dispersion is given in Table 9.

**Table 9**: Entrapment efficiency and relative recovery of liposomes with CAM for probe sonication method.

| Exp. Ordered* | EE (% ± SD) | Polydispersity index (P.I.) | Mean vesicle diameter (nm) | RR (% ± SD) |
|---|---|---|---|---|
| **1** | 30.13 ± 0.15 | 0.512 | 836.07 | 88.09 ± 0.39 |
| **2** | 31.05 ± 0.06 | 0.352 | 67.10 | 101.51 ± 0.51 |
| **9** | 26.00 ± 0.02 | 0.479 | 834.93 | 104.02 ± 0.69 |
| **10** | 22.98 ± 0.12 | 0.498 | 768.60 | 101.47 ± 0.67 |

* Refers to empty liposomes in full factorial design given in Table 8.

As it is shown in Table 9, three of the four liposome dispersions were, as expected, larger in size in the presence of CAM, than for the corresponding experiments we had with empty liposomes. CAM has low aqueous solubility thus will incorporated in the lipid bilayer, and has therefore been described in the literature to make liposome vesicles more resistance to the size reduction (Anderson et al., 2012). Experiment number 2 gave unexpected small vesicle size at 67.10 nm, knowing that empty liposomes with the same experimental conditions had a mean vesicle size 252.58 nm. If we compared the four experiments results in Table 9, all experiment had the same lipid concentration, sample volume and drug concentration. The only factors that differ between these experiments were number of sonication cycles (experiment 1 and 9 had 2 sonication cycle, whereas experiment 2 and 10 had 6 sonication cycles), and added volume of PG (for experiment 1 and 2 was 50 µl, whereas exp. 9 and 10 had 200 µl). For more details you can have a look in Appendix 2. The high number of sonication cycles of 6, relative to experiments 1 and 9 with only 2 sonications cycles could explain the relative smaller size. However, experiment 2 had less PG volume 50 µl then experiment 10 had 200 µl which the oppsite of what we would expect. The conclusion that had to be related to experimantal errors during probe sonication such as probe position that might influence the ablity to minimize the vesicle size as mentioned earlier by Antimisiaris (Antimisiaris et al., 2008).

As the results showed in Table 9, experiment 1 gave higher entrapment efficiency at 30.13 % in comparison with experiment 9 that had 26 % entrapment efficiency, and these two experiments were sonicated in 2 sonication cycles, whereas the experiments were containing different amount of PG volume. Experiment number 2 gave the highest entrapment efficiency of 31.05 % even if this experiment had low mean vesicle size. Generally we observed the four experiments had quit similar entrapment efficiency between 23-31 %.

According to literature (Bhalekar et al., 2009) a high amount of drug could be incorporated in liposome dispersion due to lipid solubility of the drug. And according to our result the larger liposomes have more capacity of drug incorporation than the smaller liposomes. When using DAC method the vesicle size resulted 263.07 nm, while probe sonication method resulted larger vesicle size. We thus should expect that the sonicated liposomes had a higher drug entrapment than the DAC-liposomes. The fact that the opposite is found can however be explained by the higher lipid content in the DAC-liposomes.

DAC resulted more EE % according to when the lipid film was hydrated by PBS pH 7.4 there was low volume of PBS and low amount of CAM in the aqueous medium. Therefore, it was more incorporation of CAM in lipid membrane, while the sonication method had more PBS volume and the most of the drug (CAM) was in PBS (aqueous medium) than in lipid membrane. The more CAM in aqueous medium lead to the more dissolving of CAM and the less capacity of CAM incorporation in the lipid membrane. Due to the results, DAC method seemed to be found gentler than sonication method.

The recovery of the drug was determined for the all probe-sonicated samples, and it was found to be between 88.09 and 104.02 % of the total amount that was used for preparation of the formulations. The drug recovery was found to be lower in sample 8 in comparison to the other three formulations Table 9.

## 5. Conclusion

We have demonstrated that both DAC and probe sonication are suitable methods for making liposomes with a desired size range of between 200 and 300 nm. DAC is favored by a higher incorporation efficiency for the model drug applied here, chloramphenicol (CAM). Statistical experimental design and multivariate analysis were applied successfully, and confirmed that samples volume and number of sonication cycles are the most responding variable with regard to vesicle size when using probe sonication and the variable ranges chosen for the study.

## 6. Perspectives

- New experiments with less gentle sonication conditions should be performed with CAM-liposomes to reach the aimed size distribution.
- Propylene glycol concentrations in sonication should be increased to look for effect on needed sonication time for sufficient size reduction and to investigate effect drug entrapment.
- Liposomes with the bet size distribution and drug entrapment should be combined with a suitable vehicle for topical application.
- A wider range for some of the variables used might give more information on their impact on vesicle size, such as amount of PG-added.

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Appendix

Appendix 1 *

| Experiment (Ordered) | Experiment (Randomized) | Mean diameter (nm) | SD (nm) | Variance (P.I) | Fit error |
|---|---|---|---|---|---|
| **1** | 8 | 305.47 | 8.32 | 0.38 | 1.97 |
| **2** | 14 | 252.58 | 1.90 | 0.34 | 2.61 |
| **3** | 16 | 133.59 | 5.18 | 0.32 | 3.04 |
| **4** | 11 | 99.82 | 1.23 | 0.33 | 2.63 |
| **5** | 12 | 407.79 | 7.57 | 0.39 | 2.22 |
| **6** | 4 | 96.74 | 1.65 | 0.31 | 2.55 |
| **7** | 2 | 112.31 | 1.31 | 0.31 | 2.35 |
| **8** | 13 | 68.37 | 1.35 | 0.37 | 3.09 |
| **9** | 17 | 254.30 | 3.21 | 0.39 | 2.05 |
| **10** | 6 | 302.68 | 5.08 | 0.35 | 2.16 |
| **11** | 9 | 121.87 | 2.35 | 0.31 | 2.31 |
| **12** | 15 | 95.79 | 2.15 | 0.31 | 2.95 |
| **13** | 10 | 876.65 | 36.37 | 0.59 | 2.34 |
| **14** | 18 | 85.84 | 2.37 | 0.32 | 2.73 |
| **15** | 19 | 106.55 | 4.96 | 0.33 | 3.19 |
| **16** | 1 | 55.84 | 0.87 | 0.33 | 2.83 |
| **CP 1** | 3 | 105.94 | 1.52 | 0.32 | 2.36 |
| **CP 2** | 5 | 75.87 | 0.36 | 0.36 | 2.77 |
| **CP 3** | 7 | 89.38 | 0.24 | 0.35 | 2.65 |

Appendix* Liposome size results of 19 experiments PCS measurement carried out day after sonication volume weighted Gaussian (n= 3)

Appendix 1

Overview over the composition of the CAM- and PL-solution prior to making the lipid films, and amount of every component in the liposome dispersion

| Experiment Ordered | Experiment Randomized | Sonication cycle | Sample volume (ml) | CAM (mg/ml) | Lipid conc. (mg/ml) | Volume PG (µl) |
|---|---|---|---|---|---|---|
| **1** | 11 | 2 | 2 | 1 | 10 | 50 |
| **2** | 12 | 6 | 2 | 1 | 10 | 50 |
| **9** | 16 | 2 | 2 | 1 | 10 | 200 |
| **10** | 8 | 6 | 2 | 1 | 10 | 200 |

Appendix 2

ANOVA mean particle size and analysis of mean particles size as response

| ANOVA | DF | MS | F-ratio | p-value |
|---|---|---|---|---|
| **Summary** | | | | |
| **Model** | 4.0000 | 78511.7000 | 3.0595 | 0.0525 |
| Error | 14.0000 | 25662.0200 | | |
| Corr. Total | 18.0000 | | | |
| **Variables** | | | | |
| Sonication cycle | 1.0000 | 99362.0800 | 3.8720 | 0.0692 |
| Sample volume | 1.0000 | 199788.9000 | 7.7854 | 0.0145 |
| Lipid concentration | 1.0000 | 3720.6960 | 0.1450 | 0.7091 |
| PG volume | 1.0000 | 11175.1300 | 0.4355 | 0.5200 |
| **Model check** | | | | |
| Mean | | | | |
| Linear | 4.0000 | 78511.7000 | 3.0595 | 0.0525 |
| **Lack of Fit** | | | | |
| Lack of fit | 12.0000 | 299901.2200 | 131.8242 | 0.0076 |
| Pure error | 2.0000 | 226.8264 | | |
| Error | 14.0000 | 25662.0200 | | |

## Appendix 3

summary of ANOVA results of full factorial design

| | B Coefficients | | | | |
|---|---|---|---|---|---|
| Beta Coefficient | Mean_particle_size | | | | |
| | B-coefficient | Low uncertainty limit | High uncertainty limit | p-value | t-value |
| B0 | 191,9674 | | | | |
| Sonication_cycles (A) | -78,8044 | -163,4117 | 5,8029 | 0,0640 | -2,3406 |
| Sample_volume (B) | -111,7444 | -196,3517 | -27,1371 | 0,0159 | -3,3189 |
| Lipid_concentration (C) | 15,2494 | -69,3579 | 99,8567 | 0,6886 | 0,4529 |
| PG_volume (D) | 26,4281 | -58,1792 | 111,0354 | 0,4918 | 0,7849 |
| Sonication_cycles*Sample_volume (AB) | 59,4919 | -25,1154 | 144,0992 | 0,1436 | 1,7670 |
| Sonication_cycles*Lipid_concentration (AC) | -70,7594 | -155,3667 | 13,8479 | 0,0899 | -2,1016 |
| Sample_volume*Lipid_concentration (BC) | -28,7494 | -113,3567 | 55,8579 | 0,4559 | -0,8539 |
| Sonication_cycles*PG_volume (AD) | -23,5981 | -108,2054 | 61,0091 | 0,5381 | -0,7009 |
| Sample_volume*PG_volume (BD) | -30,6831 | -115,2904 | 53,9242 | 0,4273 | -0,9113 |
| Lipid_concentration*PG_volume (CD) | 28,5306 | -56,0766 | 113,1379 | 0,4592 | 0,8474 |