**Charles University in Prague**

**Faculty of Pharmacy in Hradec Králové**

**Department of Pharmaceutical Technology**

**Disposed with Institute of Pharmacy and Molecular Biotechnology of Heidelberg University**

# Characterization of liposomal formulations for brain-targeting

**Diploma Thesis**

**Heidelberg & Hradec Králové 2015** **Andrea Labajová**

Hereby I declare, this thesis is my original copyrighted work. All literature and other sources that I used while processing are listed in bibliography and properly cited. To my knowledge, this thesis has not been submitted for obtaining the same or any other degree.

Hradec Králové, 25.8.2015 Andrea Labajová

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# 1 Abstract

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This paper deals with characterization of liposomal formulations with encapsulated triethylenetetramine (TETA), which is selective $Cu^{II}$- chelator used in the treatment of Wilson's disease for decades. Liposomal formulations were prepared by a film hydration method with subsequent dual asymmetric centrifugation with the addition of 2.5 mol/l TETA solution dissolved at pH3 or/and pH7, respectively. Size exclusion chromatography (SEC) was performed to separate free-TETA from the encapsulated-one at day 1, 2, 3, 4, 5 and 8. Two methods of liposome purification, the one column method and the two columns method, were used. The size and the size distribution of prepared liposomes were measured by photon correlation spectroscopy (PCS) at each day of storage. The concentration of encapsulated TETA, as well as the concentrations of cholesterol before the SEC and after the SEC were determined by HPLC in order to express the encapsulation efficiency. No influence of pH or the method of purification on the liposome stability (the average size) were found. However, the higher polydispersity was observed for TETA-liposomes with pH7 purified by the two columns method. The pH value and/or the method of purification significantly influenced the variability of the obtained data or/and the encapsulation efficiency (EE), respectively. The more consistent results of EE during the days of storage were observed with the dissolution of TETA at pH3 value; the higher EE was obtained for the two columns method of purification.

# 2 Abstrakt

Univerzita Karlova v Prahe, Farmaceutická fakulta v Hradci Králové

| | |
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Táto práca sa zaoberá charakterizáciou lipozomálnych formulácii s enkapsulovaným triethylentetraamínom, ktorý sa používa ako selektívny $Cu^{II-}$ chelátor k liečbe Wilsonovej choroby už po desaťročia. Liposomálne formulácie boli pripravené filmovou metódou s následnou duálnou asymetrickou centrifugáciou s prídavkom 2.5 mol/l triethylentetraamínu rozpusteného jednotlivo v pH3 alebo/a pH7. Bola prevedená gélová chromatografia za účelom separácie voľného triethylentetramínu od enkapsulovaného v deň 1, 2, 3, 4, 5 a 8. Boli použité dve metódy čistenia: jednokolonková a dvojkolonková. Veľkosť a distribúcia veľkosti častíc bola určená pomocou fotón korelačnej spektroskopie. Pomocou HPLC bola určená koncentrácia enkapsulovanej TETY, rovnako ako aj koncentrácie cholesterolu pred a po gélovej chromatografii za účelom vyjadrenia enkapsulačnej účinnosti. Nebol zistený vplyv hodnoty pH, ani metódy čistenia na stabilitu lipozómov (priemernú veľkosť). Avšak, vyššia polydisperzita bola pozorovaná pre TETA-lipozómy s pH7 čistené dvojkolonkovou metódou. Hodnota pH alebo/a metóda čistenia významne ovplyvnili variabilitu získaných výsledkov a/alebo enkapsulačnú účinnosť. Boli pozorované konzistentnejšie výsledky enkapsulačnej účinnosti pre triethylentetramín rozpustený v pH3, vyššia EE bola zistená pri použití dvojkolonkovej metódy čistenia.

# 3 The aim of study

As the theoretical background of this work, liposomes, its classification, materials used for preparing of liposomes, preparation techniques including dual asymmetric centrifugation, the principle of size exclusion chromatography and photon correlation spectroscopy, the drug triethylenetetramine and its uses will be described.

The purpose of this thesis is to prepare liposomes with triethylenetetramine (TETA) dissolved at pH3 or/and pH7 by a film hydration method followed by a dual asymmetric centrifugation. Photon correlation spectroscopy is used to evaluate the stability of prepared liposomes, i.e. their size and polydispersity.

The concentration of encapsulated TETA and the concentrations of cholesterol before the size exclusion chromatography (SEC) and after the SEC are determined by HPLC in order to calculate the encapsulation efficiency of TETA in liposomes.

The influence of the method of purification and the value of pH used on the properties of the prepared liposomes and the encapsulation efficiency will be estimated.

# 4 List of abbreviations

| | | |
|---|---|---|
| BBB | | Blood-brain barrier |
| Conc | (mM) | Concentration |
| d | (nm) | diameter |
| D | ($m^2 \cdot s^{-1}$) | translational diffusion coefficient |
| DAC | | Dual asymmetric centrifugation |
| DAT | | N1, N10-diacetyltriethylenetetramine |
| d (H) | (m) | hydrodynamic radius |
| EE | (%) | Encapsulation efficiency |
| EPC | | Egg-phosphatidylcholine |
| Fig | | Figure |
| FMOC | | 9-flouorenylmethylchloroformate |
| GUV | | Giant unilamellar vesicles |
| GM1 | | Monosialoganglioside |
| HPLC | | High performance liquid chromatography |
| Chol | | Cholesterol |
| k | ($1.38 \cdot 10^{-23} J \cdot K^{-1}$) | Boltzmann's constant |
| LUV | | Large unilamellar vesicles |
| LMV | | Large, multilamellar vesicles |
| MAT | | N1-acetyltriethylenetetramine |
| MLV | | Multilamellar vesicles |
| ŋ | ($N \cdot s \cdot m^{-2}$) | viscosity of suspending liquid |
| OLV | | Oligolamellar vesicles |
| PBS | | Phospate Buffered Saline |
| PCS | | Photon correlation spectroscopy |
| PdI | | Polydispersity index |

| | | |
|---|---|---|
| PEG- PE | | Polyethylene glycol modified with polyethanolamine |
| PLs | | Phospholipids |
| P1 | | Purification 1 |
| P2 | | Purification 2 |
| SEC | | Size exclusion chromatography |
| SD | | Standard deviation |
| S1 | | Sample 1 |
| S2 | | Sample 2 |
| SUV | | Small unilamellar vesicles |
| T | (K) | Absolute temperature |
| TETA | | Triethylenetetramine |
| WD | | Wilson disease |

# 5 Introduction

Development of drug delivery systems for brain-targeting represents one of the most challenging research topics in the pharmaceutical field, mainly due to the presence of the blood-brain barrier (BBB), which separates the blood from the cerebral parenchyma thus limiting the brain uptake of the majority of drugs. Among the several carriers, which have been developed to overcome this problem, liposomes have attracted significant attention as promising agents for brain-targeted drug delivery.[1]

Triethylenetetramine (TETA) is drug commonly used for the treatment of Wilson's disease, which is manifested by copper accumulation in tissues, brain including. Although TETA has been used for decades, there is just one medical product on the market: Capsules SYPRINE and pharmacologic or clinical information are very limited or unavailable.[2]

This thesis aims to investigate and determine the size, size distribution and encapsulation efficiency of TETA (dissolved at pH3 or/and pH7) in liposomes prepared from cholesterol (chol), egg-phospatidylcholine (EPC) and polyethylene glycol modified phospatidyl ethanolamine (PEG-PE). It was already determined that PEG-PE extends circulation lifetimes when incorporated into liposomes.[3]

The following techniques were used for this project:

- Liposome production and formation by the film hydration method
- Dual asymmetric centrifugation
- Size-exclusion chromatography
- Photon correlation spectroscopy
- High performance liquid chromatography

# 6 Theoretical section

## *6.1 Liposomes*

The word liposome comes from two Greek words: ‘Lipos’ meaning fat and ‘Soma’ meaning body. Since their discovery in mid-60s, the field of liposome research has progressed enormously, increasing applications area from drug and gene delivery to cosmetics, diagnostics or food and chemical industry.[4] They were first described by British haematologist Bangham at the Babraham Institute in Cambridge as a model of cellular membranes. According to legend, Bangham and his colleague were testing the institute's new electron microscope, when they made a noted observation about phospholipids forming closed multilamellar vesicle spontaneously in an aqueous solution.[5]

Liposomes are sphere-shaped nanovesicles consisting of a central aqueous compartment surrounded by one or more membraneous lipid bilayers. Membranes are usually made of phospholipids, which are molecules with a hydrophilic head group and a hydrophobic tail group, which is made of a long hydrocarbon chain. In the presence of water, the head is attracted to water and the tail is repelled by water.[5] Furthermore, the choice of bilayer components estimates the ‘fluidity’ and the charge of the bilayer. For example, unsaturated phosphatidylcholine species from natural sources (for instance, egg phosphatidylcholine) help create much more permeable and less stable bilayers, whereas the saturated phospholipids with long acyl chains (dipalmitoylphosphatidylcholine) form a rigid, impermeable bilayer structure.[6]

Phospolipids impulsively create closed structure when are hydrated in aqueous solution. Because of liposomes unique structure, hydrophilic, hydrophobic and amphiphilic substances may be incorporated within these vesicles. Hydrophilic molecules are encapsulated in the interior aqueous compartments, while liphophilic drugs can be accommodated in the lipid phase.[4, 5] The surface of liposomes can be modified by the incorporation and covalent linkage of glycoproteins or synthetic polymers.[7]

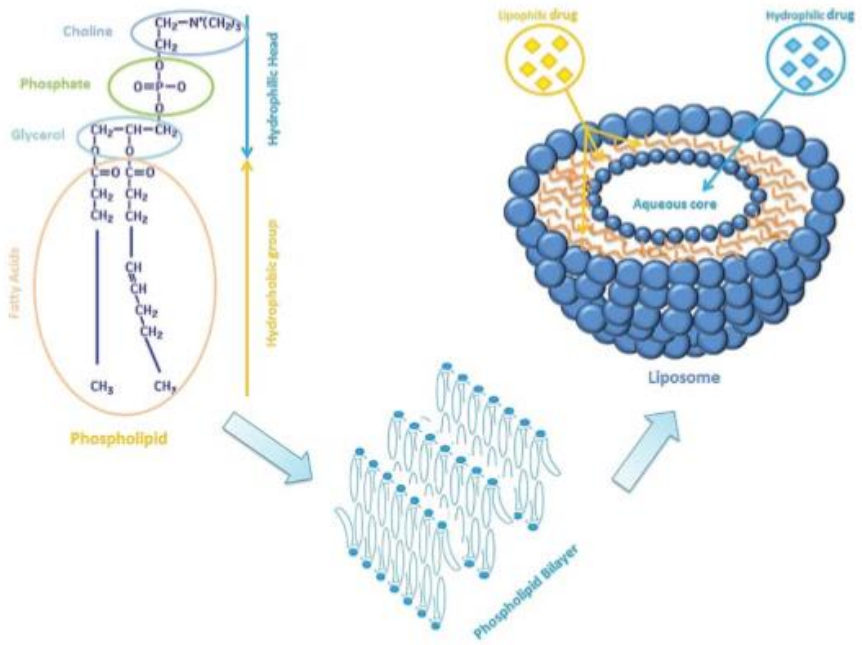


Fig. 1. Schematic illustration of liposome structure and liphophilic and hydrophilic drug entrapment models[4]

Liposomes are used as drug delivery systems due to their excellent characteristics like biodegradability, biocompatibility, flexibility, increasing therapeutic index of drug and efficacy, increasing stability of entrapped drug from hospital environment, reducing side effects, providing sustained release and acting as reservoir of drug.[8]

They can be formulated and processed to differ in size, composition, lamellarity and charge and can be administered by many routes e.g. oral, nasal, pulmonary, intravenous, intramuscular, topical, and ocular.[8, 9]

### 6.1.1 Classification of Liposomes

Liposomes can be classified on the basis of various characteristics, such as size, lamellarity, lipid composition, or applications, to name a few.[7]

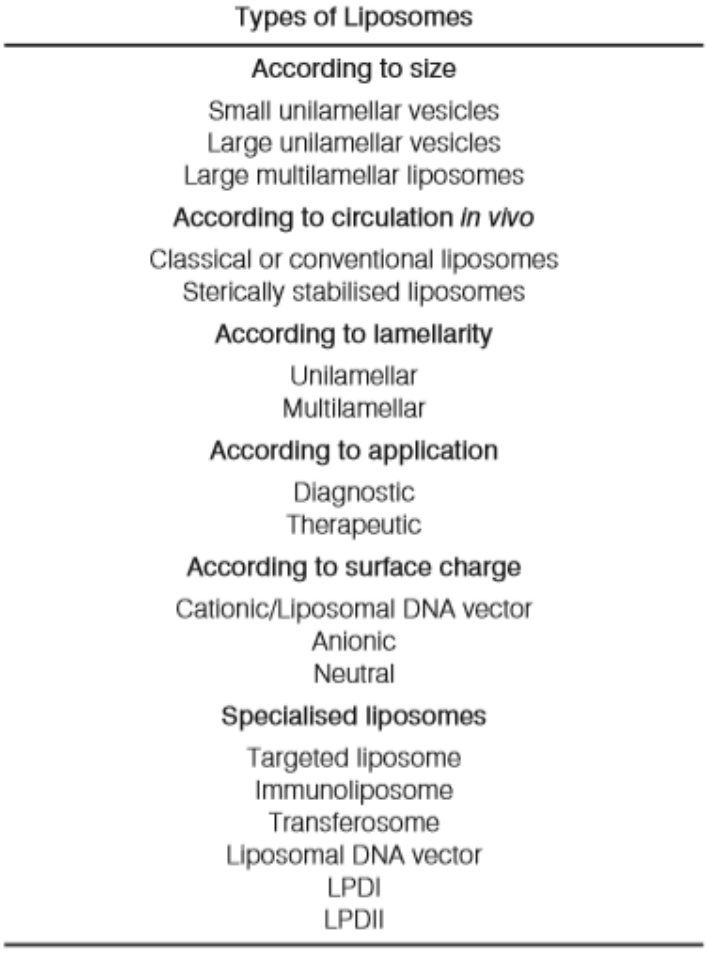


Fig. 2. Classification of liposomes[7]

#### 6.1.1.1 Classification based on size and lamellarity

Liposomes are most commonly classified by their size and the number of lipid bilayers. Unilamellar vesicles have a single phospholipid bilayer sphere enclosing aqueous solution and are divided into three size types:[7]

- small unilamellar vesicles (SUV): 20–100 nm
- large unilamellar vesicles (LUV): > 100 nm
- giant unilamellar vesicles (GUV): > 1000 nm

Oligolamellar vesicles (OLV), 100–500 nm, are rare, although an example of a polymer-induced transformation of unilamellar to bilamellar vesicles has been reported.[4, 9]

Multilamellar vesicles (MLV), > 500 nm, have onion structure which form one inside the other, creating a multilamellar structure of concentric phospholipid spheres separated by layers of aqueous solution.[4, 9, 10]

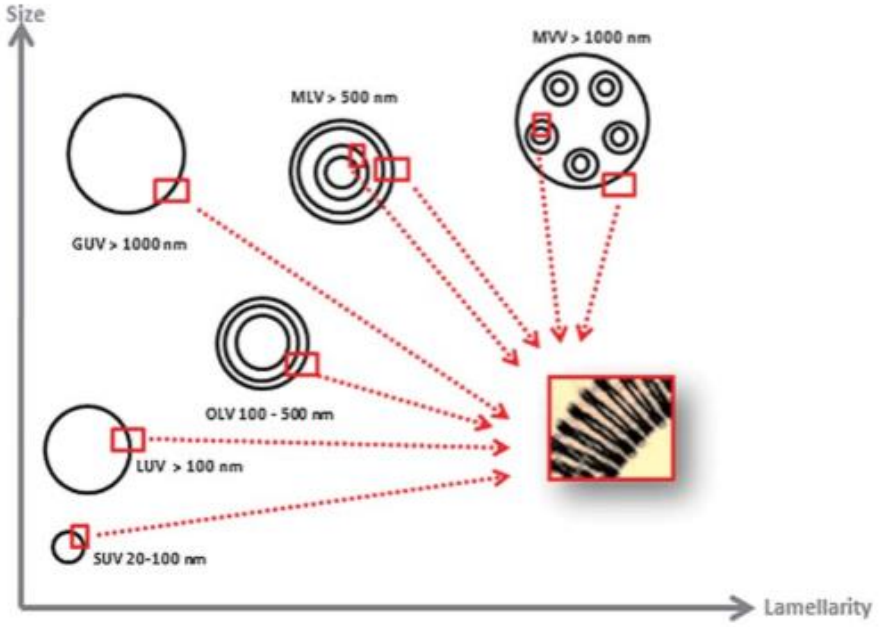


Fig. 3. Liposomes classification based on size and lamellarity[4]

The size of liposomes plays a significant role in their clearance—the extent of its importance varies for conventional and sterically stabilised ("stealth") liposomes. Strong size dependence can be found in clearance of conventional liposomes following i.v. administration; as the liposome diameter increases the rate at which the liposomes are eliminated from blood into the reticuloendothelial system also increases.[7]

In case of sterically stabilised liposomes with average size around 250.0 nm, it was observed that they are slowly filtered out by liver Kupffer cells. At size above 300 nm, increased uptake into the spleen occurs, apparently by a mechanism of passive filtration.[7]

#### *6.1.1.2* Classification based based on circulation *in vivo*

The conventional liposomes are typically composed of neutral phospholipids like phosphatidylcholine along with varying amounts of cholesterol and sometimes small amount of an acidic phospholipid. Cholesterol is important for increasing the stability of liposomes in the presence of plasma, and the negative charge is added to avoid aggregation and to increase encapsulation efficiency. These liposomes are cleared

from the blood stream rapidly thanks to phagocytic cells of the reticuloendothelial system. Their half-life decreases with increasing diameter, negative charge and fluidity.[7]

Within last decades, many experiments have been performed to improve plasma stability of liposomes by the addition of specific mixtures to their surface. These so-called sterically stabilised liposomes extend circulation time in blood due to a reduced recognition rate by cells and macrophages. Most of the initial studies were made with monosialoganglioside (GM1) or phosphatidyl inositol as a stabilising component. Best results were achieved by modifying the liposomal surface with hydrophilic polymers like polyethylene glycol.[3, 11] The effect of glycolipids and polysaccharides on liposome circulation time was investigated after consideration of the structure of the red cell outer membrane, which surface consists of glycolipids and sialic acid.[12]

#### 6.1.1.3 Classification based based on surface charge

The charge of classic liposomes is originally neutral, therefore charge inducers are used for modification of the net surface charge of liposomal formulation. Stearylamine can be used as a cationic charge inducer, while diacetylphospate or phosphatidyl serine to prepare the negatively charged liposomes.[9] These charged liposomes are removed more rapidly than the classical ones. The splenic uptake is typical for the negative charged liposomes and is about twice faster than for the positively charged ones. Liposomes with positive surface charge are playing a significant role in recombinant DNA-technology and are also called as liposomal DNA delivery vectors.[7]

#### 6.1.1.4 Classification based based on clinical applications

Applications of liposomes in medicine and pharmacy can be divided according to the therapeutic and diagnostic purpose.

***Diagnostic application***

Large liposomes are removed quickly via uptake by the reticuloendothelial system of the liver and spleen. This fact can be used in passively targeting for delivering diagnostic imaging agents into these organs.[7] The ability of liposomes to carry different compounds make them suitable for all contrast procedures such as

sonography, gamma scintigraphy, magnetic resonance imaging or computed tomography imaging.[8]

***Therapeutic application***

Liposomes have shown tremendous therapeutic potential as drug carriers. However, the benefits and limitations of liposome depend on the interaction of liposomes with cells and their faith in vivo after administration. Studies of these interactions between liposomes and cells have shown that there are four possible mechanisms of adsorption: simple adsorption, subsequent endocytosis, fusion, which is rare or exchange of bilayer components.[13]

Liposomes can offer several advantages over conventional dosage forms especially for parenteral, topical and pulmonary route of administration. The possibility of modulating the technological characteristics of the vesicles makes them highly versatile both as carriers of different kinds of drugs (from conventional chemotherapeutics to proteins) and in therapeutic application (from vaccination to cancer therapy). In previous years, several important formulation for the treatment of different disease have been developed or are currently undergoing clinical trials.

Doxorubicin, daunorubicin, amphotericin B or vincristine can be mentioned as an example of the most successful encapsulated drugs in the liposomal formulation.[4,13]

## *6.2 Material used for liposome preparation*

Liposomes can be formed from a variety of lipids and their mixtures.

### 6.2.1 Phospholipids

Phospolipids (PLs) can be found in high proportions in cell membranes of living matter. PLs are amphipathic molecules consisting of two fatty acids linked to a polar head group and as the back bone glycerol or sphingomyeline is used. The two hydrocarbon chains constitute the hydrophobic tails, while the phosphate group and its polar attachment constitute the hydrophilic tail. PLs can consist of different head and tail groups that affect the surface charge and bilayer permeability of the liposomes. Phospatidyl ethanolamine, phospatidyl glycerol, phospatidyl serine, phosphatidylinositol and phospatidylcholine (see Fig 4.) belong to most common used

glycerophospholipids. Phosphatidylcholine can be derived from natural or synthetic sources.[14]

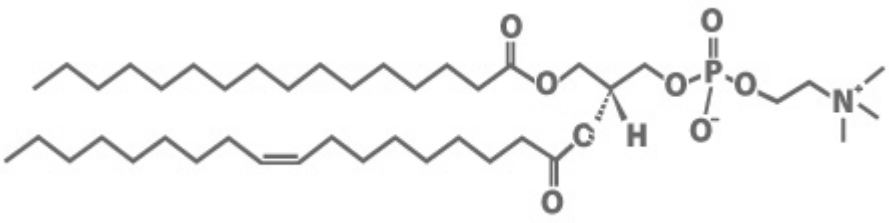

Fig. 4. Structure of EPC molecule[15]

The packing of the hydrocarbon chains of lipid molecules influences the stability of the liposome. The membrane transforms from a fully extended and closely packed "gel phase" to a liquid crystalline disordered at the main transition temperature, which is dependent on the hydrocarbon chain length and degree of saturation of the acyl chains. In general, rigid bilayers are less permeable to solutes than fluid membranes.[16]

### 6.2.2 Cholesterol

Cholesterol (Fig 5) does not form a bilayer on its own, but can be incorporated into the lipid bilayers at concentrations up to 1:1 molar ratio. Due to its amphiphatic properties, chol itself inserts in the bilayer with its OH-groups oriented towards the aqueous core and the rigid hydrophobic tail toward the bilayers. The presence of chol in the lipid bilayer increases the stability and form highly ordered and rigid membrane with fluid like characteristics.[17]

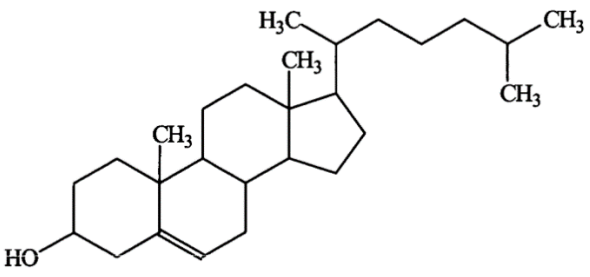

Fig. 5. Structure of cholesterol molecule[18]

### 6.2.3 Polyethylene glycol

As mentioned above, sterically stabilized liposomes in which the surface has been altered offer significant advantages over conventional liposomes. The resulting liposomes are more hydrophilic and less able to bind opsonins from plasma.

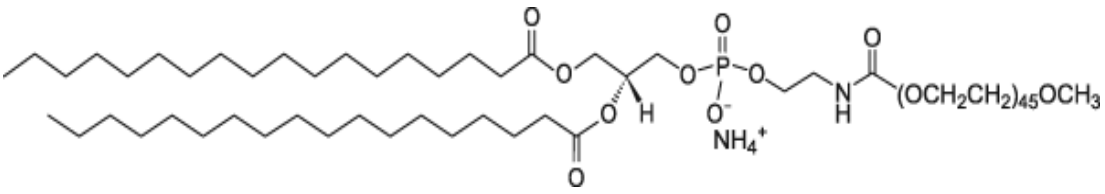


Fig. 6. Structure of 1, 2- distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt) (PEG- 2000PE)[19]

One solution how to achieve this goal is to use polyethylene glycol, which can be incorporated in different ways: by the physical adsorption of the polymer onto the surface of liposomes, by incorporation of PEG-lipid conjugate during liposome preparation, or by covalent attachment of reactive groups onto the surface of performed liposomes.[12] Coating of liposomes with polyethylene glycol (PEG) further allows a prolonged circulation time in plasma allowing prolonged dosing intervals.[20]

## 6.3 Preparation techniques of liposomes

The following parameters are important for the correct choice of liposome preparation method:[5]

1. The physicochemical characteristics of the substance to be entrapped and those of the liposomal mixtures
2. The nature of the medium in which the lipid vesicles are mixed
3. The concentration of entrapped substance and its potential toxicity
4. Additional processes involved during application
5. Size, polydispersity and shelf-life of the vesicles
6. Batch-to-batch reproducibility and possibility of large scale production

### 6.3.1 Mechanical methods

#### Method of film lipid hydration

The origin of this method was developed by Bangham[21] and still remains the simplest procedure for liposome preparation, however having some difficulties thanks to its low encapsulation efficiency.

Liposomes are prepared by hydrating the thin lipid film in an organic solvent following with the remove of the solvent to yield a lipid film. A dry nitrogen or argon steam in fume hood are used to evaporate of small volumes of organic solvent; for larger volumes, the solvent can be eliminated by rotary evaporation, where thin lipid film is formed on the sides of round bottom flask. To remove the residual organic solvent, the vial or flask is placed for 1 hour in the exsiccator under vacuum.[5]

When all the solvent is removed, the lipid film is hydrated using aqueous medium. Hydration time may differ slightly among lipid structure and composition of a mixture. It is recommended to use hydration time around 1 hour with additional mixing, shaking or stirring. The lipids swell and hydrate forming a heterogenous sized population of large, multilamellar vesicles (LMV).[5, 22] For converting MLV suspension to liposomes of smaller size, the extrusion, sonication or French pressure cell method can be used.[8]

#### Sonication method

This method is used for preparation of SUVs from MLVs prepared by the conventional method. The sonication is based on pulsed, high frequency sound waves (sonic energy), which agitate a suspension of MLV. Suspension is sonicated either with a bath type sonicator which is more suitable for a large volume of a sample or a probe sonicator recommended for the small volume of a sample.[23]

#### French pressure cell method

This method was originally developed for disrupting cells under better conditions compared to the ultrasound method, because lipid, as well as proteins or other sensitive compounds might be degraded during the sonication. This method involves the extrusion of MLVs at 20, 000 Pa at 4 °C through a small orifice, however system can be used only in the volume of 1–40 ml.[24]

### 6.3.2 Methods based on replacement of organic solvent

Generally, lipids are dissolved in organic solvents, which is then dispersed into aqueous medium containing substance to be entrapped.[22]

#### Ether infusion method

A solution of lipids dissolved in diethylether or ether-methanol mixture is carefully injected to an aqueous solution of the substance to be encapsulated at 55 to 65 °C. The following removal of ether under vacuum leads to formation of liposomes. Disadvantages of this method include the exposure of compounds to be encapsulated to the organic solvent, the use of high temperature and the preparation of liposome formulation having large size distribution (70–190nm).[8]

#### Ethanol Injection method

An ethanolic solution of lipids is injected to a vast excess of a buffer. As a result, the MLVs are formed. The main disadvantage of this method is heterogeneous population of liposomes (30–110nm).[9]

#### Reverse phase evaporation technique

First, the water-in-oil emulsion is shaped by brief sonication of a two-phase system containing phospholipids in an organic solvent and an aqueous buffer. The organic solvents are removed under reduced pressure by a rotary evaporator, resulting in the formation of a semi-solid gel. Liposomes are formed when residual solvent is eliminated by continued evaporation. In a medium of low ionic strength, a high encapsulation efficiency up to 65 % can be achieved. The main drawback is the exposure of the material to be encapsulated to organic solvents and to brief periods of sonication.[25]

### 6.3.3 Other methods

#### High pressure extrusion method

This method is widely used process for converting MLVs to SUVs suspensions, in which liposomes prepared by conventional method are repeatedly forced under high

pressure through polycarbonate membrane filters with the defined pore size. The mechanism of action appears to be like peeling onion.[23]

### Dual asymmetric centrifugation

Dual asymmetric centrifugation (DAC) is a special kind of centrifugation. While in conventional centrifugation, vials turn around the main rotation axis at defined distance and speed, vials in the DAC process turn around their own centre as well as around the centre of centrifuge similarly to the normal centrifugation process. This result in two overlying movements of the sample material in the centrifugation vial. The main rotation pushes the sample material in an outward direction in response to centrifugal forces, while the additional rotation of the centrifugation vial around its own centre constantly pushes the sample material in the opposite direction due to adhesion between the sample material and the rotating vial (see Fig. 7, Fig 8).[26]

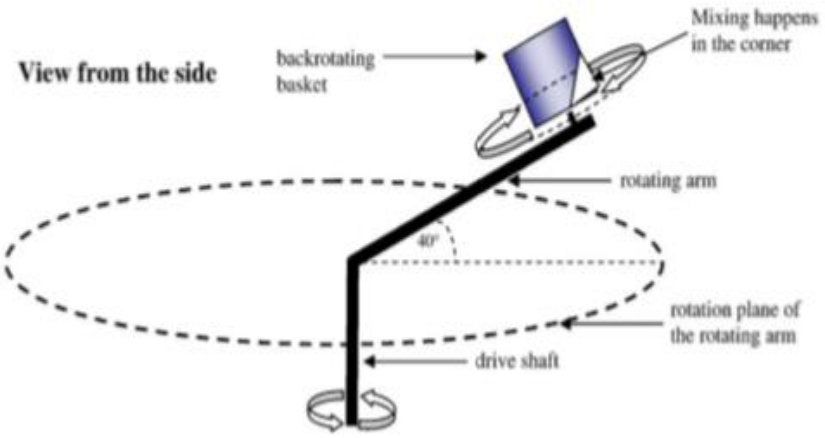


Fig. 7. Schematic illustration of DAC principle[26]

If sufficient adhesion of the sample material on the vial material is presented and the sample material is viscous enough, the latter movement, the inward transport of the sample material, is effective. Both of these factors can affect the transference of energy into the sample material. Therefore, DAC is suitable for homogenization of the viscous material. The product of homogenization is vesicular phospholid gel, which can be diluted to a conventional liposome dispersion.[26]

It was already shown, that the DAC speed, the homogenization time, the lipid concentration and the addition of glass beads are the critical factors for the liposomes size.[26]

The optimal lipid concentration in range from 350 up to 450 mg/ml, the homogenization time of 30 minutes or longer at maximum speed 3540 and the addition of 50 up to 125 weight percent of glass beads (related to total mass of lipid dispersion) result in the desired small liposomes in a highly reproducible manner.[26]

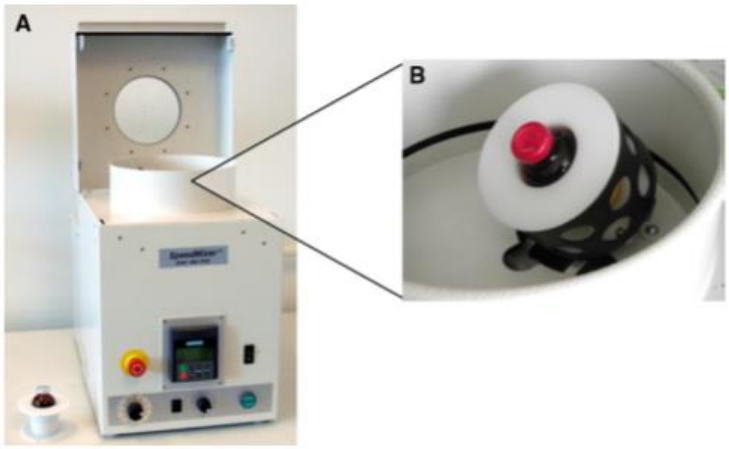


Fig. 8. Dual asymmetric centrifugation, A- DAC with open lid, B- view into the rotation chamber[26]

### 6.3.4 Drug loading into liposomes

Drug loading can be achieved either passively, which means that drug is encapsulated during the liposome formation, or actively, after the liposome formation. Hydrophobic drugs like amphotericin B or taxol, can be incorporated into liposomes during their formation, and the range of uptake and retention is managed by drug-lipid interactions. The trapping efficiency is dependent on the solubility of the drug in the liposome membrane. The passive encapsulation of hydrophilic drugs depend on the ability of liposomes to trap the aqueous medium containing a dissolved drug during the liposome formation. The trapping efficiency is limited by the trapped volume contained in the liposomes and solubility of drug.[27]

## *6.4 Methods used for purification and characterization of liposomes*

### 6.4.1 Size exclusion chromatography

In order to determine encapsulation efficiency of a drug and/or the amount of a drug incorporated within liposomes over time, the external drug in the outer aqueous phase of the liposome dispersion must be removed. One of the most suitable method for this is size exclusion chromatography (gel filtration chromatography).[28]

Gel filtration, or size exclusion chromatography, is a simple chromatography technique, separating molecules according to difference in size as they pass through a gel filtration medium packed in a column. It is based on the fact that in a gel, sufficiently small species (such as ions or drug) have the tendency to enter the pores of gel particles while bigger molecules such as liposomes, may not be able to enter into the pores of the gel at all, see Fig 9. When not entering the pores, the large molecules and particles instead move through the column at the same speed as the aqueous medium used for elution of the column.[29]

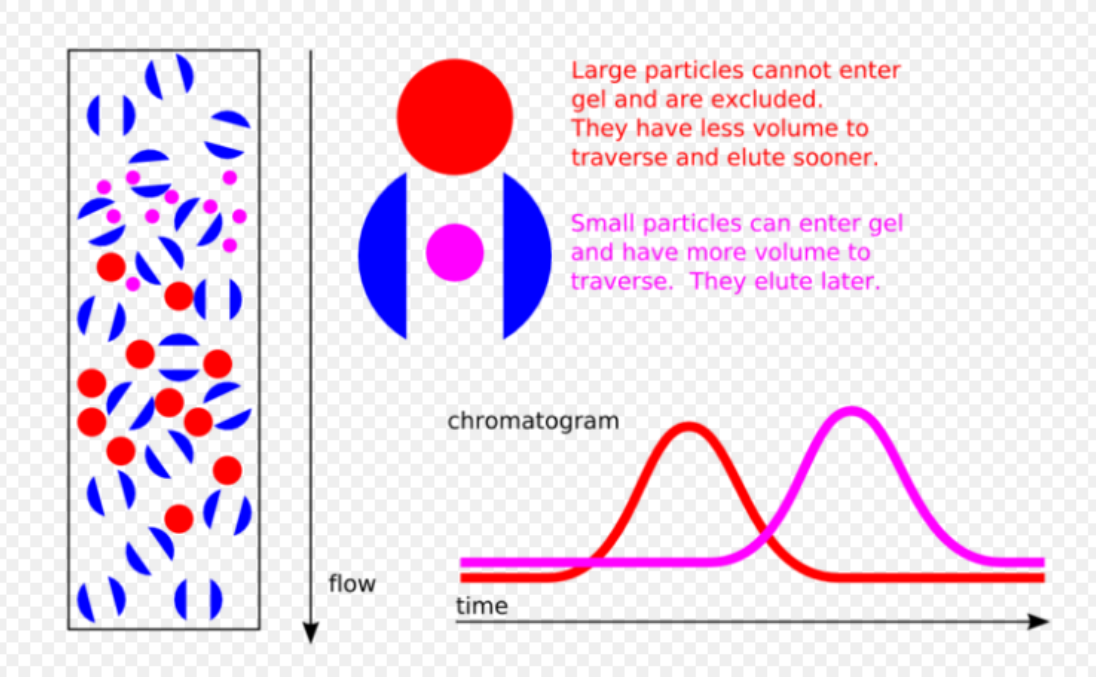


Fig. 9. Description on how SEC works[30]

Sephadex is a gel made from cross-linked dextran with. Different type of sephadex gel vary in degree of cross linking, and hence in their degree of swelling, and their

selectivity for the molecules of different sizes. The sephadex gels have names that start whit a G (i.e. gel), and ends with a number. For example, in Sephadex G-50 "50" stands for the water regain of the gel, in the instance of G-50, 5.0 g water per g dry gel. Sephadex G-50 is suitable for separating large molecules, relative molecular weight (Mr) >30 000, from molecules with Mr under 1 500 mg/ml. That makes it suitable for separating liposomes from a substance in the outer phase.[29]

### 6.4.2 Photon correlation spectroscopy

There are two methods for determining the size of liposomes: the electron microscopic examination and Dynamic Light Scattering, sometimes referred as Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering.

Electron microscopy is considered as one of the most precise method, because it permits to view each individual liposome and enables to obtain accurate information about the profile of a liposome population over the whole range of sizes. However, this technique can be very time-consuming and requires equipment that may not be immediately at hand.[31] In contrast, PCS is simple, automatized and rapid to perform.

PCS measures the Brownian motion of particles, which is a random movement of microscopic particles suspended in liquid caused by collisions with molecules of the surrounding medium. Shining a monochromatic light beam, such as a laser, onto a suspension with particles in this motion causes a Doppler Shift when the light hits the moving particles, changing the wavelength of the light source. Then, it is possible to calculate the size distribution and give a description of the particles motion in the suspending liquid, measuring the diffusion coefficient of the particle and using the autocorrelation function.[31]

This dimension is usually expressed as translational diffusion coefficient, signed as the symbol D. The hydrodynamic radius of the particles is then calculated by the Stokes-Einstein relationship:[31]

$$d(H) = \frac{k \cdot T}{6. \cdot \pi \cdot \eta \cdot D} \qquad (1)$$

Where:

d (H) = hydrodynamic radius (m)

k = Boltzmann's constant ($1.38 \cdot 10^{-23}$ J·K$^{-1}$)

T = absolute temperature (K)

ŋ = viscosity of suspending liquid ($N \cdot s \cdot m^{-2}$)

D = translational diffusion coefficient ($m^2 \cdot s^{-1}$)

It is necessary to keep a constant temperature during the measurement, because the viscosity is strongly temperature-dependent. The translational diffusion coefficient is dependent on the size of particle "core", on the surface structure of the particle and on the concentration and type of ions in medium.[32, 33]

A typical PCS instrument consists of six main components Fig 10. Laser (1) provides the light source for illuminating the sample particles within a cell (2). The scattered light is measured by the detector (3), which is positioned at 173° or 90° (according to different kind of instrument). The intensity of scattered light must be within a certain range, otherwise the detector is not able to measure it correctly. The intensity of the laser source and the intensity of scattering are regulated by attenuator (4). The scattering intensity signal is then passed to a correlator, which compares the scattering intensity at successive time intervals to derive it the rate at which intensity is varying. Data from correlator are sent to the computer (6). Special software analyses these data and calculates the size of particles in the sample.[34]

In this thesis, Zetasizer Nano ZS was used.

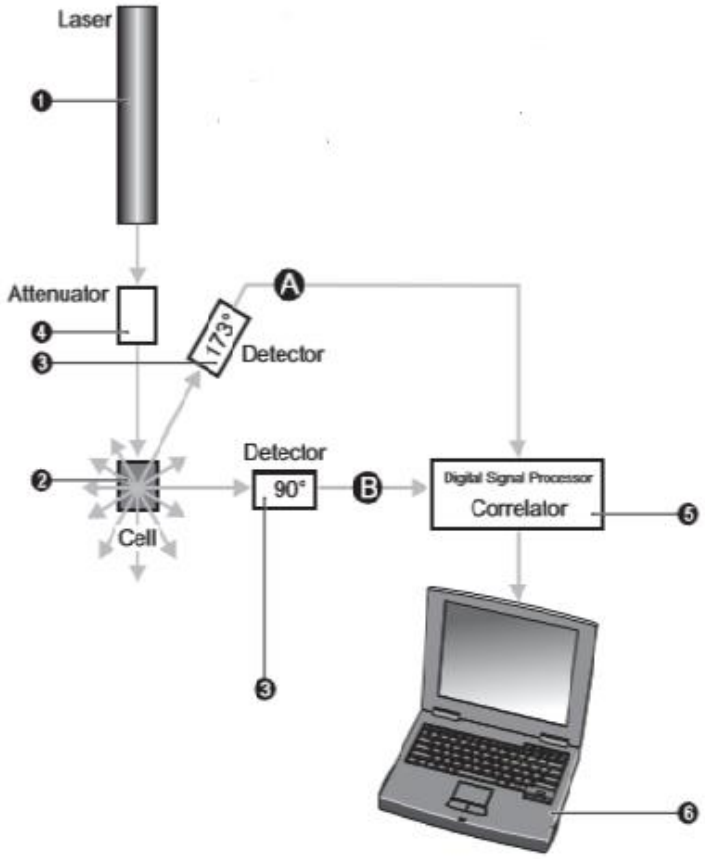


Fig. 10. Main components of Zetasizer Nano ZS[34]

## 6.5 Wilson's disease and triethylenetetramine

Triethylenetetramine (TETA) is an established orphan drug commonly used for the treatment of Wilson's disease (WD). [35] Wilson's disease is an autosomal recessive inherited disorder caused by abnormal cooper metabolism and is also lethal if left without diagnosis and prompt treatment. Illness is manifested by cooper accumulation in many organs of patient, mainly the liver, cornea and brain. Depending on what parts are affected, illness presents as various psychiatric or neurologic symptoms and hepatic dysfunction (acute or chronic hepatitis, cirrhosis, the Kayser-Fleischer ring, cardiomyopathy, Parkinson-like symptoms, etc). Treatment is focused on achieving a negative cooper balance either with chelators, currently available drugs include D-Penicillamine, TETA and Ammonium tetrathiomolybdate, or Zinc which helps prevent cooper absorption from intestinal tract or both.[36, 37]

TETA is $Cu^{II}$- selective chelator, with promising clinical applications and implications. It has been used as the second line treatment for patients with WD, who

developed toxic reactions to penicillamine. TETA increases urinary Cu excretion and also decreases intestinal cooper absorption. TETA is poorly absorbed with relatively low bioavailability (from 8 % to 30 %) and is widely distributed into different tissues with higher concentration measured in liver, kidney and heart. Two metabolites of TETA have been identified in human plasma and urine, being N1-acetyltriethylenetetramine (MAT) and N1, N10-diacetyltriethylenetetramine (DAT). Although TETA has been used in treatment for decades, information about its pharmacology is still limited and any comprehensive review does not exist to this date.[35, 38]

However, it has been suggested, that TETA may be a promising anticancer agent because it inhibits telomerase and has anti-angiogenesis properties.[39, 40] TETA has also been used in clinical trials for the treatment of diabetic heart failure and has shown to be effective in patients with diabetes type 2 presenting cardiac complication.[41]

### 6.5.1 Detection of TETA

TETA is a synthetic analog of polyamine compounds spermidine and spermine (Fig. 11) and with four nitrogen groups acts as a perfect $Cu^{II}$- selective chelator.

TETA itself has a very polar structure, does not elute efficiently from HPLC column and possesses no UV-VIS absorption or emits fluorescence. Thus for its analysis using high performance liquid chromatography (HPLC) with conventional UV or fluorescence detectors, the drug has to be derivatized with a suitable reagent.[35] Derivatization is a chemical process that leads to new products with improved chromatographic properties and can be done prior to the separation step (prederivatization) or as a postcolumn derivatization.[42] Recently, the reaction with the derivatizing agent 9-fluorenylmethyl chloroformate (FMOC-Cl) has shown to be as the most suitable precolumn derivatizing method. In this process, substitution of four hydrogen atoms in the amino group occurs in the TETA molecule by aromatic rings containing alternating double bonds, Fig. 12. The main advantage of using FMOC, is in its capability to label not only TETA, but also its two major metabolites. Any other to this date published quantification procedure is not capable of simultaneous determination of TETA, MAT, DAT.[43, 44]

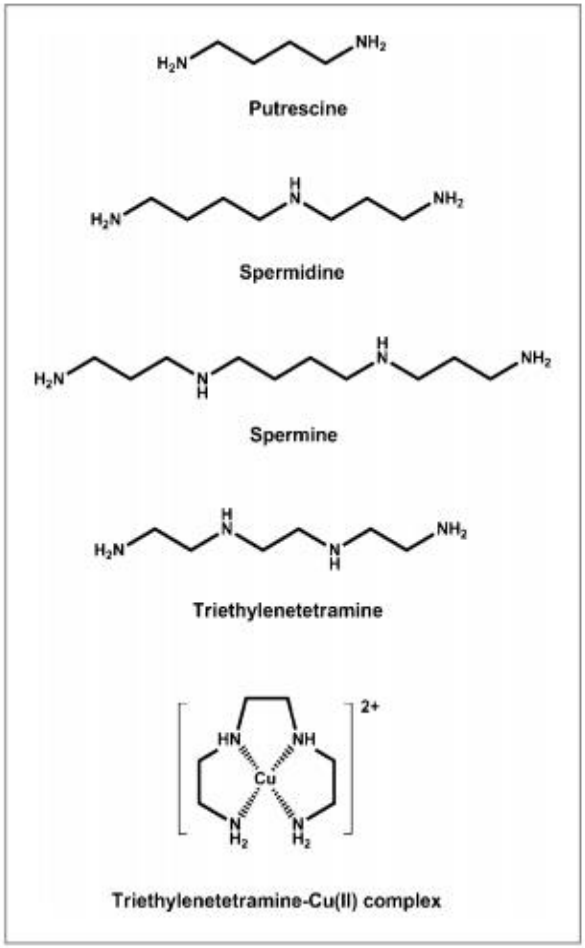


Fig. 11. Structure of polyamines, TETA and TETA-$Cu^{II}$ complex[35]

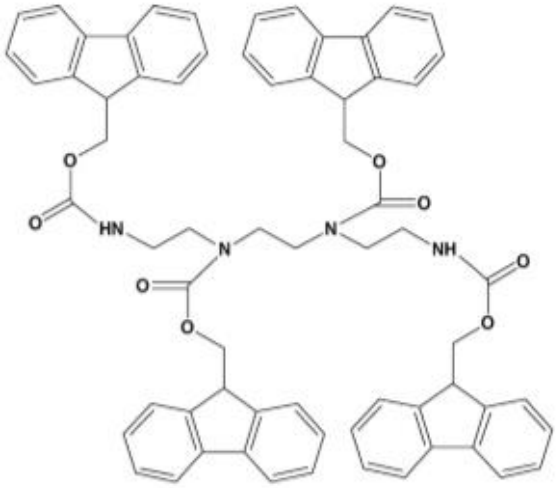

Fig. 12. Completely derivatized TETA[44]

# 7 Experimental section

## *7.1 Chemicals*

| Name | Company | Purity |
|---|---|---|
| 1,2- distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG-2000-PE) | Avanti Polar Lipids, Inc. | >99 % |
| 9-Fluorenylmethylchloroformate chloride | Sigma-Aldrich Chemie GmbH | 97 % |
| Acetonitrile | Sigma-Aldrich Chemie GmbH | >99 % |
| Chloroform | central supply of Univeristy | ≥ 99 % |
| Cholesterol | Cholesterol Sigma-Aldrich Chemie GmbH | ≥ 99 % |
| Dubbeco's Phospate Buffered Saline (DPBS) | Gibco® by life technologies | n.a. |
| Egg-phosphatidylcholine | Lipoid GmbH | ~80 % |
| Ethanol | central supply of University | 99 % |
| Isopropanol | central supply of University | 99,9 % |
| Methanol | VWR International GmbH | 99,8 % |
| Triethylentetramin Monohydrat (TETA) | Sigma-Aldrich Chemie GmbH | 98 % |
| Trifluoroacetic acid | Merck KGaA | ≥ 98 % |
| Water for HPLC | VWR International GmbH | n.a. |

**Buffers and solutions**

| Name | Ingredients | Concentration |
|---|---|---|
| Borax ph9 | NaOH<br>H3BO3 | 10 M |
| Buffer pH3 | Citric acid<br>NaCl<br>NaOH | 0,1 M |
| FMOC | 9-flouorenylmethylchloroformate<br>Acetonitrile | 50 mM |

## *7.2 Instruments and materials*

| Designation | Details | Producer |
|---|---|---|
| Eppendorf tubes | 1.5 ml, 2 ml | Eppendorf, Hamburg, Germany |
| Glass beads | ø 0,75-1 mm | Roth GmbH & Co. KG |
| HPLC-Column | Acclaim® 120, c18 5µm, 120 Å, 4,6 x 250 mm | Dionex Corporation |
| HPLC-Column | Luna 3µ C18 150 × 3.0-mm | Phenomenex |
| Plastic cuvettes | Rotilabo® , PS | Roth GmbH & Co. KG |
| Polyethylen frits | ø 0,5 & 2 cm | Sigma-Aldrich Chemie GmbH |
| Sephadex | G-50 Fine | GE Healthcare GmbH |
| Speedmixer | DAC 150 FVZ | Hauschild Engineering GmBH |
| Ultimate 3000 | HPLC device | Dionex Corporation |
| Zetasizer Nano ZS | Particle Analyser | Malvern Instruments Ltd |

## *7.3 Methods used in formulation and evaluation of liposomes*

### 7.3.1 Production of liposomes by film method

For preparing liposomes, egg-phosphatidylcholine (EPC), cholesterol and 1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt), (PEG-2000-PE) were used.

I prepared stock solution of each lipid in the volume of 10 ml in advance. Each lipid was separately dissolved in solvent mixture of chloroform and methanol (9:1) to achieve the desired concentration as shown in Tab. 1.

Tab. 1. Parameters of stock solutions

| Lipid | $M_w$ (g/mol) | Concentration (mmol/l) |
|---|---|---|
| EPC | 760 | 100 |
| Cholesterol | 386.7 | 100 |
| PEG-2000-PE | 2805.5 | 10 |

Using the stock solutions, I prepared the mixture of lipids in the 2 ml eppendorf tube containing 55 % of EPC, 40 % of Chol and 5 % of PEG-2000-PE calculated for the final volume of liposomal solution with TETA after dual asymmetric centrifugation (DAC), which was 400 µl as shown in Tab. 2.

Tab. 2. Parameters of the lipid mixture

| Volume of the resulting liposomal dispersion after DAC 400 µl | | | |
|---|---|---|---|
| Lipid | Ratio (%) | Volume (µl) | Weight (mg) |
| EPC | 55 | 220 | 16.72 |
| Chol | 40 | 160 | 6.18 |
| PEG-2000-PE | 5 | 200 | 5.61 |

Eppendorf tubes were positioned into the water bath at 50° C and the tip of nitrogen stream was placed near the liquid level. The solvent was vaporized in the range of 10 to 15 minutes. After evaporation of the organic solvent a lipid film was created on the

wall of the tube. For removing residual solvent, the film lipid was positioned under the vacuum in the desiccator for one hour.

### 7.3.2 Passive loading of triethylenetetramine into liposomes

The method of dual asymmetric centrifugation (DAC) was used for the encapsulation of TETA into liposomes.

Triethylenetetramine Monohydrat ($M_w$ -146.23 g/mol) was dissolved in 5 ml volumetric flask in Phospate Buffered Saline (PBS) and/or in a buffer solution pH3, respectively, to achieve the concentration of 2.5 mol/l.

The mass of the dried lipid film was calculated and an equal amount of glass beads (ø 0.75–1 mm) was added. I added the solution of TETA in buffer (PBS and/or pH3). Then, the mixture was subjected in three steps to dual asymmetric centrifugation (DAC) according to the schedule described in Tab. 3.

In the first step, the 1.5fold of the mass of dried lipid film (43 µl) of 2.5 mol/l TETA solution in the buffer solution pH3 and/or in PBS, respectively, was added and centrifuged for 30 min at 3540 rpm.

Subsequently, the 2.5fold mass of the mass of dried lipid film (71 µl) of the same triethylenetetramine (TETA) solution was added and subjected to another DAC run for 5 min at 3540 rpm.

In the last (third) centrifugation step, the 2.5fold mass of the mass of dried lipid film (71 µl) of the PBS buffer (without TETA) was added and centrifugated for 5 min at 3540 rpm again.

Tab. 3. Parameters of DAC procedure

| | |
|---|---|
| 1st Run of DAC | 43 µl of Buffer solution with TETA |
| 2nd Run of DAC | 71 µl of Buffer solution with TETA |
| 3rd Run of DAC | 71 µl of PBS |

The resulting liposomal dispersion was diluted (filled up) with PBS buffer to obtain 400 µl of the final dispersion.

### 7.3.3 Purification of liposomal dispersion

Separation of the free triethylenetetramine and the liposome-encapsulated TETA was achieved with the size exclusion chromatography (SEC).

The small amount of Sephadex™ G-50 Fine was mixed with PBS and kept to swell for 10 minutes. The column was filled with this mixture and polyethylene membrane filters were placed at the both ends of the column. The column was flushed with 15 ml of PBS after every using.

The liposomal dispersion with TETA was gently added to the column followed by PBS flow. The movement of this dispersion was possible to watch visually thanks to its white colouring. The middle of eluate, containing only liposomal TETA, was collected in the eppendorf tube as illustrated in Fig. 1 and stored in the refrigerator at 2- 8° C.

I used two different methods for the purification of liposomes: a one column method, and a two columns method.

In the one column method (called **purification 1** in this thesis), I prepared 400 µl of the liposomal dispersion and then I purified 60 µl of this dispersion at day 1 using the size exclusion chromatography (SEC). The same procedure, I repeated at time 2, 3, 4, 5, and 8 day.

In the two columns method (called **purification 2** in this thesis), I prepared 400 µl of the liposomal dispersion and then I purified 360 µl of this dispersion at day 1 using the SEC. 60 µl of this purified dispersion was taken and purified at time 2, 3, 4, 5, and 8 day.

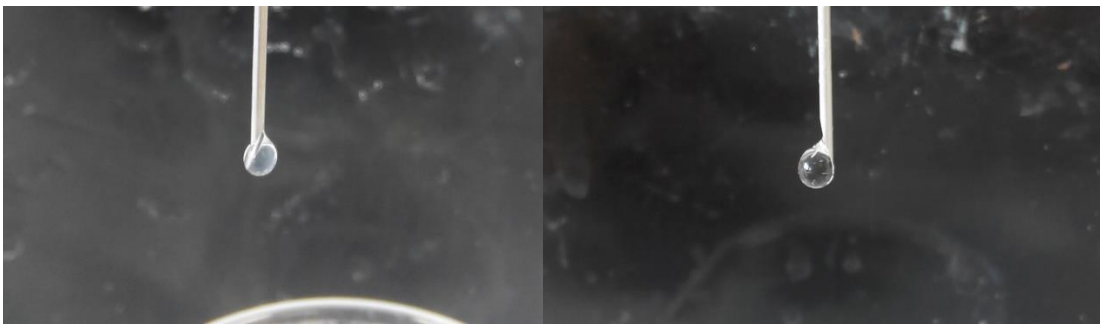

Fig. 13. The eluate of liposomes with TETA and without TETA

I repeated this procedure three times for each experimental run. For better orientation, I marked samples with codes, where S means sample (S1 – S3), PH means pH

(3 or 7), P means purification (P1 or P2). For example: S1PH3P1 means sample 1, pH3 and purification 1.

Tab. 4. The Scheme of experiment

| | | |
|---|---|---|
| pH3 | P1 | S1 |
| | | S2 |
| | | S3 |
| | P2 | S1 |
| | | S2 |
| | | S3 |
| ph7 | P1 | S1 |
| | | S2 |
| | | S3 |
| | P2 | S1 |
| | | S2 |
| | | S3 |

### 7.3.4 Evaluation of the size and the size distribution of liposomes

Liposome dispersions after the SEC were analysed by photon correlation spectroscopy (PCS) with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom) using a 633 nm red laser.

Samples were diluted in PBS prior to measurement in the ratio 1:1000. To determine the size and the size distribution of the liposomal dispersions in the PBS buffer, the automatic mode with temperature of 25° C was used and the average of three measurements was taken.

The results were described as Z-average and polydispersity index (PdI) in Tab. 5-16.

## *7.4 Methods used in evaluation of encapsulation efficiency*

### 7.4.1 High performance liquid chromatography (HPLC)

The concentration of cholesterol before the SEC, cholesterol after the SEC and TETA after the SEC were quantitatively analysed using HPLC ((Ultimate 3000, Dionex) with UV-VIS detection. To manipulate data, the program Chromeleon Client Version 6.80 was used.

The relationships between absorbance and the concentration (calibration curves) were linear over the concentration range of 1-500 μM for TETA and 0.01- 5000 μM for cholesterol, in both cases with a correlation coefficient of 0.99 or better and an intercept value not statistically different from zero. The lower limit of qualification for TETA was 1 μM, for cholesterol 0.01 μM .The upper limit of qualification was 500 μM in case of TETA and 5000 μM for cholesterol.

#### 7.4.1.1 Determination of cholesterol concentration in liposomes

The separation was performed in Reversed-Phase-18 (Acclaim® 120, c18, 5μm, 120 Å, 4.6 x 250 mm, Dionex) at 45° C and the peak was detected at 205 nm. The retention time was approximately 10.5 min and overall runtime was 16. Methanol and water, each of them with 0.05 % trifluoroacetic acid, were used as mobile phases with isocratic elution and the flow rate was 2ml/min.

In order to detect the cholesterol concentration in liposomes, the liposomes must first be destroyed. This was achieved by dilution with methanol. For estimation of the chol concentration before the SEC, the original liposomal dispersion was diluted in ratio 1:100 to achieve the total volume of 500 μl.

To estimate concentration after the SEC, the dispersion of the cleaned liposomes was diluted 1:10 to the total volume of 500 μl again. This procedure was repeated at each out of the days according to the methods of purification mentioned above in the Section 6.3.3. The amount of cholesterol in every sample was determined.

I took 100 μl of this diluted dispersion and placed it into the HPLC glass vials three times. Before the beginning of chromatography, the samples were heated at 35° C and an aliquot of 50 μl of each sample solution was directly injected into the HPLC system. The average of 3 measurements was taken, Tab. 17–20. The detected concentrations of cholesterol before the SEC and after the SEC were used for the estimation of the encapsulation efficiency of TETA in the liposomes.

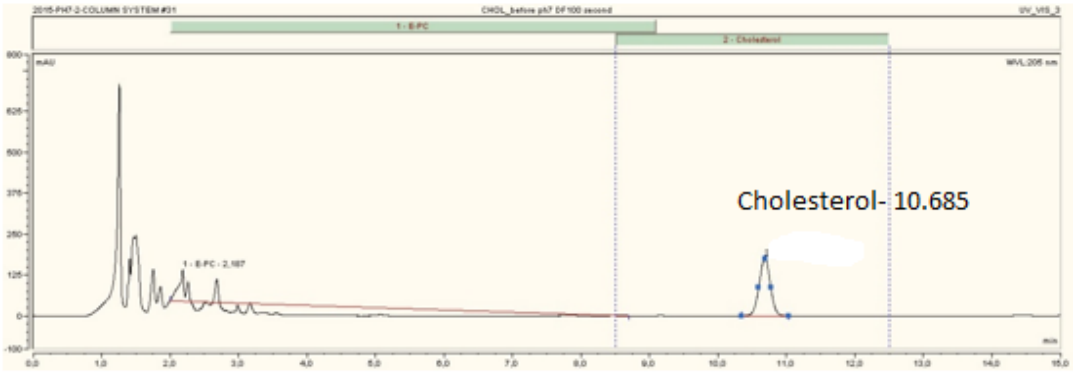


Fig. 14. An example of a typical chromatogram of cholesterol

### 7.4.1.2 Determination of TETA concentration in liposomes

The chromatographic separation of TETA was achieved using a Luna 3µ C18 150 × 3.0-mm reversed phase column (Phenomenex, Auckland) maintained at 25°C with a linear gradient mobile phase. Acetonitrile (ACN, mobile phase A) and water (mobile phase C), again each with 0.05 % trifluoroacetic acid, were used. The linear gradient program was: 0 min, 50 % A; 7 min, 70 % A; 7.5 min, 80 % A; 17.0 min, 80 % A; 19 min, 95 % A; 23 min, 95 % A; 25 min, 50 % A; 35 min, 50 % A. Overall runtime was 36 minutes with the flow rate being maintained at 0.5 mL/min. The retention time of TETA was approximately 25.5 min and the peak was detected at 295 nm.

The samples were treated as follows: to 45 µl of borax buffer in an eppendorf tube, 5 µl of the liposomal dispersion with TETA and 450 µl of the FMOC solution in ACN (50 mmol/) were added. The samples were shaken for 30 minutes to complete derivatization reaction. After shaking, I took 100 µl three times and placed it into the HPLC glass vials.

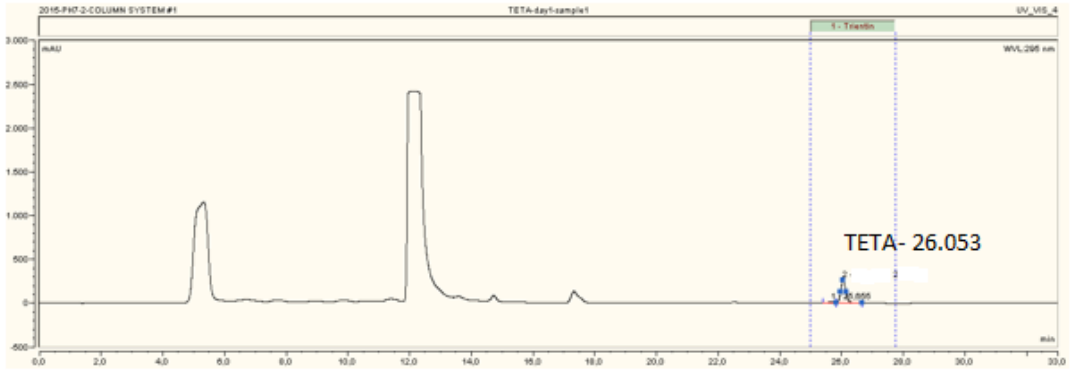


Fig. 15. An example of typical chromatogram of TETA

Again, the amount of TETA in the sample is proportional to the area under the peak. The detected concentrations were used for the estimation of the encapsulation efficiency of TETA in the liposomes.

### 7.4.2 Estimation of the encapsulation efficiency of TETA in liposomes

From the detected concentrations mentioned above, I calculated the ratio of TETA/ cholesterol before the SEC and the ratio of TETA/cholesterol after the SEC at each day of purification. The encapsulation efficiency (loading) of TETA in liposomes at day 1, 2, 3, 4, 5 and 8 was determined as the percentage ratio of the ratio TETA encapsulated in liposomes before the size exclusion chromatography and the ratio TETA encapsulated in liposomes after the SEC.

The encapsulation efficiency (EE) was calculated with the following formula:

$$EE\ (\%) = \frac{ratio\ of\ TETA\ in\ liposomes\ after\ SEC}{ratio\ of\ TETA\ in\ liposomes\ before\ SEC} \cdot 100 \qquad (2)$$

The original concentration of TETA in liposomes was calculated using the following formula:

$$c = \frac{a+b}{d} \cdot e \qquad (3)$$

Where: C = theoretical (calculated) concentration of TETA expected in liposomes (mmol/l)

a = the volume of TETA solution used in the first run by DAC (µl)

b = the volume of TETA solution used in the second run by DAC (µl)

d = the total volume of the original liposomal dispersion (µl)

e = the concentration of TETA in the buffer solution (mmol/l) = 2.5 mol/l

# 8 Results

## *8.1 The measured values of liposome size and polydispersity*

All liposomal formulation I prepared for each experiment 3 times. Each sample was measured by PCS to verify the size and polydispersity of the liposomes after the SEC. During a single measuring each sample was measured 3 x 10 times. The results in Tables are expressed as a Z-average diameter d (nm) and a mean (n = 30) with standard deviation SD. The same way, polydispersity index PdI was estimated. The mean for three samples (S1-S3) are shown in Fig. 16–17.

Tab. 5. The measured values of liposome size and polydispersity for S1PH3P1

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 151.7 | 155.3 | 3.412 | 0.116 | 0.16 | 0.038 |
| 2 | | 158.5 | | | 0.181 | | |
| 3 | | 155.6 | | | 0.182 | | |
| 4 | Day2 | 146.5 | 145.1 | 1.250 | 0.109 | 0.13 | 0.025 |
| 5 | | 144.5 | | | 0.133 | | |
| 6 | | 144.2 | | | 0.159 | | |
| 7 | Day3 | 170.6 | 163.7 | 6.709 | 0.189 | 0.19 | 0.011 |
| 8 | | 163.3 | | | 0.179 | | |
| 9 | | 157.2 | | | 0.200 | | |
| 10 | Day4 | 146.0 | 143.1 | 2.610 | 0.169 | 0.14 | 0.023 |
| 11 | | 142.2 | | | 0.135 | | |
| 12 | | 141.0 | | | 0.124 | | |
| 13 | Day5 | 147.3 | 142.1 | 4.620 | 0.162 | 0.15 | 0.012 |
| 14 | | 140.7 | | | 0.159 | | |
| 15 | | 138.4 | | | 0.140 | | |
| 16 | Day8 | 138.2 | 137.2 | 0.839 | 0.141 | 0.15 | 0.004 |
| 17 | | 136.8 | | | 0.149 | | |
| 18 | | 136.7 | | | 0.147 | | |

Tab. 6. The measured values of liposome size and polydispersity for S2PH3P1

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 141.7 | 139.4 | 1.966 | 0.113 | 0.14 | 0.020 |
| 2 | | 138.2 | | | 0.145 | | |
| 3 | | 138.4 | | | 0.149 | | |
| 4 | Day2 | 142.8 | 138.4 | 3.790 | 0.130 | 0.13 | 0.012 |
| 5 | | 136.5 | | | 0.120 | | |
| 6 | | 136.0 | | | 0.143 | | |
| 7 | Day3 | 140.3 | 136.4 | 3.568 | 0.162 | 0.16 | 0.006 |
| 8 | | 135.6 | | | 0.170 | | |
| 9 | | 133.3 | | | 0.159 | | |
| 10 | Day4 | 144.2 | 140.3 | 3.460 | 0.170 | 0.17 | 0.004 |
| 11 | | 139.1 | | | 0.163 | | |
| 12 | | 137.6 | | | 0.164 | | |
| 13 | Day5 | 136.6 | 135.1 | 2.100 | 0.136 | 0.13 | 0.008 |
| 14 | | 136.0 | | | 0.140 | | |
| 15 | | 132.7 | | | 0.125 | | |
| 16 | Day8 | 136.7 | 135.0 | 1.572 | 0.122 | 0.13 | 0.017 |
| 17 | | 133.6 | | | 0.153 | | |
| 18 | | 134.7 | | | 0.126 | | |

Tab. 7. The measured values of liposome size and polydispersity for S3PH3P1

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 151.2 | 148.7 | 2.914 | 0.169 | 0.16 | 0.018 |
| 2 | | 149.4 | | | 0.142 | | |
| 3 | | 145.5 | | | 0.177 | | |
| 4 | Day2 | 154.4 | 148.6 | 5.437 | 0.160 | 0.17 | 0.011 |
| 5 | | 147.9 | | | 0.168 | | |
| 6 | | 143.6 | | | 0.182 | | |
| 7 | Day3 | 145.3 | 142.6 | 2.810 | 0.147 | 0.15 | 0.018 |
| 8 | | 142.9 | | | 0.135 | | |
| 9 | | 139.7 | | | 0.170 | | |
| 10 | Day4 | 144.8 | 141.9 | 2.685 | 0.163 | 0.17 | 0.017 |
| 11 | | 139.5 | | | 0.151 | | |
| 12 | | 141.4 | | | 0.185 | | |
| 13 | Day5 | 138.9 | 137.2 | 1.582 | 0.145 | 0.16 | 0.011 |
| 14 | | 136.8 | | | 0.161 | | |
| 15 | | 135.8 | | | 0.166 | | |
| 16 | Day8 | 145.2 | 143.0 | 2.150 | 0.135 | 0.16 | 0.023 |
| 17 | | 143.0 | | | 0.153 | | |
| 18 | | 140.9 | | | 0.180 | | |

Tab. 8. The measured values of liposome size and polydispersity for S1PH3P2

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 154.7 | 151.4 | 2.996 | 0.15 | 0.16 | 0.031 |
| 2 | | 150.5 | | | 0.14 | | |
| 3 | | 148.9 | | | 0.20 | | |
| 4 | Day2 | 151.3 | 148.5 | 2.458 | 0.15 | 0.15 | 0.018 |
| 5 | | 147.7 | | | 0.16 | | |
| 6 | | 146.6 | | | 0.13 | | |
| 7 | Day3 | 154.2 | 150.2 | 3.534 | 0.15 | 0.14 | 0.011 |
| 8 | | 148.9 | | | 0.13 | | |
| 9 | | 147.5 | | | 0.15 | | |
| 10 | Day4 | 162.7 | 154.1 | 7.565 | 0.14 | 0.13 | 0.009 |
| 11 | | 150.9 | | | 0.12 | | |
| 12 | | 148.6 | | | 0.13 | | |
| 13 | Day5 | 152.9 | 149.4 | 3.083 | 0.15 | 0.14 | 0.004 |
| 14 | | 148.4 | | | 0.14 | | |
| 15 | | 147.0 | | | 0.14 | | |
| 16 | Day8 | 165.3 | 151.1 | 12.324 | 0.12 | 0.14 | 0.016 |
| 17 | | 144.8 | | | 0.16 | | |
| 18 | | 143.2 | | | 0.14 | | |

Tab. 9. The measured values of liposome size and polydispersity for S2PH3P2

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 194.4 | 185.7 | 8.554 | 0.16 | 0.19 | 0.032 |
| 2 | | 185.4 | | | 0.22 | | |
| 3 | | 177.3 | | | 0.21 | | |
| 4 | Day2 | 179.6 | 174.7 | 4.272 | 0.24 | 0.20 | 0.028 |
| 5 | | 172.2 | | | 0.18 | | |
| 6 | | 172.2 | | | 0.20 | | |
| 7 | Day3 | 179.9 | 173.9 | 5.254 | 0.20 | 0.19 | 0.018 |
| 8 | | 171.4 | | | 0.17 | | |
| 9 | | 170.3 | | | 0.20 | | |
| 10 | Day4 | 173.9 | 170.0 | 4.110 | 0.17 | 0.18 | 0.013 |
| 11 | | 170.3 | | | 0.20 | | |
| 12 | | 165.7 | | | 0.19 | | |
| 13 | Day5 | 178.3 | 173.5 | 4.222 | 0.29 | 0.25 | 0.056 |
| 14 | | 170.5 | | | 0.19 | | |
| 15 | | 171.6 | | | 0.29 | | |
| 16 | Day8 | 165.1 | 161.2 | 4.004 | 0.15 | 0.18 | 0.021 |
| 17 | | 161.4 | | | 0.19 | | |
| 18 | | 157.1 | | | 0.19 | | |

Tab. 10. The measured values of liposome size and polydispersity for S3PH3P2

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 179.6 | 171.8 | 6.829 | 0.13 | 0.11 | 0.019 |
| 2 | | 168.9 | | | 0.10 | | |
| 3 | | 166.9 | | | 0.10 | | |
| 4 | Day2 | 165.3 | 163.5 | 1.800 | 0.12 | 0.13 | 0.024 |
| 5 | | 163.5 | | | 0.15 | | |
| 6 | | 161.7 | | | 0.11 | | |
| 7 | Day3 | 173.1 | 167.9 | 4.660 | 0.12 | 0.13 | 0.010 |
| 8 | | 166.5 | | | 0.14 | | |
| 9 | | 164.1 | | | 0.13 | | |
| 10 | Day4 | 164.3 | 162.6 | 2.916 | 0.13 | 0.14 | 0.009 |
| 11 | | 164.2 | | | 0.14 | | |
| 12 | | 159.2 | | | 0.15 | | |
| 13 | Day5 | 165.4 | 163.4 | 1.721 | 0.13 | 0.14 | 0.005 |
| 14 | | 162.2 | | | 0.14 | | |
| 15 | | 162.7 | | | 0.14 | | |
| 16 | Day8 | 158.3 | 156.2 | 1.819 | 0.14 | 0.13 | 0.025 |
| 17 | | 155.1 | | | 0.16 | | |
| 18 | | 155.2 | | | 0.11 | | |

Tab. 11. The measured values of liposome size and polydispersity for S1PH7P1

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 153.9 | 149.8 | 3.629 | 0.18 | 0.16 | 0.014 |
| 2 | | 148.5 | | | 0.15 | | |
| 3 | | 147.0 | | | 0.16 | | |
| 4 | Day2 | 151.2 | 148.9 | 2.787 | 0.16 | 0.16 | 0.013 |
| 5 | | 149.7 | | | 0.17 | | |
| 6 | | 145.8 | | | 0.15 | | |
| 7 | Day3 | 152.5 | 149.6 | 2.762 | 0.15 | 0.14 | 0.013 |
| 8 | | 149.3 | | | 0.12 | | |
| 9 | | 147.0 | | | 0.15 | | |
| 10 | Day4 | 161.0 | 155.4 | 4.850 | 0.13 | 0.14 | 0.012 |
| 11 | | 152.6 | | | 0.14 | | |
| 12 | | 152.6 | | | 0.16 | | |
| 13 | Day5 | 156.4 | 150.4 | 5.254 | 0.14 | 0.15 | 0.019 |
| 14 | | 147.9 | | | 0.13 | | |
| 15 | | 146.8 | | | 0.17 | | |
| 16 | Day8 | 161.6 | 158.8 | 4.706 | 0.15 | 0.17 | 0.031 |
| 17 | | 161.5 | | | 0.15 | | |
| 18 | | 153.4 | | | 0.20 | | |

Tab. 12: The measured values of liposome size and polydispersity for S2PH7P1

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 144.6 | 141.8 | 3.262 | 0.16 | 0.16 | 0.006 |
| 2 | | 142.5 | | | 0.17 | | |
| 3 | | 138.2 | | | 0.16 | | |
| 4 | Day2 | 146.6 | 142.4 | 3.731 | 0.17 | 0.16 | 0.014 |
| 5 | | 141.3 | | | 0.17 | | |
| 6 | | 139.4 | | | 0.14 | | |
| 7 | Day3 | 138.4 | 138.4 | 0.252 | 0.16 | 0.16 | 0.005 |
| 8 | | 138.7 | | | 0.16 | | |
| 9 | | 138.2 | | | 0.16 | | |
| 10 | Day4 | 144.1 | 141.1 | 2.676 | 0.13 | 0.15 | 0.018 |
| 11 | | 138.9 | | | 0.17 | | |
| 12 | | 140.4 | | | 0.15 | | |
| 13 | Day5 | 156.9 | 149.6 | 6.700 | 0.16 | 0.15 | 0.014 |
| 14 | | 148.3 | | | 0.15 | | |
| 15 | | 143.7 | | | 0.14 | | |
| 16 | Day8 | 139.7 | 138.0 | 1.480 | 0.15 | 0.15 | 0.008 |
| 17 | | 137.0 | | | 0.16 | | |
| 18 | | 137.3 | | | 0.15 | | |

Tab. 13: The measured values of liposome size and polydispersity S3PH7P1

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 143.6 | 140.7 | 2.572 | 0.29 | 0.21 | 0.068 |
| 2 | | 139.6 | | | 0.17 | | |
| 3 | | 138.8 | | | 0.17 | | |
| 4 | Day2 | 141.3 | 138.5 | 2.905 | 0.15 | 0.15 | 0.010 |
| 5 | | 138.7 | | | 0.16 | | |
| 6 | | 135.5 | | | 0.14 | | |
| 7 | Day3 | 142.7 | 140.6 | 1.960 | 0.17 | 0.16 | 0.015 |
| 8 | | 138.8 | | | 0.14 | | |
| 9 | | 140.4 | | | 0.17 | | |
| 10 | Day4 | 147.1 | 144.1 | 2.574 | 0.13 | 0.14 | 0.010 |
| 11 | | 142.8 | | | 0.15 | | |
| 12 | | 142.5 | | | 0.14 | | |
| 13 | Day5 | 141.3 | 137.6 | 3.201 | 0.14 | 0.14 | 0.004 |
| 14 | | 135.4 | | | 0.14 | | |
| 15 | | 136.2 | | | 0.14 | | |
| 16 | Day8 | 136.3 | 135.6 | 0.907 | 0.14 | 0.14 | 0.004 |
| 17 | | 136.0 | | | 0.14 | | |
| 18 | | 134.6 | | | 0.14 | | |

Tab. 14: The measured values of liposome size and polydispersity for S1PH7P2

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 151.7 | 150.3 | 1.350 | 0.19 | 0.17 | 0.021 |
| 2 | | 150.3 | | | 0.18 | | |
| 3 | | 149.0 | | | 0.15 | | |
| 4 | Day2 | 187.1 | 163.9 | 20.206 | 0.36 | 0.24 | 0.098 |
| 5 | | 154.1 | | | 0.20 | | |
| 6 | | 150.4 | | | 0.18 | | |
| 7 | Day3 | 288.8 | 264.6 | 27.112 | 0.39 | 0.33 | 0.048 |
| 8 | | 269.7 | | | 0.30 | | |
| 9 | | 235.3 | | | 0.31 | | |
| 10 | Day4 | 149.0 | 147.5 | 1.419 | 0.20 | 0.20 | 0.007 |
| 11 | | 146.2 | | | 0.20 | | |
| 12 | | 147.2 | | | 0.19 | | |
| 13 | Day5 | 159.1 | 157.9 | 6.834 | 0.04 | 0.11 | 0.110 |
| 14 | | 164.0 | | | 0.23 | | |
| 15 | | 150.5 | | | 0.05 | | |
| 16 | Day8 | 162.8 | 162.5 | 3.113 | 0.16 | 0.20 | 0.048 |
| 17 | | 165.4 | | | 0.20 | | |
| 18 | | 159.2 | | | 0.25 | | |

Tab. 15: The measured values of liposome size and polydispersity for S2PH7P2

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 142.3 | 140.2 | 1.888 | 0.19 | 0.19 | 0.006 |
| 2 | | 138.6 | | | 0.19 | | |
| 3 | | 139.8 | | | 0.18 | | |
| 4 | Day2 | 141.5 | 138.5 | 2.706 | 0.24 | 0.19 | 0.043 |
| 5 | | 137.6 | | | 0.16 | | |
| 6 | | 136.3 | | | 0.16 | | |
| 7 | Day3 | 171.2 | 171.0 | 12.252 | 0.31 | 0.29 | 0.027 |
| 8 | | 158.6 | | | 0.30 | | |
| 9 | | 183.1 | | | 0.26 | | |
| 10 | Day4 | 143.8 | 141.1 | 2.401 | 0.21 | 0.18 | 0.030 |
| 11 | | 140.1 | | | 0.16 | | |
| 12 | | 139.3 | | | 0.16 | | |
| 13 | Day5 | 136.4 | 135.4 | 0.850 | 0.15 | 0.16 | 0.015 |
| 14 | | 135.1 | | | 0.15 | | |
| 15 | | 134.8 | | | 0.17 | | |
| 16 | Day8 | 154.4 | 149.9 | 5.272 | 0.22 | 0.19 | 0.027 |
| 17 | | 151.2 | | | 0.20 | | |
| 18 | | 144.1 | | | 0.16 | | |

Tab. 16: The measured values of liposome size and polydispersity for S3PH7P2

| Record Number | | Z-Average | | | PdI | | |
|---|---|---|---|---|---|---|---|
| | | d (nm) | Mean (nm) | SD | | Mean | SD |
| 1 | Day1 | 144.2 | 143.5 | 0.611 | 0.18 | 0.18 | 0.009 |
| 2 | | 143.0 | | | 0.18 | | |
| 3 | | 143.4 | | | 0.19 | | |
| 4 | Day2 | 165.3 | 162.8 | 3.270 | 0.24 | 0.22 | 0.018 |
| 5 | | 159.1 | | | 0.20 | | |
| 6 | | 164.0 | | | 0.22 | | |
| 7 | Day3 | 172.9 | 177.4 | 15.699 | 0.29 | 0.29 | 0.010 |
| 8 | | 164.5 | | | 0.29 | | |
| 9 | | 194.9 | | | 0.28 | | |
| 10 | Day4 | 150.1 | 146.4 | 5.541 | 0.19 | 0.21 | 0.048 |
| 11 | | 149.0 | | | 0.27 | | |
| 12 | | 140.0 | | | 0.18 | | |
| 13 | Day5 | 143.9 | 143.3 | 2.011 | 0.20 | 0.19 | 0.008 |
| 14 | | 145.0 | | | 0.20 | | |
| 15 | | 141.1 | | | 0.18 | | |
| 16 | Day8 | 150.4 | 152.8 | 2.227 | 0.21 | 0.26 | 0.049 |
| 17 | | 154.8 | | | 0.31 | | |
| 18 | | 153.2 | | | 0.27 | | |

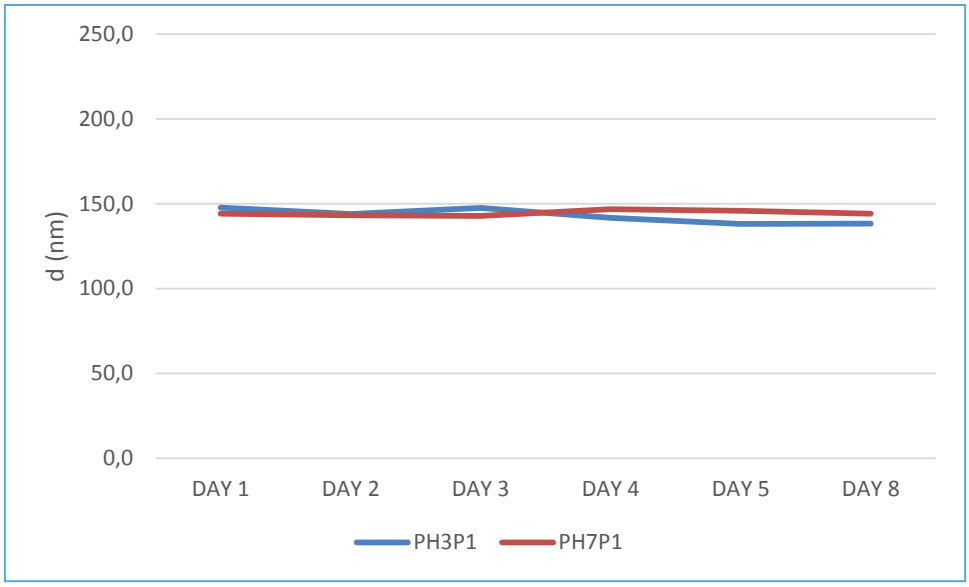


Fig. 16. The influence of storage time (days) and pH value on the particle size d (mm) for liposomes purified by the one column method (P1)

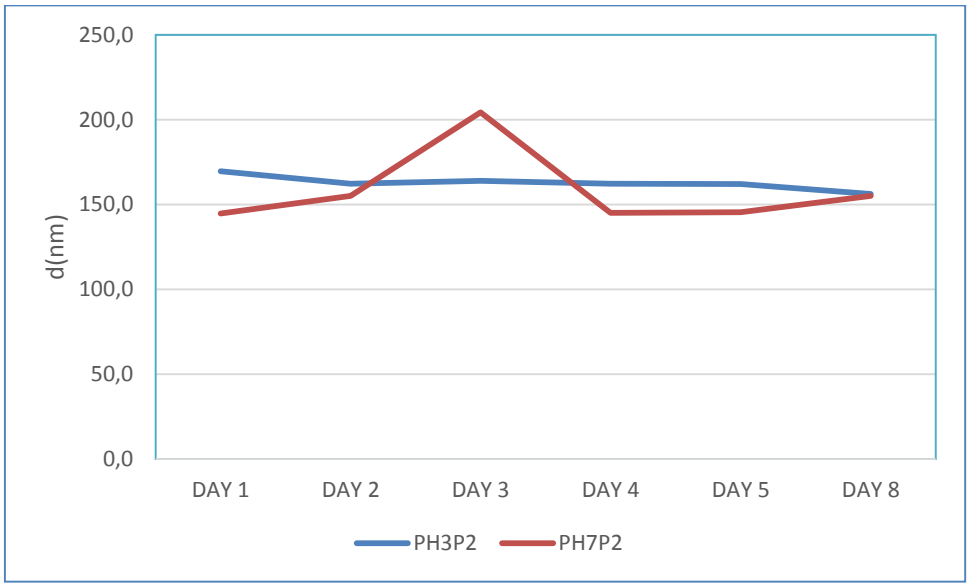


Fig. 17. The influence of storage time (days) and pH value on the particle size d (mm) for liposoms purified by the one two method (P2)

## 8.2 Estimation of encapsulation efficiency of TETA in liposomes

Tab. 17. Results of EE (%) for S1-S3PH3P1

| PH3P1 | | | | | | | |
|---|---|---|---|---|---|---|---|
| DAY | C (mM/l) | Conc of chol before SEC (mM) | Ratio of TETA/Chol before SEC | Conc of TETA after SEC (mM) | Conc of chol after SEC (mM) | Ratio of TETA/Chol after SEC | EE (%) |
| S1 | | | | | | | |
| 1 | 712. 500 | 50. 667 | 1406.250 | 2. 110 | 2.111 | 99. 964 | 7.11 |
| 2 | | | | 2.314 | 2.178 | 106.250 | 7.56 |
| 3 | | | | 1.569 | 1.917 | 81.854 | 5.82 |
| 4 | | | | 2.273 | 2.285 | 99.462 | 7.07 |
| 5 | | | | 2.446 | 2.149 | 113.807 | 8.09 |
| 8 | | | | 1.889 | 2.228 | 84.755 | 6.03 |
| S2 | | | | | | | |
| 1 | 712.500 | 45. 363 | 1570.652 | 1.687 | 2.239 | 75.355 | 4.80 |
| 2 | | | | 1.655 | 2.307 | 71.721 | 4.57 |
| 3 | | | | 1.936 | 2.612 | 74.130 | 4.72 |
| 4 | | | | 2.124 | 2.766 | 76.794 | 4.89 |
| 5 | | | | 1.597 | 2.046 | 78.060 | 4.97 |
| 8 | | | | 1.563 | 2.073 | 75.396 | 4.80 |
| S3 | | | | | | | |
| 1 | 712.500 | 46.427 | 1534.678 | 2.133 | 1.762 | 121.036 | 7.89 |
| 2 | | | | 2.257 | 2.241 | 100.690 | 6.56 |
| 3 | | | | 2.228 | 2.118 | 105.230 | 6.86 |
| 4 | | | | 2.794 | 2.604 | 107.297 | 7.00 |
| 5 | | | | 1.808 | 1.799 | 100.515 | 6.55 |
| 8 | | | | 1.837 | 2.276 | 80.695 | 5.26 |

Tab. 18. Results of EE (%) for S1-3PH3P2

| PH3P2 | | | | | | | |
|---|---|---|---|---|---|---|---|
| DAY | C (mM/l) | Conc of chol before SEC (mM) | Ratio of TETA/Chol before SEC | Conc of TETA after SEC (mM) | Conc of chol after SEC (mM) | Ratio of TETA/Chol after SEC | EE (%) |
| S1 | | | | | | | |
| 1 | 712.500 | 39.877 | 1786.759 | 6.697 | 7.465 | 89.712 | 5.20 |
| 2 | | | | 3.465 | 0.429 | 808.404 | 45.24 |
| 3 | | | | 3.679 | 0.585 | 628.456 | 35.17 |
| 4 | | | | 3.362 | 0.485 | 693.109 | 38.79 |
| 5 | | | | 3.739 | 0.545 | 686.420 | 38.42 |
| 8 | | | | 3.671 | 0.523 | 702.339 | 39.31 |
| S2 | | | | | | | |
| 1 | 712. 500 | 35.287 | 2019.176 | 5.863 | 5.131 | 114.260 | 5.66 |
| 2 | | | | 3.711 | 0.426 | 871.086 | 43.14 |
| 3 | | | | 3.723 | 0.474 | 785.512 | 38.90 |
| 4 | | | | 3.618 | 0.431 | 839.773 | 41.59 |
| 5 | | | | 3.705 | 0.343 | 1080.106 | 53.49 |
| 8 | | | | 3.564 | 0.369 | 965.039 | 47.79 |
| S3 | | | | | | | |
| 1 | 712.500 | 36.003 | 1978.983 | 8.949 | 6.554 | 136.534 | 6.90 |
| 2 | | | | 4.616 | 0.477 | 967.641 | 48.90 |
| 3 | | | | 4.702 | 0.604 | 778.840 | 39.36 |
| 4 | | | | 4.233 | 0.530 | 798.089 | 40.33 |
| 5 | | | | 4.254 | 0.528 | 805.178 | 40.69 |
| 8 | | | | 4.295 | 0.548 | 783.370 | 39.58 |

Tab. 19. Results of EE (%) for S1-3PH7P1

| PH7P1 | | | | | | | |
|---|---|---|---|---|---|---|---|
| DAY | C (mM/l) | Conc of chol before SEC (mM) | Ratio of TETA/Chol before SEC | Conc of TETA after SEC (mM) | Conc of chol after SEC (mM) | Ratio of TETA/Chol after SEC | EE (%) |
| S1 | | | | | | | |
| 1 | 712. 500 | 47.807 | 1490.378 | 1.789 | 1.392 | 128.577 | 8.63 |
| 2 | | | | 1.670 | 1.392 | 119.985 | 8.05 |
| 3 | | | | 1.627 | 1.881 | 86.477 | 5.80 |
| 4 | | | | 1.953 | 1.970 | 99.120 | 6.65 |
| 5 | | | | 4.703 | 2.334 | 201.511 | 13.52 |
| 8 | | | | 0.729 | 1.458 | 50.004 | 3.36 |
| S2 | | | | | | | |
| 1 | 712. 500 | 46.123 | 1544.771 | 1.537 | 2.435 | 63.116 | 4.09 |
| 2 | | | | 1.616 | 2.435 | 66.345 | 4.29 |
| 3 | | | | 1.444 | 1.551 | 93.127 | 6.03 |
| 4 | | | | 4.044 | 2.540 | 159.221 | 10.31 |
| 5 | | | | 3.810 | 2.135 | 178.465 | 11.55 |
| 8 | | | | 1.165 | 1.755 | 66.389 | 4.30 |
| S3 | | | | | | | |
| 1 | 712. 500 | 47.687 | 1494.128 | 1.667 | 1.888 | 88.310 | 5.91 |
| 2 | | | | 1.710 | 1.888 | 90.562 | 6.06 |
| 3 | | | | 2.369 | 2.085 | 113.602 | 7.60 |
| 4 | | | | 4.457 | 2.271 | 196.270 | 13.14 |
| 5 | | | | 4.753 | 2.22 | 214.138 | 14.33 |
| 8 | | | | 1.594 | 2.361 | 67.520 | 4.52 |

Tab. 20. Results of EE (%) for S1-3PH7P2

| PH7P2 | | | | | | | |
|---|---|---|---|---|---|---|---|
| DAY | C (mM/l) | Conc of chol before SEC (mM) | Ratio of TETA/Chol before SEC | Conc of TETA after SEC (mM) | Conc of chol after SEC (mM) | Ratio of TETA/Chol after SEC | EE (%) |
| S1 | | | | | | | |
| 1 | 712. 500 | 54.347 | 1311.028 | 5.833 | 9.440 | 61.788 | 4.71 |
| 2 | | | | 4.546 | 0.978 | 464.984 | 35.47 |
| 3 | | | | 4.519 | 0.960 | 470.766 | 35.91 |
| 4 | | | | 5.000 | 0.862 | 579.792 | 44.22 |
| 5 | | | | 6.088 | 0.769 | 792.078 | 60.42 |
| 8 | | | | 7.213 | 1.128 | 639.223 | 48.76 |
| S2 | | | | | | | |
| 1 | 712. 500 | 37.110 | 1919.968 | 10.034 | 9.914 | 101.209 | 5.27 |
| 2 | | | | 4.263 | 0.903 | 471.969 | 24.58 |
| 3 | | | | 4.861 | 0.901 | 539.711 | 24.58 |
| 4 | | | | 4.910 | 0.742 | 661.733 | 34.47 |
| 5 | | | | 6.858 | 0.531 | 1291.527 | 67.27 |
| 8 | | | | 5.616 | 0.608 | 923.192 | 48.08 |
| S3 | | | | | | | |
| 1 | 712. 500 | 41.680 | 1709.453 | 11.037 | 9.378 | 117.696 | 6.89 |
| 2 | | | | 4.412 | 0.963 | 458.165 | 26.80 |
| 3 | | | | 4.567 | 0.923 | 494.977 | 28.96 |
| 4 | | | | 4.327 | 0.716 | 603.982 | 35.33 |
| 5 | | | | 5.823 | 0.546 | 1066.566 | 62.39 |
| 8 | | | | 5.066 | 0.649 | 780.137 | 45.64 |

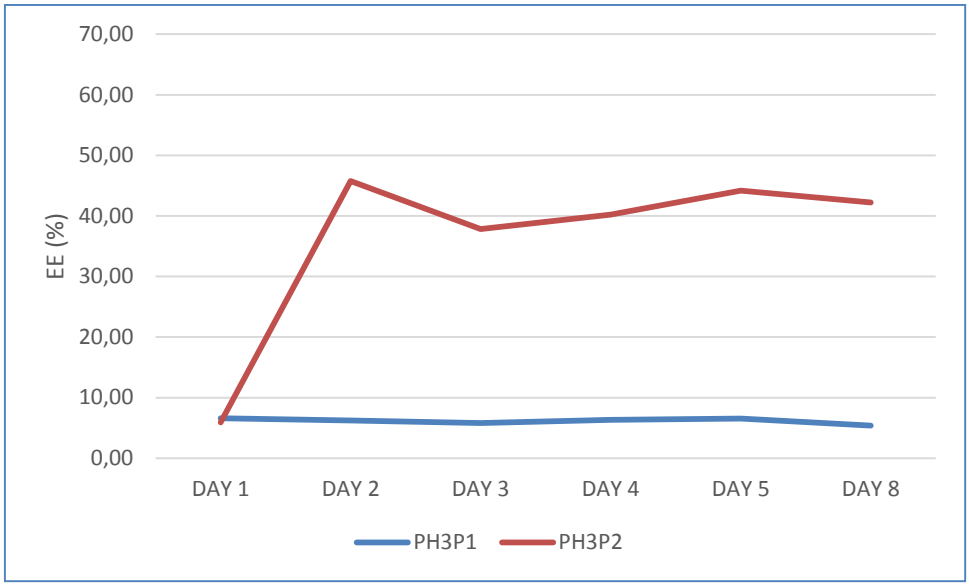


Fig. 18. The influence of the method of purification (P1, P2) on the EE (%) for liposomes with pH3

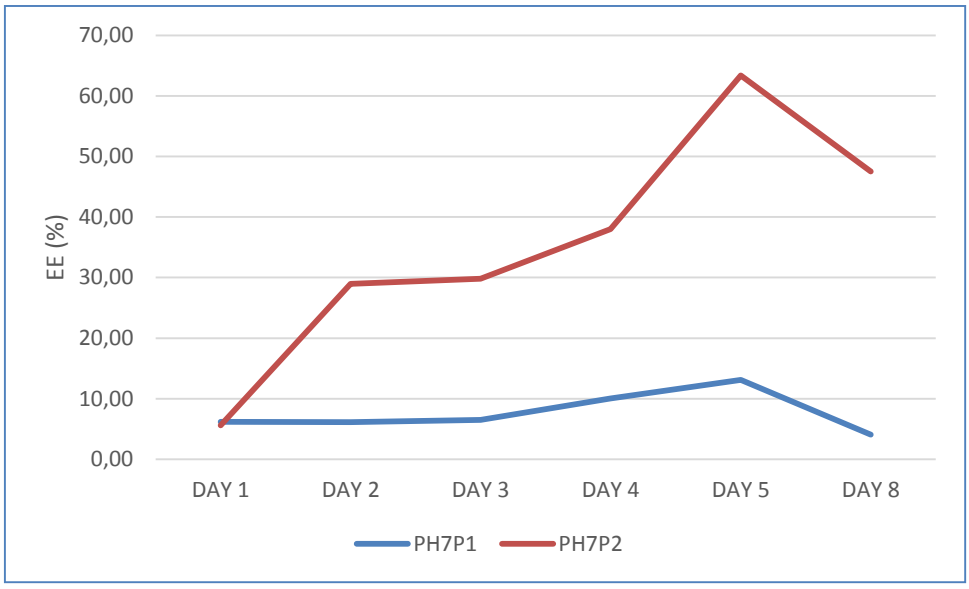


Fig. 19. The influence of the method of purification (P1, P2) on the EE (%) for liposomes with pH7

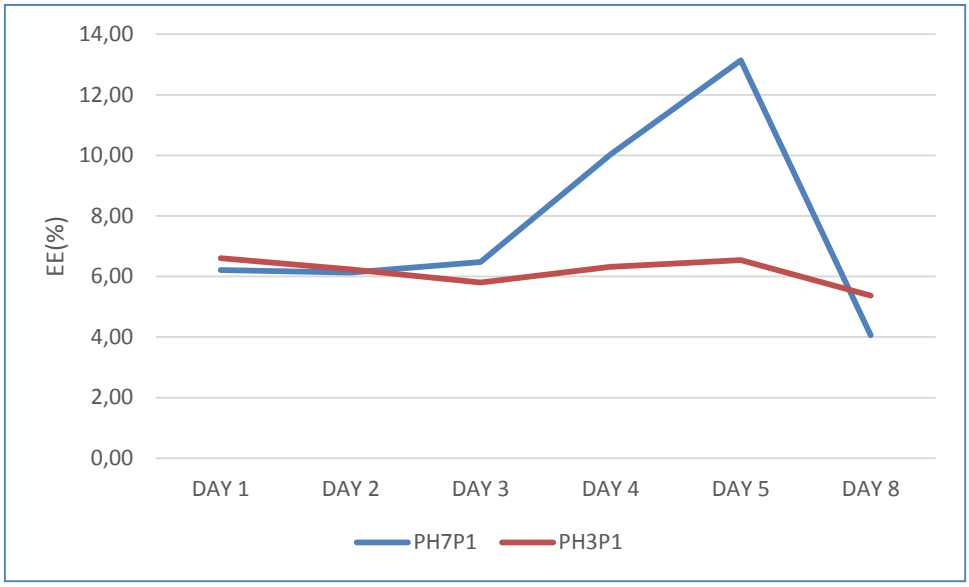


Fig. 20. The influence of pH value on the EE (%) for liposomes purified by the one column method (P1)

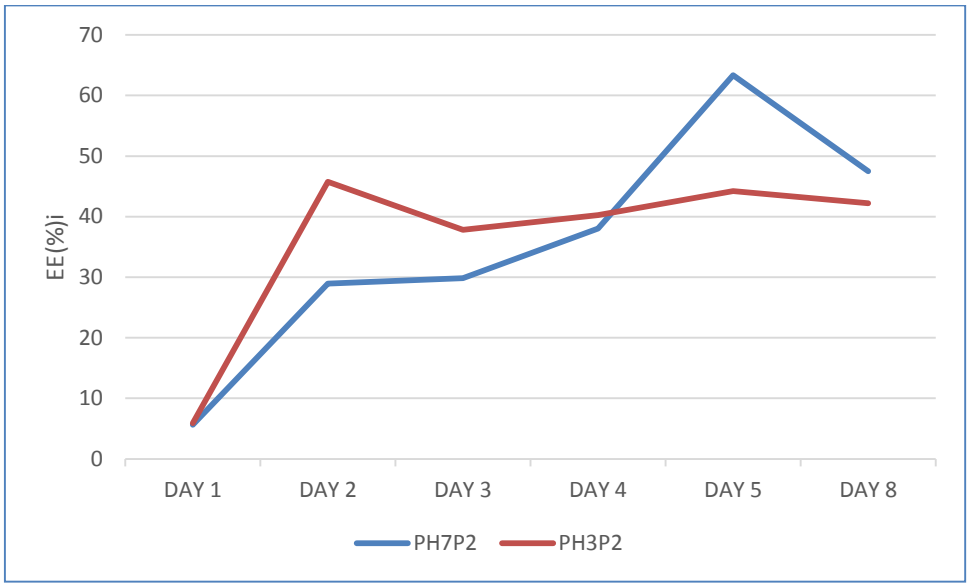


Fig. 21. The influence of pH value on the EE (%) for liposomes purified by the two columns method (P2)

# 9 Discussion

The blood brain-barrier represents the main obstacle for the treatment of central nervous system diseases. One possibility how to deliver drugs into the brain is the use of colloidal carrier systems—amongst others, the liposomes.[45] Liposomes are commonly prepared for intravenous administration in the pharmaceutical industry. The parenteral administration route is the most effective and common form of delivery for active drug substances with poor bio-availability. This route maintains its value due to special advantages like fast onset of action, targeting of the drug to the desired site of action, prevention of the first pass metabolism etc.[46]

Triethylenetetramine (TETA) is a drug used for the treatment of Wilson's disease as was described in section 6.5 and it is poorly absorbed, with relatively low bioavailability. The aim of this thesis was to prepare liposomes loaded with TETA dissolved at pH3 or/and pH7 by the film method with subsequent dual asymmetric centrifugation and to investigate their size and their size distribution by photon correlation spectroscopy and to estimate the encapsulation efficiency at day 1, 2, 3, 4, 5 and 8.

Liposomes were prepared using the film hydration method combined with dual asymmetric centrifugation (DAC). The formulations subjected to DAC usually need to contain a high amount of phospholipids to obtain a sufficient viscosity. Egg-phospatidylcholine, cholesterol and PEG - 2000- PE in ratio 55:40:5 were used to prepare the liposomes.

In this work, two different pH values were used to study the influence of pH value on the liposome stability and the encapsulation efficiency of TETA. The values were chosen based on preliminary studies in the laboratory that indicated the sufficient stability of liposomes (the average size and the polydispersity).

## *9.1 Liposome size and polydispersity*

The average size (described as Z-average in this thesis) and the size distribution of liposomes (described as polydispersity index, PdI, in this thesis) are important parameters that impact on the physical properties and biological fate of the liposomes and their entrapped substances. These parameters affect the physical stability, in vivo distribution and the size uniformity of the liposomal formulation as was described in

the theoretical section 6.1.1. The PEG-covered i.e. sterically shielded liposomes, avoid the mononuclear phagocytic system, however, this is directly associated with the size of liposomes. The best results are achieved if the liposome size is between 70 and 200 nm.[47]

The polydispersity index describes the distribution width of the particle size and is indexed from 0 to 1. The polydispersity index of 0 corresponds to a completely homogenous monodisperse sample, whereas the polydispersity index of 1 indicates a very broad size distribution. Usually, a sample with the polydispersity index <0.20 is considered to be monodisperse. A higher value of PdI normally indicates multiple sizes of particles in the given formulation.[47] The slow settling could occur for the large particles or aggregates under such circumstance.

In this work, the influence of the time of storage at days 1, 2, 3, 4, 5 and 8 after the preparation on the size and size distribution of the liposomal formulation of TETA was investigated using photon correlation spectroscopy (PCS). Obtained data are presented in Tables 5–16.

The Figures 17 and 18 summarize the influence of storage (days) and the pH value used for dissolution of TETA on the particle size (nm) for liposomes purified by the one column method (P1) and by the two columns method (P2). As we can see in Figure 18, the average size of particles complied with the stated limit of 200 nm for all the time except for pH7 at day 3 when it was above this limit. This probably resulted from the high variability of the experimental data as could be seen by the comparison of results in Tables 14–16. The larger size of liposomes detected for the only one sample (Table 14) could indicate the experimental error. If we calculate the average size for pH7 day 3 using the data from Tables 15 and 16, the size of liposomes is 174.2 nm.

The results verify good stability of liposomes. This could also be seen from Figures 17 and 18 for 8 days at both pH values used. The influence of the method of purification on the size of liposomes was not observed. However, the higher polydispersity was detected for pH7P2, which is ineligible for liposome preparation. The results displayed in Tables 5–13 indicates that the liposomes formulations could be assumed monodisperse.

## 9.2 Encapsulation efficiency

The encapsulation efficiency (EE) of a drug into a nanoparticulate drug delivery system is one out of the most important dependent variables. For the estimation of EE, it was necessary to determine the concentration of cholesterol and TETA before the size exclusion chromatography (SEC) as well as the concentration of cholesterol and TETA after the SEC. The concentration of TETA before the SEC was calculated using the equation 3 and it was always equal to 712.5 mmol/l. The concentration of TETA after the SEC was estimated using the method described in Sections 6.5.1 and 7.4.1.2. The HPLC method was used.

For the detection of cholesterol concentration before and after the SEC, it is necessary to destroy the liposomes. This was achieved by dilution of liposomal dispersion with methanol. The concentration of cholesterol differed depending on how much of cholesterol had built in liposomes during their preparation.

The encapsulation efficiency was then calculated as the ratio of the TETA/cholesterol ratios before the SEC and after the SEC in accordance to the equation 2. The results of the EE estimation are summarized in the last column of Tables 17–20. The influence of the method of purification and the pH value can be observed in Figures 18–21.

The significant influence of the method of purification on EE was found as illustrated in Figures 18 and 19. The purification by the two columns method (P2) was found to give higher encapsulation yield than the purification by one column method (P1). The values of EE for P1 in a range of 4 % to 14 % were observed, however, the EE from 24 % to 67 % was detected for P2. The lower EE values from from 4 % to 8 % for P2 referred to the first day in Table 18 and 20 are associated with the method of purification as described in the experimental section. At the first day, TETA and cholesterol concentration in liposomal formulation were determinated only by just once purification as in the procedure signed with P1.

For the one column method, the encapsulation efficiency of TETA-liposomes with pH3 did not revealed any significant changes during days of storage (Fig. 20). On the other side, the EE for pH7 showed increasing tendency reaching the maximum value of about 13.13 % at day 5.

Generally, the higher EE was obtained for the two columns method of purification. As can be seen in Figure 21, the highest encapsulation efficiency for pH3 was found at

day 2 (45.76 %) in comparison to pH7 when the highest EE was found at day 5 (63.36 %).

In a summary, the consistent values of the encapsulation efficiency during the days of storage were observed with the dissolution of TETA at pH3 value for both methods of purification. In contrast, the higher variability of resulting data were revealed dissolving TETA at pH7.

# 10 Conclusions

From the results of this thesis the following conclusions can be summarized:

- It was possible to prepare liposomes with encapsulated TETA by a film hydration method followed by dual asymmetric centrifugation. The average liposome size was below 200 nm.
- Liposomes containing TETA dissolved at pH3 or/and pH7, respectively, were stable for 8 days when they were stored in the refrigerator at 2–8° C. No effect of pH on the stability was observed. However, the size of liposomes was slowly decreasing with the time of storage.
- The method of purification did not influence the stability of liposomes; the average size remained unchanged for eight days of storage. However, the method of purification influenced the polydispersity. The higher polydispersity was observed for liposomes containing TETA dissolved at pH7 and purified by the two columns method.
- It is possible to successfully express the encapsulation efficiency based on the estimation of the concentration of TETA and the concentration of cholesterol before the size exclusion chromatography and after the size exclusion chromatography. The encapsulation efficiency was slightly influenced by the value of pH used and significantly influenced by the method of purification:
    - For the one column method, the values of the encapsulation efficiency ranged from 5 % to 14 % depending on the pH value. The higher encapsulation efficiency was observed at pH7 in comparison to pH3, but the variability of the results was high.
    - For the two columns method, the encapsulation efficiency around 67 % at pH7 was observed; however the values of the encapsulation efficiency varied in the large range from 25 % to 67 %.
    - For the two columns method, the more consistent results of the encapsulation efficiency at pH3 were observed—ranging from 35 % to 54 %.
- Obtained results should set out an efficient direction for further development of TETA liposomal dispersion for brain-targeting.

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