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„Development of ergothioneine-containing liposomes for aerosol therapy of chronic obstructive pulmonary disease“

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**„If we knew what it was we were doing,
it would not be called research, would it?“**

Albert Einstein (1879 – 1955)

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1. ABSTRACT

Background

Ergothioneine, a natural occurring amino acid, is a promising antioxidant and cytoprotective agent in the treatment of diseases with oxidative and inflammatory damage, such as COPD. Inhalation of therapeutic agents is a long-established means of pulmonary drug delivery. This thesis deals with the membrane intensity of liposomes and encapsulating the model compound ergothioneine. Thus, the aim of this work was to develop a liposomal formulation for targeted, stable and dose-dependent oral aerosol pulmonary delivery.

Methods

Within scope of this work different liposomal formulations were prepared by thin-film hydration and extrusion method and evaluated for their encapsulation efficiency by HPLC and long-term and aerosolization stability. The influence of lipid composition and the stability of vesicles was investigated by measuring the changes in mean diameter, polydispersity index and ζ -potential.

Results

The liposome composition DPPC:Chol:DSPG (7:2:1) exhibited the highest encapsulation efficiency of $13.0 \pm 5.7\%$ and delivered good long-term stability at 4-6°C. Even though, aerosolization with the Aerogen Pro nebulizer presented a retention of $8.9 \pm 4.1\%$ and occurred significant variations in heterogeneity and size, particularly associated with presence of ergothioneine.

Conclusions

This work showed that liposome composition DPPC:Chol:DSPG has good stability with encapsulation efficiency of ergothioneine and aerosolization retention of more than 5% on average. As a result, the presence of ergothioneine increases the physicochemical instability of the aerosol. This suggests a promising oral inhalation formulation for the treatment of COPD. However, further studies are needed to determine the effect of ergothioneine on resistance to nebulization exposures.

1. ZUSAMMENFASSUNG

Hintergrund

Ergothionein, eine natürliche Aminosäure, erweist sich als vielversprechend als Antioxidans und zytoprotektiven Wirkstoff bei Behandlung von Krankheiten mit oxidativen und entzündlichen Schäden, wie z.B. COPD. Die Inhalation von therapeutischen Substanzen ist ein seit langem bekanntes Mittel zur Verabreichung von Medikamenten in der Lunge.

In der vorgelegten Arbeit untersuchten wir die Membranintensität von Liposomen, die die Modelverbindung Ergothionein verkapseln. Demzufolge war das Ziel dieser Arbeit die Entwicklung einer liposomalen Formulierung für eine gezielte, stabile und dosisabhängige orale Aerosolverabreichung in der Lunge.

Methoden

Im Rahmen dieser Arbeit wurden verschiedene liposomale Formulierungen durch Dünnschichthydratation und Extrusion hergestellt und auf ihre Verkapselungseffizienz mittels HPLC sowie ihre Langzeit- und Aerosolisationsstabilität hin untersucht.

Der Einfluss der Lipidzusammensetzung und die Stabilität der Vesikel wurde durch Messung der Veränderungen der Größe, Polydispersität Index und ζ -Potentials untersucht.

Ergebnisse

Die Liposomen Zusammensetzung DPPC:Chol:DSPG (7:2:1) wies mit $13,0 \pm 5,7\%$ die höchste Verkapselungseffizienz und eine entsprechend gute Langzeitstabilität bei $4-6^\circ\text{C}$ auf. Die Aerosolisierung mit dem Aerogen Pro Vernebler ergab jedoch eine Retention von $8,9 \pm 4,1\%$ und verursachte signifikante Variationen in Heterogenität und Größe, insbesondere in Verbindung mit dem Vorhandensein von Ergothionein.

Fazit

Zusammenfassend zeigte diese Arbeit, dass die Liposomenzusammensetzung DPPC:Chol:DSPG eine gute Stabilität mit einer Verkapselungseffizienz von Ergothionein und einer Aerosolisierungsretention von durchschnittlich mehr als 5% aufweist. Das Vorhandensein von Ergothionein erhöht die physikochemische Instabilität des Aerosols. Dies deutet auf eine vielversprechende orale Inhalationsformulierung für die Behandlung chronischer Lungenkrankheiten hin, doch sind weitere Studien er-

forderlich, um die Auswirkungen von Ergothionein auf die Resistenz gegenüber Vernebelungsexpositionen zu bestimmen.

2. INTRODUCTION

2.1 Lung anatomy

The lungs are located in the anterior part of the thoracic cavity, where they occupy the largest part of it. As a major part of the respiratory system, they are responsible for the exchange of oxygen (O₂) and carbon dioxide (CO₂) in the human body. Due to a high degree of elasticity, the lungs are capable to contract and expand during inhalation and exhalation, which is needed for a sufficient gas exchange (National Heart Lung and Blood Institute, 2020).

The bronchus is divided in the lung into lung lobes. The left lung consists out of two lobes and is wider and shorter than the right lung, which is consists of three lobes (Drake *et al.*, 2020, pp. 173-177).

The main bronchus is structured into the lung into secondary bronchi (lobar bronchi), each of which provides a lobe. The lobar bronchi further are subdivided within tertiary bronchi (segmental bronchi), that provide bronchopulmonary segments (Drake *et al.*, 2020, p. 173).

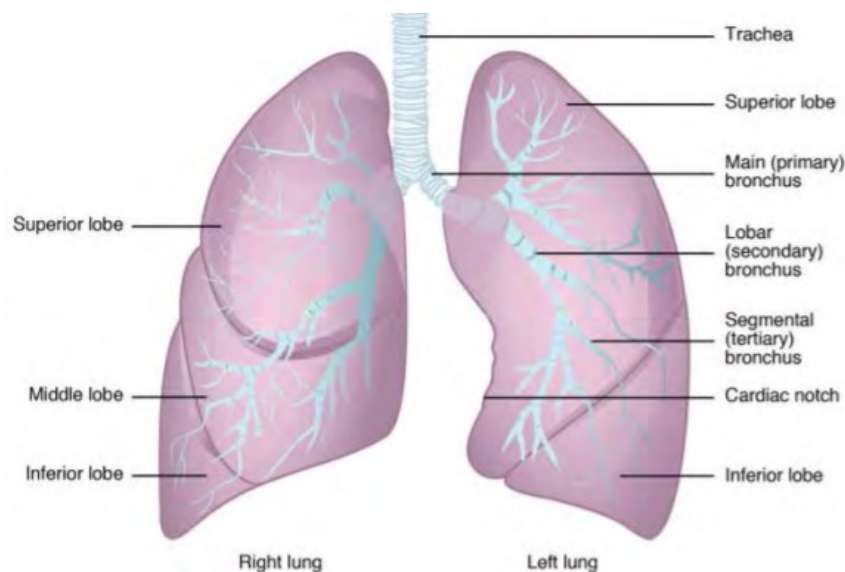


Figure 1. The right and left lung (Young *et al.*, 2013, Chapter 22, p. 994).

The trachea is a flexible tube from which the main airway splits at the carina, where it is divided into the right and left primary bronchi. From there another splitting occurs into the tertiary (segmental) and secondary (lobar) bronchi. Gas exchange is the major function of the lungs, for which the blood from the pulmonary circulation is required (Young *et al.*, 2013, Chapter 22, p. 994).

2.1.1 Physiology of the lung

GAS EXCHANGE

The lung alveoli are responsible for gas exchange. The lungs are used for exchange of gases between the respiratory air and the pulmonary capillary blood (Wagner, 2015; Knudsen *et al.*, 2018).

The respiratory membrane enables gases to cross by simple diffusion, in which the oxygen is absorbed by the blood for transport and CO₂ to be dispensed into the air out of the alveoli (Patwa *et al.*, 2015).

The alveolar wall is composed of three major cell types: alveolar macrophages, type I alveolar cells (AT1) and type II alveolar cells (AT2). The alveolar macrophages are a type of phagocytic cell of the immune system that removes pathogens that have reached the alveoli (Knudsen *et al.*, 2018; Naeem *et al.*, 2021).

The AT1 cell accounts for up to 95% of the alveolar surface area. These cells are a squamous epithelial cell of the alveoli and highly permeable to gases (Knudsen *et al.*, 2018, Helms *et al.*, 2008).

The AT2 cell segregates pulmonary surfactant that decreases the surface tension of the alveoli. Repair and renewal are performed by them (Knudsen *et al.*, 2018; Zhang *et al.*, 2019).

The alveoli expands when oxygen is inhaled, since it has a high O₂ and low CO₂ content. When exhaling, exactly the opposite happens and this process is called ventilation (Wagner, 2015; Knudsen *et al.*, 2018).

2.1.2 Chronic Obstructive Pulmonary Disease (COPD)

COPD refers to the chronic diseases of the lungs such as emphysema, which is an enlargement of alveoli and destruction of parenchyma resulting in progressive decline of FEV₁ (forced expiratory volume in 1 second), chronic obstructive bronchitis and chronic respiratory failure (Michaeloudes *et al.*, 2020; Raherison *et al.*, 2009; Decramer *et al.*, 2012).

In healthy individuals, the peak of FEV₁ and FVC occurs in average around the age of 25, with the tendency of declining slowly with aging. With time it comes to a change in flow-volume loop, due to a loss in the lung volume. This is largely attributed to the narrowing of the peripheral airways. Other factors contributing to the loss in the lung volume include: reduced lung elasticity and enlargement of alveolar spaces (Barnes *et al.*, 2019).

The diagnosis of COPD is assessed by post-bronchodilator spirometry, which confirms the presence of a limitation of the chronic airflow. The grade of severity of this limitation is labeled with the FEV₁ percent (Lange *et al.*, 2016).

The diagnostic definition of the permanent limitation of the airflow is defined by dividing the FEV₁ with FVC (<0.7) (Lange *et al.*, 2016).

Risk factors have been able to identify by an excess decline in FEV₁. In middle aged adults, the rate of acceptance in FEV₁ is some 25mL per year in women and 30mL per year in men (Vestbo *et al.*, 2016).

Spirometry is used in COPD to measure the severity of the airflow limitation and thus confirming the persistent airflow limitation. However, spirometry has some disadvantages. First, it only has a limited relationship with patient-originated outcomes such as symptoms. Furthermore, it is also relatively inaccurate in detecting early disease or people at risk of developing chronic airway disease (Zimmermann *et al.*, 2019).

The diseases are irreversible, airflow obstruction that is only partly reversible and are based on inflamed and permanently narrowed airways, which of 2020 was the third leading cause of death worldwide. The main risk factor still remains active smoking,

but factors like air pollution and infections also play an important role (Raheerison *et al.*, 2009; Decramer *et al.*, 2012).

2.1.2.1 Pathogenesis of airway inflammation in COPD

Chronic obstructive pulmonary disease is a common, heterogeneous, treatable and preventable disease that is identified by airflow limitation and persistent respiratory symptoms (Soriano *et al.*, 2018).

COPD is characterised due to an enhanced response of inflammation of the airway epitheliums to inhaled noxious gases and particles (e.g. tobacco smoke).

Oxidative stress, as well as imbalance between antiproteases and proteases contribute to the pathogenesis in COPD (Linden *et al.*, 2019).

The oxidative stress is increased in patients suffering from COPD, especially in the phase of acute exacerbations. Air pollution and cigarette smoke are the major exogenous sources of oxidative stress in the lungs, but the oxidative stress also persists in ex smokers, which suggests that oxidative stress also occurs endogenously. Alveolar macrophage numbers are tremendously increased in the lungs of COPD patients and are more likely to be activated in comparison to control subjects. This results in releasing greater amounts of ROS in forms such as superoxide anions or hydrogen peroxide (H₂O₂) (Barnes, 2020).

The endogenous compounds include the reactive nitrogen species (RNS) and reactive oxygen species (ROS), which have the potential to cause oxidative damage to biomolecules, which hinders normal function of the cells and activate the expression of redox-sensitive, pro inflammatory transcription factors like NF-κB, which results in the chronic pro-inflammatory state that elevates the production of cell-derived ROS like xanthine/xanthine oxidase system, heme-peroxidases, NADPH oxidase. These ROS are commonly detected in COPD patients (Magallón *et al.*, 2019).

The pathogenesis of COPD may begin much earlier, even before birth, due to the explosion to passive smoke *in utero*, which is associated with enhanced risk of COPD in adulthood (Soriano *et al.*, 2018).

The inhalation of the smoke leads to infiltration of inflammatory cells of the submucosa, mucosa, and glandular tissue. Enhanced mucus content, disturbed tissue repair with wall thickening and epithelial-cell hyperplasia in the airways are key features of COPD (Decramer *et al.*, 2012).

A corresponding role is given to autoimmunity in COPD. The designation of COPD as an autoimmune disease opens new horizons for treatment strategies and lines of research to be developed (Bagdonas *et al.*, 2015).

The adaptive immune system plays an important role in COPD. Activation of the adaptive immune system leads to a decrease in regulatory T-cells in the large and small airways and infiltration of T-helper cells (Th17), T-cells and B-cells (Wang *et al.*, 2018).

A fundamental factor in suppressing the progression of COPD is the airway inflammation, are bronchodilators such as β_2 -agonists (short acting: fenoterol, salbutamol; long acting: formoterol, salmeterol) and anticholinergics (short acting: ipratropium bromide; long acting: tiotropium). For symptomatic treatment of bronchoconstriction, bronchodilators are preferred, which are mostly combined with corticosteroids (beclomethasone, budesonide) (Brightling *et al.*, 2019; Decramer *et al.*, 2012; Terry *et al.*, 2020).

Often used as long-term treatment of symptoms are inhaled rather than oral therapies such as methylxanthines (theophylline, aminophylline), mucolytics (N-acetylcysteine), or phosphodiesterase-4 inhibitors (roflumilast) because they are more effective, faster acting, and have fewer side effects (Terry *et al.*, 2020).

2.1.2.2 Classification of COPD

In the late 1990s, the Global initiative for GOLD - Chronic Obstructive Lung Disease was established. The aim of the initiative was to raise awareness of COPD as an important lung health problem and to facilitate its treatment, as well as prevention (Terry *et al.*, 2020).

The classification according to GOLD (I-IV) indicates how affected the lung function is. The chronic airflow obstruction, for which the Tiffeneau index claims that the postbronchodilator ratio of forced expiratory volume in 1 second (FEV₁) to forced vital capacity (National Clinical Guideline Centre, 2010; Decramer *et al.*, 2012).

Severity of airflow obstruction are distinguished by four GOLD stages: stage I describes itself as mild course with FEV₁ ≥80% of predicted. The stage II describes itself as moderate course with FEV₁ 50–80%. Stage III describes itself as severe course with FEV₁ 30–50% of predicted and stage IV describes itself as very severe course with FEV₁ <30% of predicted (Decramer *et al.*, 2012).

2.1.2.3 Epidemiology

Exacerbations can be precipitated by bacterial and viral infections, and by usual pollutants such as air pollution and tobacco. In around 30% of cases, an aetiological diagnosis cannot be confirmed. Furthermore, 25–50% of COPD patients are contracted with potential respiratory pathogens, principal *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* (Lacoma *et al.*, 2009).

Sadly, no information on the impact of COPD of recent onset is available and as such the data needs to be extrapolated from what is known for the mild progress of COPD. The EPISCAN (Epidemiological Study of COPD in Spain), a Spanish study of prevalence of COPD, confirmed that the mild COPD accounted for a majority (56%) of the diagnosed patient with COPD, albeit 73% of COPD subjects remain undiagnosed. This study showed that the prevalence of COPD in smokers, as well as ex-smokers, aged <50 was 4.1% (95% CI 2.9–5.4%) (Soriano *et al.*, 2018).

2.2 Ergothioneine (ESH)

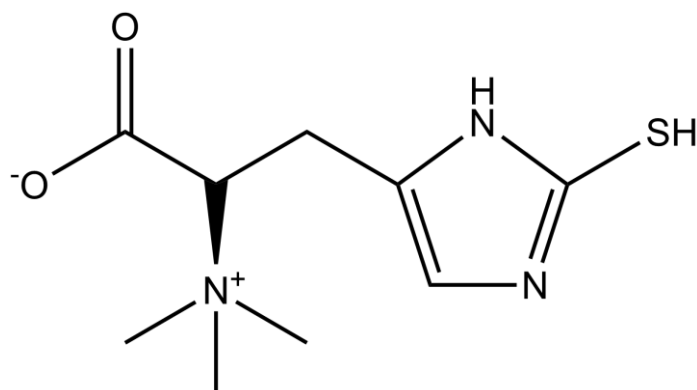


Figure 2. Structure of ergothioneine, L-(+)-ergothioneine 98+% (structure drawn with ChemDraw Ultra 12.0).

Ergothioneine (ESH) is an odourless, colourless compound and a sulfur containing histidine derivative. It is produced by bacteria and fungi as an important antioxidant and plays a major role as a cytoprotective compound (Stampfli *et al.*, 2020; Williamson *et al.*, 2020; Cumming *et al.*, 2018). In the year 1909, the ESH was identified for the first time in the ergot fungus by Charles Tanret (Kerley *et al.*, 2018).

The sulphur-based chemistry of thiols plays a major role in protection of cellular components, as well as, in the normal function of cells (Cheah *et al.*, 2021).

Although it was discovered more than a century ago, lately there has been a renewed interest in ergothioneine owing to its unique chemistry like its unusual stability, high redox potential, *etc.* The thiol-disulfide function group of ESH has a higher electropositive standard redox potential at (-60mV) in comparison to other naturally occurring thiols at pH 7 (Cheah *et al.*, 2021; Cumming *et al.*, 2018).

Various cell culture and animal model studies have been confirmed that ergothioneine plays a physiological role in immune regulation and inflammation modulation (Ehrhardt *et al.*, 2019; Cheah *et al.*, 2021).

Studies using a radiolabeled sulfur confirmed, that the amino acid L-histidine, through an intermediate precursor named hercynine (which incorporates sulfur from cystine), is responsible for the synthesis of ESH. Two main reactions are responsible for the

biosynthesis of ESH – the oxidative C-S bond formation and the C-S bond cleavage reaction, which results the transfer of sulfur from cysteine (or γ -glutamyl cysteine) to side chain of histidine – the imidazole (Cumming *et al.*, 2018).

In addition, the transporter OCNT1 was proposed to be responsible for the uptake and accumulation of ESH from our diet in several tissues, whilst also selectively distributing to specific tissues with the help of differential expression of transporters (Cheah *et al.*, 2021).

Many studies showed that the consumption of mushrooms can be linked to the decrease of the aging chronic diseases like dementia, prostate cancer and risk of diabetes. The mushroom consumption also correlated the blood ESH level, so it is postulated, that the mushrooms also have other beneficial health effects. However, to support this speculation, further observation of this correlation is required (Beelman *et al.*, 2020).

2.2.1 Organic cation transporter novel-type-1 (OCTN1)

In 1997, organic cation transporter novel-type-1 (OCTN1) was identified from a sample of a human fetal liver library. The human organic cation transporter novel-type-1 is a part of a small sub-family of organic cation transporters and is encoded by the membrane transporters of the SLC22 family genes (Pochini *et al.*, 2020; Frigeni *et al.*, 2016).

The SLC22 transporters play important role in the distribution and absorption of drugs (Qi *et al.*, 2020).

The SLC22 family consists of organic cation transporters, organic cation transporters novel and organic anion transporters. The organic cation transporters novel (OCTNs) includes OCTN1, OCTN2 and OCTN3. The OCTN subfamily is limited to OCTN1 and OCTN2, as OCTN3 is only found in mice (Qi *et al.*, 2020).

OCTN1 and OCTN2 are produced in bronchial epithelial cells and alveolar epithelial cells and their role is to transport organic cations such as carnitine and ergothioneine. OCTN1 and OCTN2 are involved in the transport of drugs such as predniso-

lone and tiotropium bromide, used for treatment of COPD, chronic obstructive bronchitis and other pulmonary inflammatory diseases (Qi *et al.*, 2020).

Organic cation transporter novel-type-1 encodes a protein consisting out of 551 amino acids with 12 transmembrane domains and is commonly found in the lungs, fetal liver, kidney, trachea, prostate, bone marrow, placenta, heart and other organs (Frigeni *et al.*, 2016).

2.2.2 Organic cation transporter novel-type-1 (OCTN1) and ergothioneine

In 2005, it was first published that OCTN1, encoded by the gene *slc22a4*, is the physiological transporters for ESH and as such the OCTN1 presence is responsible due to the accumulation of ESH (Cheah *et al.*, 2021; Halliwell *et al.*, 2018; Gründemann *et al.*, 2005).

The human OCTN1 is a pH- and Na-dependent transporter of ergothioneine and pH-dependent and Na-independent transporter of tetraethylammonium (Nakamura *et al.*, 2008).

2.3 Liposomes

One of the first nanostructures produced were liposomes. (Benedini *et al.*, 2017). Liposomes are small, spherical, and artificial vesicles that consist of phospholipids and cholesterol surrounding an aqueous core, such as natural plasma membrane (Powers *et al.*, 2019). They are surrounded by one or more lipid bilayers and have various sizes in the nm range. The most important constituents of the cell membrane are phospholipids, therefore, the liposomes are used in the cosmetics, food and drug delivery industries. They can deliver drugs specifically to the site of action, thereby reducing side effects and preventing drug degradation. They are popular as a means of delivering drugs to the lungs by nebulization (Prasad *et al.*, 2014; Swaminathan *et al.*, 2011). The bilayers consist of phospholipids from polar hydrophilic head groups and nonpolar lipophilic tails and have thereby an amphiphilic character (Inglut *et al.*, 2020). The properties variate considerable with size, the method of preparation, surface charge and lipid composition (Akbarzadeh *et al.*, 2013).

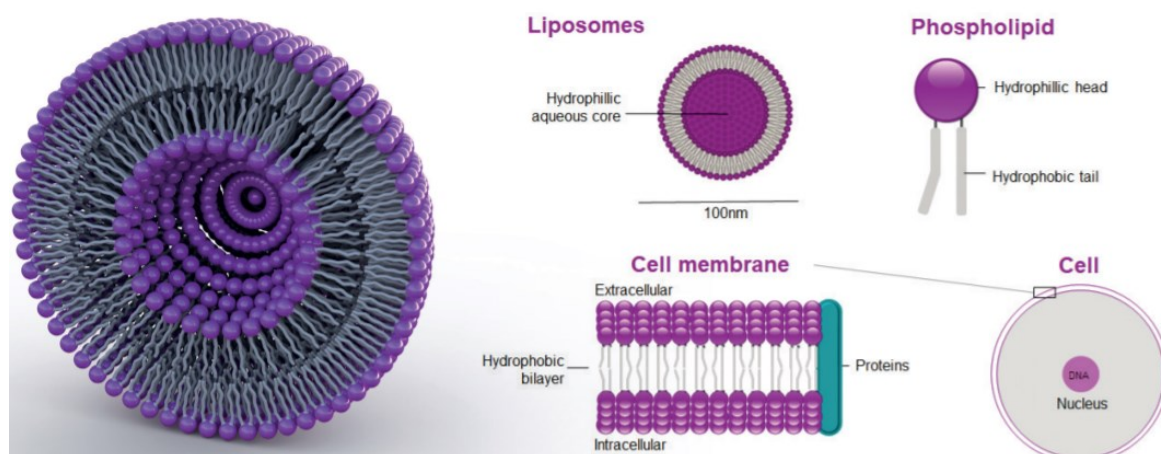


Figure 3. Liposome and its bilayer consisting of hydrophobic acyl chains and an aqueous core.

Figure 4. Liposomes imitate naturally occurring and stable vesicles exhibiting a high biological compatibility and cell-like morphology. Phospholipids consist out of hydrophilic head and hydrophobic tail.

(From formulation to manufacturing: lipid nanoparticle mRNA vaccines, gene therapies & other nano-medicines)

Four groups of liposomes are distinguished by their lamellarity and size, and as such classified in:

-) small unilamellar vesicle (SUV): 20-100 nm
-) large unilamellar vesicle (LUV): >100 nm
-) multilamellar vesicle (MLV): >500 nm
-) multivesicular vesicle (MVV): >1000 nm (Leitgeb *et al.*, 2020)

Phospholipids - primary building blocks of liposomes. They have a hydrophobic, non-polar fatty acid tail and a hydrophilic, polar phosphate head therefore they owned amphipathic character (Inglut *et al.*, 2020).

Cholesterol - major lipid component of the plasma membrane (Yun-Ui *et al.*, 2016; Kheirloomoom *et al.*, 2007).

2.3.1 Pulmonary drug delivery

The absorption through the barrier of lung epithelial is the most important factor in the efficacy of the inhaled drug. In the case of locally acting drugs, absorption into the bloodstream can lead to the drug being removed from the lungs and thus no longer exerting its effect. For systemically acting drugs, on the contrary, the absorption profile of the drug in the lungs determines the onset, duration and intensity of the drug's effect. Even though the inhalation is a well-established mean of drug delivery, drug absorption kinetics in the lungs have not been comprehensively researched (Gaspar *et al.*, 2008).

To improve the efficacy of pulmonary drug delivery systems, it is important to have an understanding of the lung physiology and the deposition mechanisms to determine the appropriate particle size. Furthermore, the technique of atomization must be considered for the increase in the effectiveness of drug delivery (Chattopadhyay, 2013).

Liposomes for pulmonary delivery have an attracting prevalence in the recent years, since liposome vesicles entrap therapeutic molecules and after inhalation can localize the effect of the drug in the pulmonary system for a longer period of time. They are made using phospholipids mostly with cholesterol. These components are very similar to mammalian pulmonary surfactants (Rudokas *et al.*, 2016).

Liposomes are largely used in various types of respiratory diseases. Liposomal aerosol has more advantages in comparison to ordinary aerosol such as: reduced toxicity, improved stability in large aqueous core, prevention of local irritation and sustained release (Daraee *et al.*, 2016).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Table 1. List of used chemicals

Name	Supplier
Acetonitrile for HPLC, gradient grade, 99,9%	Sigma-Aldrich
Ammoniumthiocyanate puriss. p. a. (Ammoniumthiocyanat)	Sigma-Aldrich
Chloroform 99,8%	Sigma-Aldrich
Cholesterol	Sigma-Aldrich
3 β -Hydroxy-5-cholestene*5-cholesten-3 β -ol	
DPBS	Sigma-Aldrich
Dulbecco's Phosphate Buffered Saline	
DPPC	Anatrace Products
1,2-Dipalmitoyl-sn-glycero-3-phosphocoline	
DSPG	Avanti Polar Lipids
1,2-Distearoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt	
DSPG	Sigma Aldrich
1,2-Distearoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt	
Ergothioneine (L-(+)-ergothioneine 98+%)	Alfa Aesar
Formic acid 98/100%	Fisher Chemical
Iron(III) chloride hexahydrate	Sigma-Aldrich
Nitrogen	BOC
PEA	Avanti Polar Lipids
1,2-distearoyl-sn-glycero-3-phosphoethanolamine	
Sodium chloride	Sigma-Aldrich
Triton X-100	Fisher Scientific
Whatman Nuclepore™ polycarbonate Filters 800 nm	Sigma-Aldrich/Lennox Chemicals Ltd.
Whatman Nuclepore™ polycarbonate Filters 600 nm	Sigma-Aldrich/Lennox Chemicals Ltd.
Whatman Nuclepore™ polycarbonate Filters 400 nm	Sigma-Aldrich/Lennox Chemicals Ltd.

Whatman Nuclepore™ polycarbonate Filters 200 nm	Sigma-Aldrich/Lennox Chemicals Ltd.
Whatman Nuclepore™ polycarbonate Filters 100 nm	Sigma-Aldrich/Lennox Chemicals Ltd.
Whatman polyester drain disc, 25 mm, PE, membrane filter accessories	Sigma-Aldrich/Lennox Chemicals Ltd.

3.1.2 Technical equipment

Table 2. List of used technical equipment

Name	Supplier
Aerogen Pro Nebuliser with a USB controller	Aerogen
Atlantis dC18 column, 100 Å, 5 µm, 4.6 x 150 mm	Waters
Branson Ultrasonics™ M Series Ultrasonic Cleaning Bath	Fisher Scientific
Centrifuge Tubes Polycarbonate 32 mL (25 x 89 mm)	Beckman Coulter
Hettich Zentrifugen Centriuge Universal 32R Max 4000 RPM D-78532	Hettich Zentrifugen GmbH & Co. KG
Lipex thermobarrel extruder	Northern Lipids
Optima XPN-100 ultracentrifuge	Beckman Coulter
pH/ATC electrode	Fisher Scientific
Swinging-Bucket Rotor SW 21 Ti	Beckman Coulter
Thermo GENESYS™ 10 UV Scanning UV-Vis, Spectrophotometer 120 V	Fisher Scientific
Vortex mixer protected by BioCote	Cole-Parmer
Water bath Grant FH15 Flowheater	Grant Instruments Cambridge
Waters 2487 Dual λ UV/Vis absorbance detector	Waters
Waters Alliance 2695 HPLC Separations Module	Waters
Zetasizer Nano ZS90	Malvern Panalytical, Malvern

3.2 Experimental methods

3.2.1 Liposomes preparation

Liposomes were prepared by the thin-film hydration and extrusion method. First, the lipid mixtures consisted of DPPC; 1,2-Dipalmitoyl-sn-glycero-3-phosphocoline and cholesterol; 3 β -Hydroxy-5-cholestene*5-cholesten-3 β -ol (66.7:33.3), then DPPC, cholesterol and PEA; 1,2-distearoyl-sn-glycero-3-phosphoethanolamine or DPPC, cholesterol and DSPG; 1,2-Distearoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (7:2:1), whereby the mixtures of lipids 40 mg total mass amount. Respective lipids were dissolved in 4 mL chloroform using a 50 mL round bottomed flask and placed in an ultrasonic bath for 5 min (Swaminathan *et al.*, 2014). Subsequent chloroform solvent was evaporated under vacuum in a rotary evaporator for 30 min at 45°C until a homogeneous and thin lipid film was formed. After that the dry lipid was rehydrated in 6.5 mL PBS for the first try liposomes, 6.5 mL after 5.5 mL distilled water was used for the other liposomes followed by ultrasonic bath for 15 min. Ergothioneine 10 mg/mL final concentration was dissolved in the PBS for the first try later then distilled water used for rehydration to allow for encapsulation. Unilamellar vesicles were received by seven until twenty-two times extrusion through one Whatman polyester drain disc membrane filter 25 mm with two (later one) Whatman Nucleopore polycarbonate filters of between 800 nm, 600 nm, 400 nm and 200 nm pore size using a Lipex thermo-barrel extruder (Northern Lipids, Burnaby, British Columbia, Canada) at 42-43°C.

Liposome suspension was ultracentrifuged using centrifuge tubes polycarbonate 32 mL (25 x 89 mm, Beckman Coulter) by ultracentrifuge (Optima XPN-100, Beckman Coulter) at 32,000 x *g*, with a rotor SW 21 Ti (Swinging-Bucket, Beckman Coulter) for 1 h and 4°C. After ultracentrifuged the pellet was resuspended with distilled water and split for aerosolization by Aerogen Pro nebulizer, encapsulation efficiency by HPLC and phospholipid content (Stewart assay).

Chemical Structures

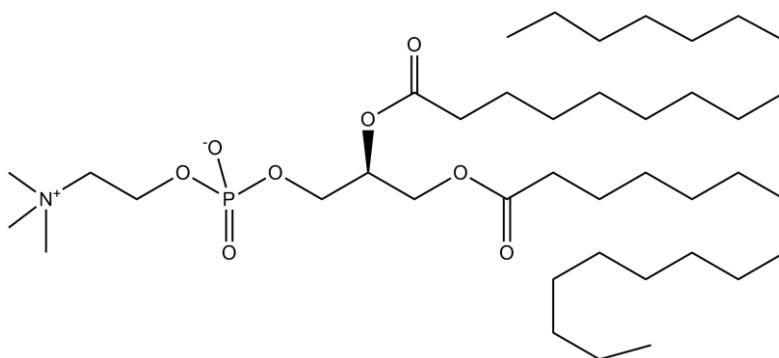


Figure 5. Structure of DPPC; 1,2-Dipalmitoyl-sn-glycero-3-phosphocoline (structure drawn with ChemDraw Ultra 12.0)

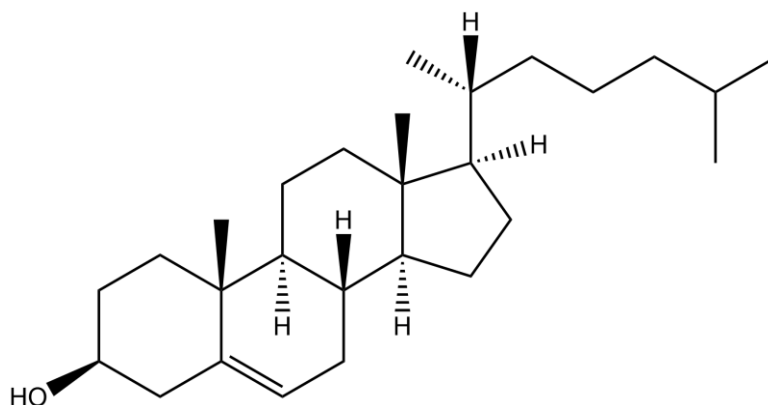


Figure 6. Structure of cholesterol; 3β -Hydroxy-5-cholestene*5-cholesten-3 β -ol (structure drawn with ChemDraw Ultra 12.0)

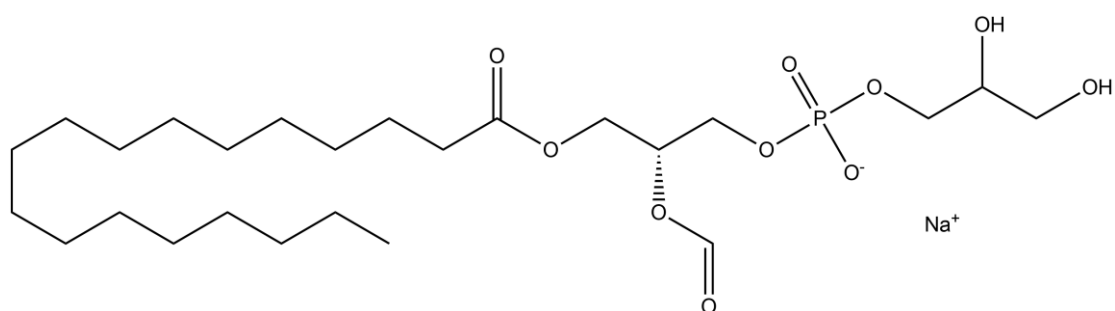


Figure 7. Structure of DSPG; 1,2-Distearoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (structure drawn with ChemDraw Ultra 12.0)

3.2.2 Physicochemical characterisation of liposomes

When liposomes are administered by pulmonary route, attention must be given to liposomes stability. After extrusion, Dynamic Light Scattering (DLS) technique were characterised performed to determine size, polydispersity index (Pdl) and ζ -potential of final liposome suspensions by using a Zetasizer Nano ZS90 (Malvern Panalytical, Malvern, UK). The liposome suspensions were diluted 10-fold with distilled water and the parameters were analysed at day 1 after the production, then day 7, 15 and 30 to determine the stability under refrigerated storage in a refrigerator (4-6°C). Therefore, the liposome suspensions were measured after extrusion by Lipex thermobarrel extruder (Northern Lipids, Burnaby, British Columbia, Canada) to determine size, polydispersity index (Pdl) and ζ -potential using Zetasizer Nano ZS90 (Malvern Panalytical, Malvern, UK).

The encapsulation efficiency ergothioneine content was analysed via high performance liquid chromatography (HPLC) and phospholipid content of liposomes was analysed via Stewart assay (Stewart, 1980; Swaminathan *et al.*, 2014), before and after aerosolization respectively after ultracentrifuge.

Phospholipid concentrations in the liposome suspensions were determined following the colorimetric assay described by Stewart. The method is based of phospholipids to form a coloured complex with 2 mL ammonium ferrothiocyanate which is soluble in 3 mL chloroform and exhibits by ultraviolet absorbance at 485 nm using a Thermo GENESYS™ 10 UV Scanning UV-Vis, Spectrophotometer 120 V (Fisher Scientific GmbH, Thermo Fisher Scientific Inc., Germany).

Phospholipid mass were obtained to a DPPC calibration curve were the absorbance values ranging from 0.02 mg to 0.24 mg.

All the measurements were performed in n=3 triplicate.

pH measurement

The pH of the ergothioneine solution (with a concentration of 10 mg/L) was measured with the help of the Fisherbrand Accumet AB250 pH/ISE benchtop meter with a TRIS-compatible double junction electrode (refillable epoxy-body pH/ATC). The readings were recorded three times, with measurements of the blanks (distilled H₂O) in between.

3.2.3 Aerosolization of Liposomes

After the ultracentrifugation, the liposome samples were prepared with 0.9% (w/v) sodium chloride and measured to determine size, polydispersity index (PDI) and ζ -potential by Zetasizer Nano ZS90 (Malvern Panalytical, Malvern, UK). An Aerogen Pro nebulizer with a USB controller (Aerogen, Dangan, Ireland) was attached to a 50 mL centrifuge tube and sealed with parafilm. Liposome samples were added into the nebuliser filler cap and left to aerosolize for 10 - 15 min at approximately 21°C room temperature. The aerosolized samples were ultracentrifuged again and the pellet resuspended and split again.

3.2.4 ESH quantification

The ergothioneine concentration was determined using an Alliance 2695 HPLC Separations Module with coupled to a 2487 Dual λ Absorbance detector (Waters, Milford, Massachusetts, USA) set at 254 nm and an Atlantis dC18 Column (100 Å, 5 μ m, 4.6 x 150 mm, Waters, Milford, Massachusetts, USA). The optimized mobile phase was composed of HPLC water with 1% formic acid and acetonitrile with 1% formic acid. A gradient from 95% A and 5% B to 5% A and 95% B for 3 min and 6 min than 95% A and 5% B for 6.1 min and 10 min was applied where A is acetonitrile with 0.1% formic acid and B is water with 0.1% formic acid at the flow rate was set at 0.5 mL/min with a total runtime of 11 min. The samples injection volume of 1 μ L were using an auto sampler and a pump with degasser. This analyse was performed at column temperature 25°C.

Reverse-phase HPLC method for quantification of encapsulated and free ergothioneine contact was determined using an Alliance 2695 HPLC Separations Module system coupled to a 2487 Dual λ UV/Vis absorbance detector (Waters, Milford, Massachusetts, USA) set at 254 nm with a degasser, pump, Atlantis dC18 column (100 Å, 5 μ m, 4.6 x 150 mm, Waters, Milford, Massachusetts, USA), Solvent Reservoirs and Control System. The Solvents were two mobile phases: 0.1% formic acid in HPLC water and 0.1% formic acid in acetonitrile. A gradient elution method was used, and the measurements take place at a constant flow rate of 0.6 mL/min with a total runtime of 11 min with an injection volume of 10 μ L and without a column temperature control. A Waters Empower Software was using for acquisition and processing the data.

After ultracentrifuged, the liposome samples were prepared whereby the supernatant was separated and pellet was dissolved in an equivalent volume of 1% Triton X-100 (Fisher Scientific) and left overnight in a refrigerator at 4-6°C to disrupt the membranes. In the end all samples were filtered through a Syringe Filter (MCE 0.45 μ m 13 mm diameter, UltraCruz, Santa Cruz Biotechnology, Inc., Germany) three times into vials (1.5 mL 32 x 11.6 mm Clear Flacon ND9, Avantor, by VWR, Belgium) before integrate into an auto sampler.

3.2.5 Stewart assay

0.1 mL to 0.2 mL of sample was added to a centrifuge tube polycarbonate and diluted to 0.5 mL with distilled H₂O, subsequent a coloured complex with 2 mL ammonium ferrothiocyanate and 3 mL chloroform were added. The centrifuge tubes were then shaken, vortexed for 30 s, shaken again and then vortexed for another 30 s. The samples were centrifuged by Hettich Zentrifugen Centrifuge Universal 32R Max 4000 RPM D-78532 (Hettich Zentrifugen GmbH & Co. KG, Germany) at 1000 rpm for 5 min and left to stand for 10 min before the top coloured layer was removed.

The chloroform organic layer was transferred into a quartz cuvette for ultraviolet absorbance at 485 nm using a Thermo GENESYS™ 10 UV Scanning UV-Vis, Spectrophotometer 120 V (Fisher Scientific GmbH, Thermo Fisher Scientific Inc., Germany). The DPPC calibration curve was obtained by measuring the absorbance of 1,2-Dipalmitoyl-sn-glycero-3-phosphocoline ranging from 0.02 mg to 0.24 mg dissolved in 3 mL chloroform (Figure 15).

Data analysis and statistical evaluation

Unless otherwise stated, the values are expressed as a mean $\pm$ standard deviation of triplicate readings of one sample or a single reading for three samples. The significant difference between two groups was assessed using an unpaired parametric t-test with statistical significance defined as $p < 0.05$. The significant difference between more than two groups was assessed using Brown-Forsythe and Welch ANOVA tests with statistical significance defined as $p < 0.05$.

Correlation was assessed using linear regression analysis, with a correlation coefficient (R^2) of 0.99 deemed acceptable. Finally, variation over time was rated using regression analysis with a p -value < 0.05 shown significant variance from the null hypothesis.

All statistical evaluation and data plotting was done using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, California, USA).

4. RESULTS

4.1 HPLC development

The following chromatograms (Figures 9, 10, 11 and 14) were taken with the use of a sensitive and rapid method for the detection of ergothioneine by reverse-phase HPLC (RP-HPLC) (Liu *et al.*, 2016; Zhou *et al.*, 2014). A solvent system of acetonitrile (ACN) with 1% formic acid (FA) and HPLC water with 1% FA was used and compatibility, sensitivity and solvent interference adjusted alteration of mobile phase gradient protocol, flow rate and column temperature. The first ergothioneine standard run tests were performed in phosphate-buffered saline, an aqueous medium containing liposome suspension that was later abandoned because of possible impairment with the phospholipid quantification assay. The first calibration curve was obtained with phosphate-buffered saline (PBS) (Figure 8).

For the experiments ergothioneine concentrations between 0.0008mg/mL to 0.5 mg/mL were used with a quick gradient transition of 80:20 to 5:95 HPLC water with 0.1% FA: ACN with 0.1% FA with a column with a temperature of 25°C and at the flow rate of 0.5 mL/min. The ergothioneine peak was detected at 3.4 min but interleaved with phosphate-buffered saline solvent peak (Figure 9). By increasing the column temperature to 30°C and the flow rate to 0.6 mL/min effected the peaks down to 2 min but had no separating effect as pictured in Figure 10. Whilst maintaining the flow rate of 0.6 mL/min and raising the starting mobile phase ratio to 95:5 without column temperature control effected the distinguishable signal for ergothioneine (Figure 11). Application to a distilled aqueous suspension medium this capability was retained sensitivity proof, with no ergothioneine peak identify a standard concentration of 0.00016 mg/mL. At this way, a water calibration curve was obtained for a range of 0.00016 mg/mL to 0.5 mg/mL (Figure 12).

Another calibration curve for ergothioneine in 1% Triton X-100 was also destined as the final HPLC analysis for encapsulation is dissolved in a Triton solution to break up the liposomal membranes and set free the encapsulated ergothioneine (Figure 13).

The Triton X-100 reduced the sensitivity of the HPLC and carried some peak tailing, but no interfering peaks were detected and the provided range from 0.0008 mg/mL to 0.5 mg/mL was satisfactory for the experiments performed (Figure 14).

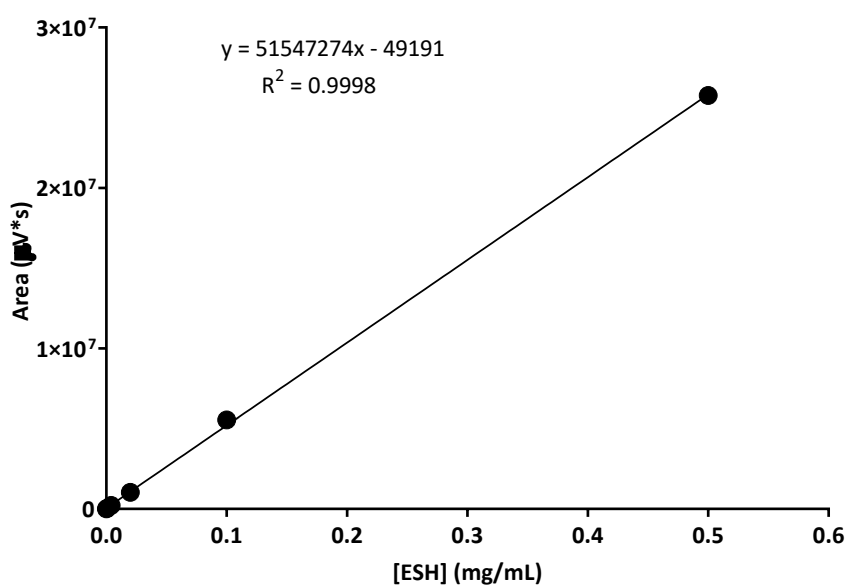


Figure 8. Calibration curve of ergothioneine dissolved in phosphate-buffered saline (PBS).

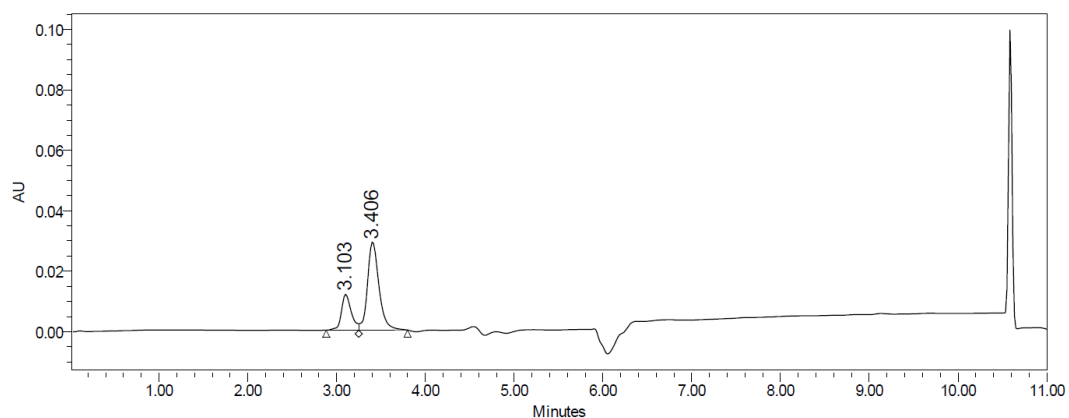


Figure 9. Ergothioneine with concentration of 0.004 mg/mL dissolved in phosphate-buffered saline.

Mobile phase: A rapid gradient transition of 80:20 to 5:95

A line – HPLC water with 1% formic acid

B line – acetonitrile with 1% formic acid

Flow rate: 0.5 mL/min

Column temperature: 25°C

Detection: 254 nm

Injection volume: 10 µL

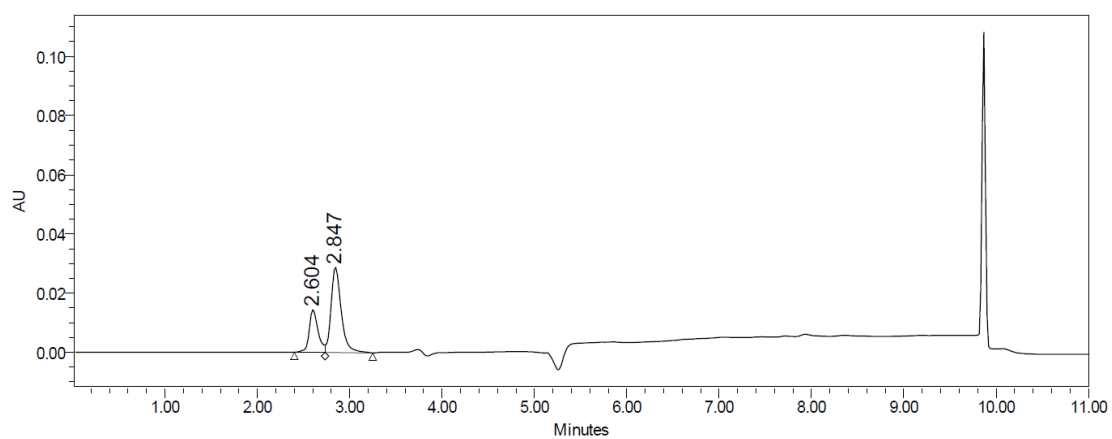


Figure 10. Ergothioneine with concentration of 0.004 mg/mL dissolved in phosphate-buffered saline.

Mobile phase: A rapid gradient transition of 80:20 to 5:95

A line – HPLC water with 1% formic acid

B line – acetonitrile with 1% formic acid

Flow rate: 0.6 mL/min

Column temperature: 30°C

Detection: 254 nm

Injection volume: 10 μ L

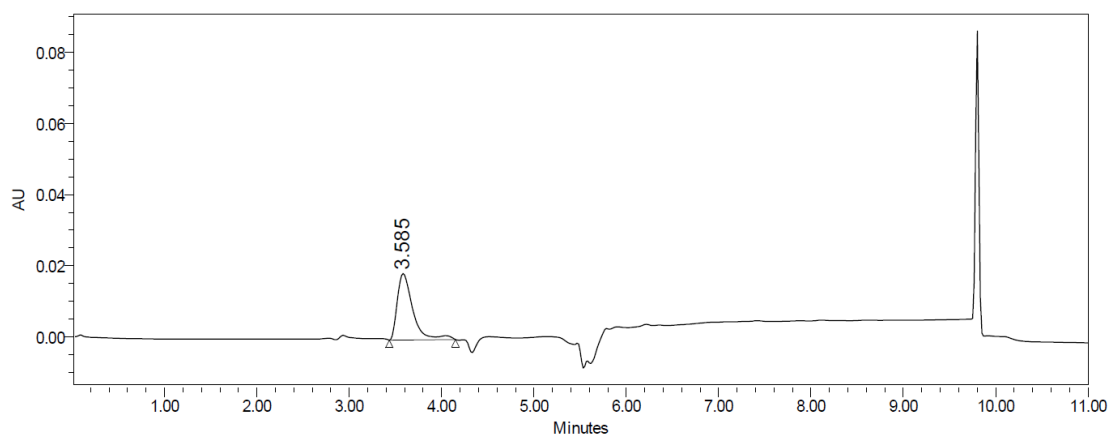


Figure 11. Ergothioneine with concentration of 0.004 mg/mL dissolved in phosphate-buffered saline.

Mobile phase: A rapid gradient transition of 95:5 to 5:95

A line – HPLC water with 1% formic acid

B line – acetonitrile with 1% formic acid

Flow rate: 0.6 mL/min

Detection: 254 nm

Injection volume: 10 μ L

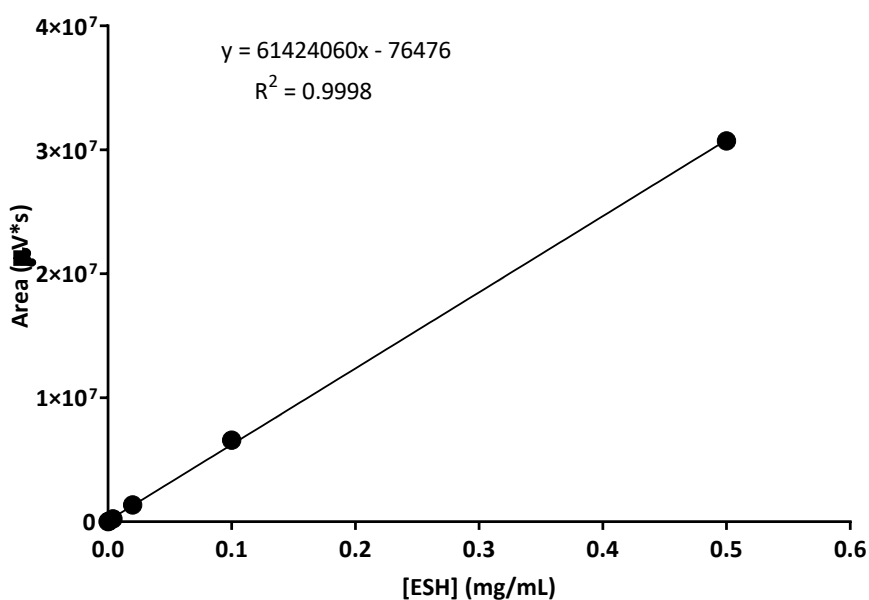


Figure 12. Calibration curve of ergothioneine (0.00016 mg/mL to 0.5 mg/mL) dissolved in HPLC water.

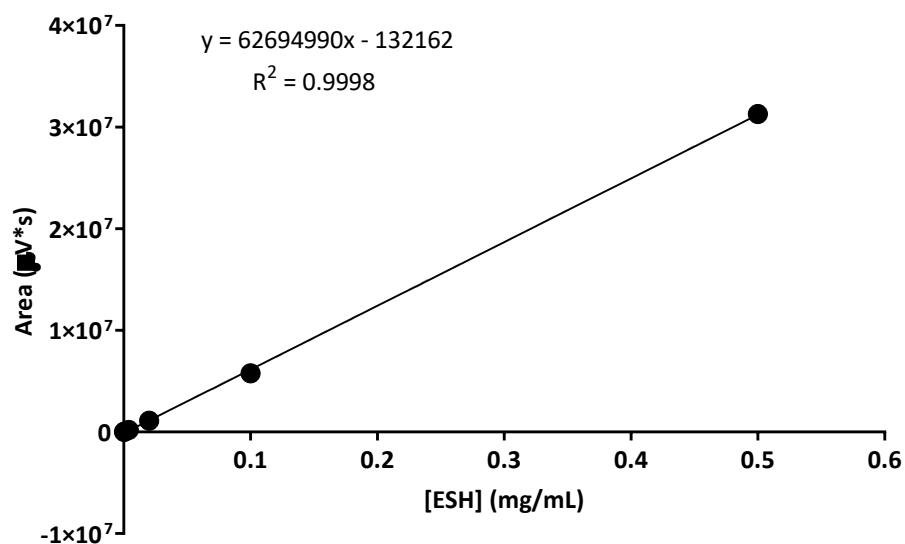


Figure 13. Calibration curve of ergothioneine dissolved in 1% Triton X-100.

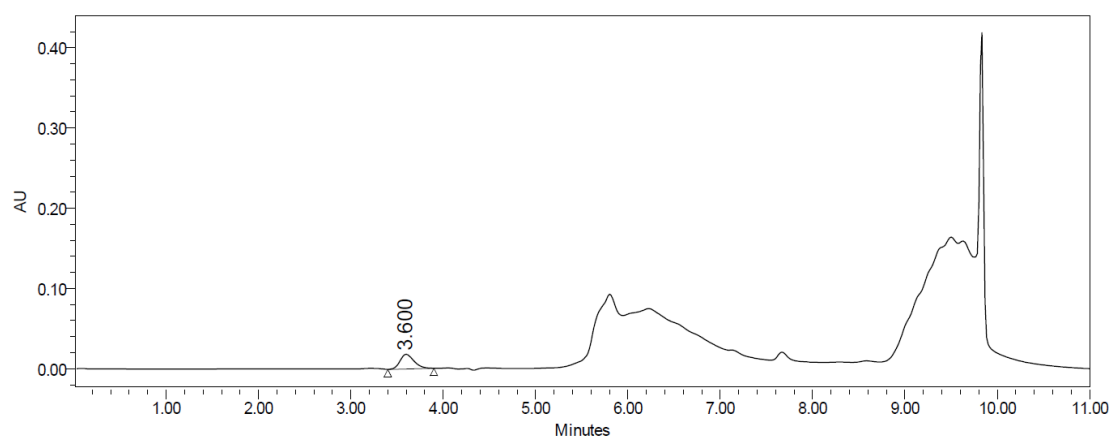


Figure 14. Ergothioneine with concentration of 0.004 mg/mL dissolved in 1% Triton X-100.

Mobile phase: A rapid gradient transition of 95:5 to 5:95

A line – HPLC water with 1% formic acid

B line – acetonitrile with 1% formic acid

Flow rate: 0.6 mL/min

Column temperature: 30°C

Detection: 254 nm

Injection volume: 10 µL

HPLC readings

Table 3. Supernatant - using H₂O calibration curve

ESH-concentration for extruded liposome samples of cholesterol and different neutral or charged phospholipid before aerosolization in distilled H₂O.

Lipid composition (molar ratio)	Area ($\mu\text{V}\cdot\text{s}$)	[ESH] (mg/mL)
DPPC:Chol (66.7:33.3)	5541763 $\pm$ 11782	1.7795 $\pm$ 0.0038
DPPC:Chol:PEA (7:2:1)	10684563 $\pm$ 4170	1.7270 $\pm$ 0.0007
DPPC:Chol:DPSG (7:2:1)	2612858 $\pm$ 11373	0.8259 $\pm$ 0.0037
DPPC:Chol:DPSG (7:2:1) ESH I	6231684 $\pm$ 13430	0.8017 $\pm$ 0.0017
DPPC:Chol:DPSG (7:2:1) ESH II	3750765 $\pm$ 14103	1.1964 $\pm$ 0.0046
DPPC:Chol:DPSG (7:2:1) ESH III	3894409 $\pm$ 3437	1.2431 $\pm$ 0.0011

Table 4. Pellet - using 1% Triton X-100 calibration curve

ESH-concentration for extruded liposome samples of cholesterol and different neutral or charged phospholipid before aerosolization in distilled H₂O.

Lipid composition (molar ratio)	Area ($\mu\text{V}\cdot\text{s}$)	[ESH] (mg/mL)
DPPC:Chol (66.7:33.3)	39838 $\pm$ 2403	0.0055 $\pm$ 0.0001
DPPC:Chol:PEA (7:2:1)	3376065 $\pm$ 22725	0.1119 $\pm$ 0.0007
DPPC:Chol:DPSG (7:2:1)	2071907 $\pm$ 35789	0.0703 $\pm$ 0.0011
DPPC:Chol:DPSG (7:2:1) ESH I	3418381 $\pm$ 23780	0.1133 $\pm$ 0.0008
DPPC:Chol:DPSG (7:2:1) ESH II	4640313 $\pm$ 11283	0.1522 $\pm$ 0.0004
DPPC:Chol:DPSG (7:2:1) ESH III	4990920 $\pm$ 11773	0.1634 $\pm$ 0.0004

Table 5. Pellet - using 1% Triton X-100 calibration curve

HPLC determined ESH-concentration for extruded liposome samples of DPPC:Chol:DSPG (7:2:1) after aerosolization in distilled H₂O.

Lipid composition (molar ratio)	Area ($\mu\text{V}\cdot\text{s}$)	[ESH] (mg/mL)
DPPC:Chol:DPSG (7:2:1) ESH I	70064 $\pm$ 1473	0.0065 $\pm$ 0.0000
DPPC:Chol:DPSG (7:2:1) ESH II	784078 $\pm$ 4030	0.0292 $\pm$ 0.0001
DPPC:Chol:DPSG (7:2:1) ESH III	495535 $\pm$ 2482	0.0200 $\pm$ 0.0001

Data are reported as means $\pm$ SD for 3 technical repetitions

4.1.1 Encapsulation efficacy

Consequently, the initial trials using phosphate-buffered saline (PBS) as the aqueous rehydration medium for the liposome samples were not considered in this chapter because the phospholipid quantification studies were not executed on the samples, as inorganic phosphate could interfere with Stewart assay. HPLC evidence of free encapsulated ergothioneine in pellet samples provided minimal fluctuation from 0.0007 mg/mL to 0.002 mg/mL from distilled H₂O samples for DPPC:Chol liposomes. At the begin three liposome formulations were reviewed, from a combination of cholesterol with a single or mixture of neural and charged phospholipids. The characterization is summed in Table 6.

Ergothioneine was encapsulated by dissolving in rehydration medium, whereby showing final medium a pH of 5.67. The encapsulation, described in Table 7, was valued with the aid of encapsulation efficiency that present final ergothioneine via phospholipid $[ESH/Lip]_f$ to initial ergothioneine via phospholipid $[ESH/Lip]_i$ (Table 7).

$$EE = \frac{[ESH/Lip]_f}{[ESH/Lip]_i} \times 100$$

Initial ergothioneine and phospholipid concentrations were determined to quantities added during preparation of 10 mg/mL ergothioneine and final ergothioneine and phospholipid concentrations were measured by HPLC and Stewart assay.

Encapsulation fluctuated significantly between samples. The smallest size, 161 nm encapsulation reached the greatest encapsulation with 20.5%. Thereupon enhancement of DSPG-formulations was observed through modification of the liposome preparation method. The liposome preparation protocol included prompt extrusion of the samples after ultrasonic bath for 15 min trough 800 nm, 600 nm, 400 nm and 200 nm pore size membrane consecutively. The extrusion was repeated seven until twenty-two times and again the size after the extrusion is analysed by means of Zetasizer Nano ZS90.

After a few attempts it was decided, that new DSPG liposome formulations after ultrasonic bath were extruded once through a Whatman Nucleopore polycarbonate filter of 600nm pore size and after then stored overnight at 4-6°C. The next day, another extrusion step was performed with 600 nm and then 200 nm pore size until an average liposome diameter +/- 200 nm was achieved, and the samples were then stored again at 4-6°C before ultracentrifuge (Table 6 and 7). The following details can be found under methods (3.2 experimental methods).

Encapsulation efficiency (EE) fluctuated significantly between formulations and from the DSPG formulation test. The Encapsulation efficiency (EE) generally decreased from the original test sample. No correlation was observed between mean diameter and determined Encapsulation efficiency (EE) with a correlation coefficient of -0.4745 indicating that size had no influence.

Table 6. Physicochemical characterization of liposome formulations after extrusion in distilled H₂O.

Lipid composition (molar ratio)	Median diameter (d.nm)	Polydispersity index	ζ-potential (mV)
DPPC:Chol (66.7:33.3)	203.6	0.138	-12.7
DPPC:Chol:PEA (7:2:1)	244.6	0.245	-13.10
DPPC:Chol:DSPG (7:2:1)	161.0	0.088	-53.00
DPPC:Chol:DSPG (7:2:1) ESH I	205.1	0.178	-68.5
DPPC:Chol:DSPG (7:2:1) ESH II	220.3	0.185	-61.6
DPPC:Chol:DSPG (7:2:1) ESH III	189.3	0.193	-60.6

Table 7. ESH encapsulation efficiency (EE) for liposome formulations after extrusion in distilled H₂O.

Lipid composition (molar ratio)	[ESH/Lip]_i	[ESH/Lip]_f	EE [%]
DPPC:Chol (66.7:33.3)	2.4074 ± 0.0000	0.0318 ± 0.0008	1.3 ± 0.0
DPPC:Chol:PEA (7:2:1)	2.0313 ± 0.0000	0.1229 ± 0.0017	6.0 ± 0.1
DPPC:Chol:DSPG (7:2:1)	2.0313 ± 0.0000	0.4169 ± 0.0101	20.5 ± 0.5
DPPC:Chol:DSPG (7:2:1) ESH I	1.7188 ± 0.0000	0.0888 ± 0.0009	5.2 ± 0.1
DPPC:Chol:DSPG (7:2:1) ESH II	1.7188 ± 0.0000	0.1840 ± 0.0046	10.7 ± 0.3
DPPC:Chol:DSPG (7:2:1) ESH III	1.7188 ± 0.0000	0.2667 ± 0.0085	15.5 ± 0.5

Data is described as single suspension sample Zetasizer measurement at 10-fold dilution.

4.1.2 Stewart Assay

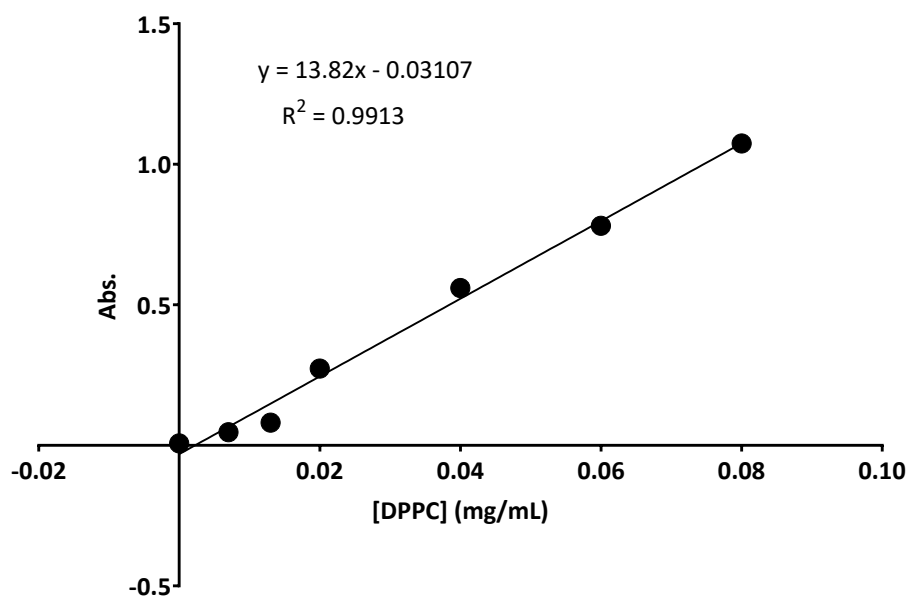


Figure 15. Calibration curve of 1,2-Dipalmitoyl-sn-glycero-3-phosphocoline (DPPC) with ammonium ferrothiocyanate complex in chloroform.

Stewart Assay readings

Table 8. Empty and ESH-loaded Sample - Phospholipid content for extruded liposome samples and UV absorbance measured at 485 nm.

Lipid composition (molar ratio)	Absorbance (Abs.)	Phospholipid con- centration in 3 mL chloroform layer (mg/mL)	Phospholipid con- centration in sample (mg/mL)
DPPC:Chol:PEA (7:2:1) Empty			
Avg	0.026	0.004	0.062
SD	0.003	0.0002	0.004
DPPC:Chol:PEA (7:2:1) ESH-loaded			
Avg	0.808	0.061	0.911
SD	0.013	0.001	0.014
DPPC:Chol:DSPG (7:2:1) Empty			
Avg	0.300	0.024	0.359
SD	0.002	0.000	0.003
DPPC:Chol:DSPG (7:2:1) ESH-loaded			
Avg	0.124	0.011	0.169
SD	0.001	0.0001	0.001
DPPC:Chol (66.7:33.3) Empty			
Avg	0.019	0.004	0.055
SD	0.003	0.0002	0.003
DPPC:Chol (66.7:33.3) ESH-loaded			
Avg	0.128	0.012	0.173
SD	0.005	0.0004	0.005

Table 9. Empty and ESH-loaded Samples I to III - Phospholipid content for extruded liposome samples and UV absorbance measured at 485 nm before aerosolization.

Lipid composition (molar ratio)	Absorbance (Abs.)	Phospholipid concentration in 3 mL chloroform layer (mg/mL)	Phospholipid concentration in sample (mg/mL)
DPPC:Chol:DSPG (7:2:1) Empty I			
Avg	0.261	0.021	0.317
SD	0.003	0.000	0.003
DPPC:Chol:DSPG (7:2:1) Empty II			
Avg	0.187	0.016	0.252
SD	0.010	0.001	0.012
DPPC:Chol:DSPG (7:2:1) Empty III			
Avg	0.069	0.007	0.120
SD	0.003	0.000	0.004
DPPC:Chol:DSPG (7:2:1) ESH I			
Avg	0.465	0.036	1.276
SD	0.018	0.001	0.018
DPPC:Chol:DSPG (7:2:1) ESH II			
Avg	0.523	0.040	0.828
SD	0.046	0.003	0.019
DPPC:Chol:DSPG (7:2:1) ESH III			
Avg	0.330	0.026	0.613
SD	0.028	0.002	0.020

Table 10. ESH-loaded Samples I to III - Phospholipid content for extruded liposome samples and UV absorbance measured at 485 nm after aerosolization.

Lipid composition (molar ratio)	Absorbance (Abs.)	Phospholipid con- centration in 3 mL chloroform layer (mg/mL)	Phospholipid con- centration in sample (mg/mL)
DPPC:Chol:DSPG (7:2:1) ESH I			
Avg	0.090	0.009	1.442
SD	0.094	0.007	1.126
DPPC:Chol:DSPG (7:2:1) ESH II			
Avg	0.164	0.014	1.550
SD	0.085	0.006	0.677
DPPC:Chol:DSPG (7:2:1) ESH III			
Avg	0.074	0.008	1.254
SD	0.028	0.002	0.330

4.2 Aerosolization

The physicochemical and ESH content were measured, before and after the aerosolization with an Aerogen Pro nebulizer and on that way the effects of the pressures caused by aerosolization on the DSPG formulation liposomal suspensions were available, which are represent in Figures 16.1, 16.2, 16.3 and Table 11. The focus is particularly on the aerosolization process of the liposomes' structures, the loaded, as well as the empty liposomes (Empty-n), which were manufactured concurrently. Unlike Figures 16.1, 16.2 and 16.3, Table 11 is focusing on the effect of the aerosolization of the encapsulation, $[ESH/Lip]_i$ ($[ESH/Lip]_f$ of Table 7 was used as result).

A significant variation is noticed in the median diameter and polydispersity index for ESH-loaded suspensions, in particular affected is the ESH III for diameter and both diameter and polydispersity index for ESH II. Generally observed the is a decrease in both factors After the aerosolization. No difference is neglectable at the surface charge of the loaded liposomes, neither each individually nor when combined.

Therefore, there was no variation detected in any measured physicochemical characteristic of the empty liposome samples.

The retention of ESH has no significant variation but is under the average a combined average across all repeats and replicates of $8.9 \pm 4.1\%$. In conclusion, the results initiate a poor stability during the aerosolization with the Aerogen Pro nebulizer.

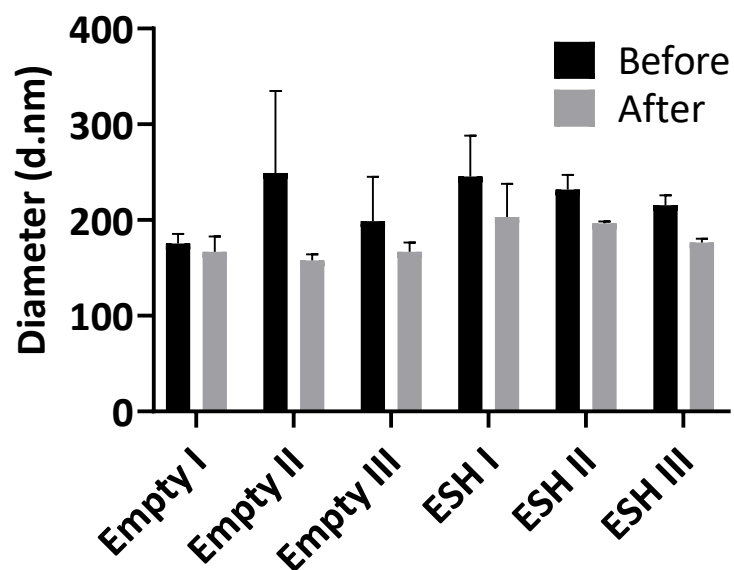


Figure 16.1. Median diameter of extruded liposome formulation (DPPC:Chol:DSPG 7:2:1) in distilled H₂O. Before and after aerosolization using an Aerogen Pro nebulizer. In this Figure you can see a little significant differences before and after aerosolization.

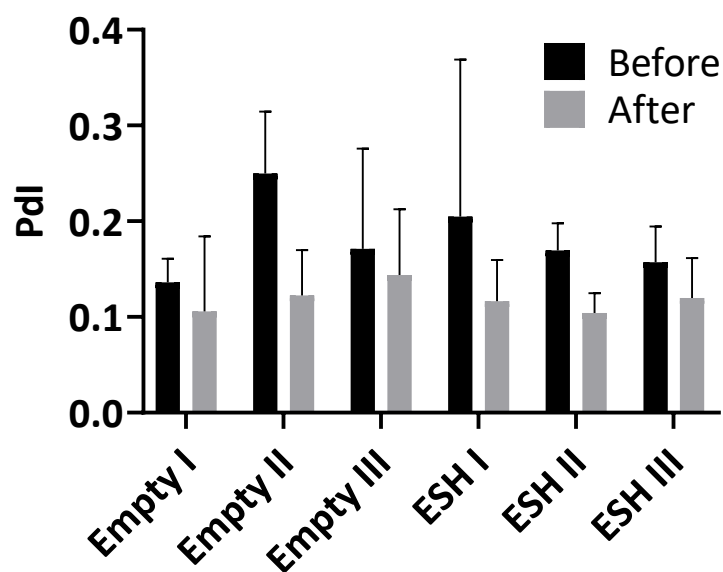


Figure 16.2. Polydispersity index (Pdl) of extruded liposome formulation (DPPC:Chol:DSPG 7:2:1) in distilled H₂O. Before and after aerosolization using an Aerogen Pro nebulizer. In this Figure you can clearly see significant differences before and after aerosolization.

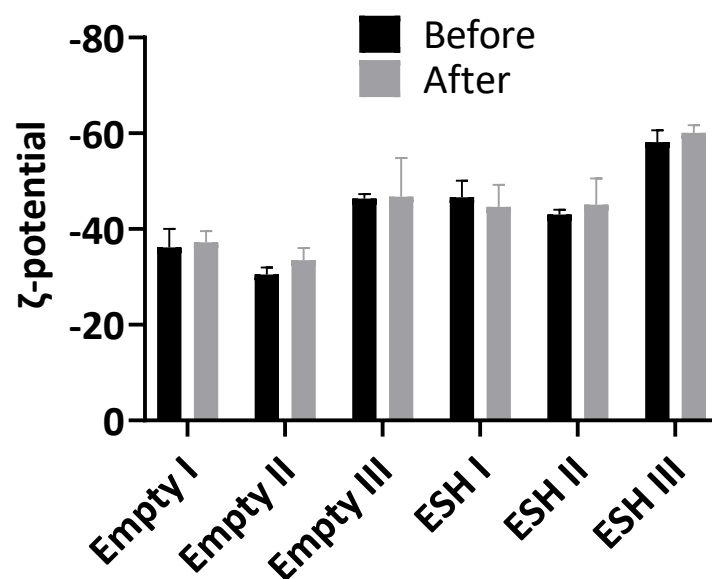


Figure 16.3. ζ-potential of extruded liposome formulation (DPPC:Chol:DSPG 7:2:1) in distilled H₂O. Before and after aerosolization using an Aerogen Pro nebulizer. There are no significant differences in this Figure.

Table 11. Ergothioneine retention before and after aerosolization using an Aerogen Pro nebulizer for extruded liposome formulations (DPPC:Chol:DSPG 7:2:1) in distilled H₂O.

Replicate	[ESH/Lip] _{before}	[ESH/Lip] _{after}	ER [%]
ESH I	0.0888 ± 0.0009	0.0074 ± 0.0037	8.3 ± 4.2
ESH II	0.1840 ± 0.0046	0.0221 ± 0.0074	11.9 ± 03.8
ESH III	0.2667 ± 0.0085	0.0170 ± 0.0038	6.4 ± 1.4

4.3 Stability

For the stability test, liposome suspensions were analysed on day 1 after preparation, then on days 7, 15, and 30 to determine stability during refrigerated storage in a refrigerator (4-6°C). Samples were diluted 10-fold with distilled H₂O and then the physicochemical properties of the liposomes were determined using Zetasizer Nano ZS90. The initial results are shown with different phospholipid formulations (Figure 17.1, Figure 17.2 and Figure 17.3).

To assess the data, a regression analysis was performed to identify whether stability changed over time. Null hypothesis was based on a linear relationship with a slope of 0. This means that there was no change over the month. The significant deviation from the null hypothesis $p < 0.05$ indicates. During the month, there were no significant changes in diameter for all formulations, despite increases for the liposome formulation DPPC:Chol (66.7:33.3) (Figure 17.1).

Although heterogenicity plot of empty liposome formulation DPPC:Chol increased, the modification was not significant with a p-value of 0.0773 and was only noted for the ergothioneine liposome formulation DPPC:Chol and DPPC:Chol:DSPG. Over the month only the ergothioneine liposome formulation DPPC:Chol resulted significant variation in ζ -potential.

All things considered, this means, the loaded liposome formulations DPPC:Chol:DSPG and DPPC:Chol:PEA (7:2:1) are stable in regard of charge and size over a month under refrigerated storage in refrigerator 4-6°C. Inclusion of ergothioneine effects instability in the liposome formulation DPPC:Chol and the polydispersity index of the liposome formulation DPPC:Chol:DSPG, whereby these modifications do not obtain when repeated with multiple sample replicates (Figure 18.1, Figure 18.2 and Figure 18.3).

Neither ANOVA tests or regression analysis indicate no significant change over month for any of the measured physicochemical properties of the empty and ergothioneine-containing liposomes.

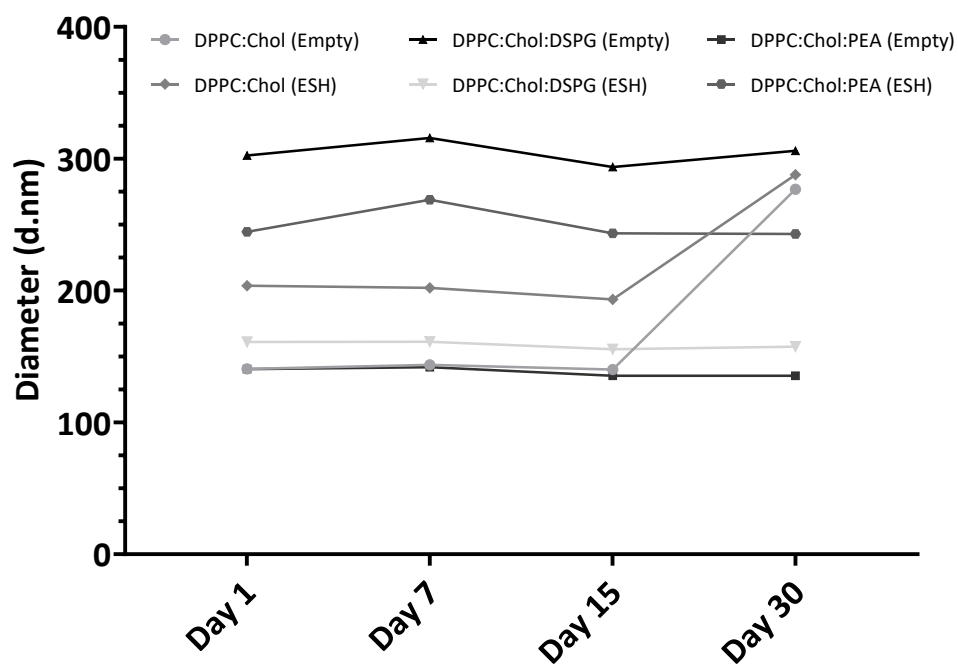


Figure 17.1. Median diameter from extruded liposome formulation of cholesterol and different neutral or charged phospholipid in distilled H₂O.

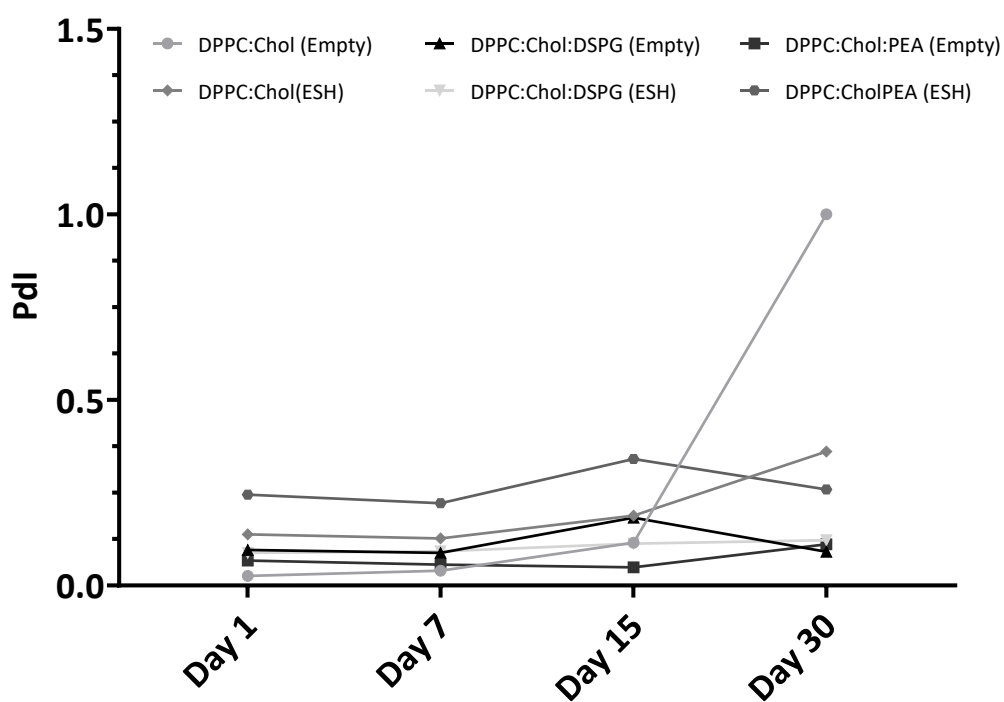


Figure 17.2. Polydispersity index (Pdl) from extruded liposome formulation of cholesterol and different neutral or charged phospholipid in distilled H₂O.

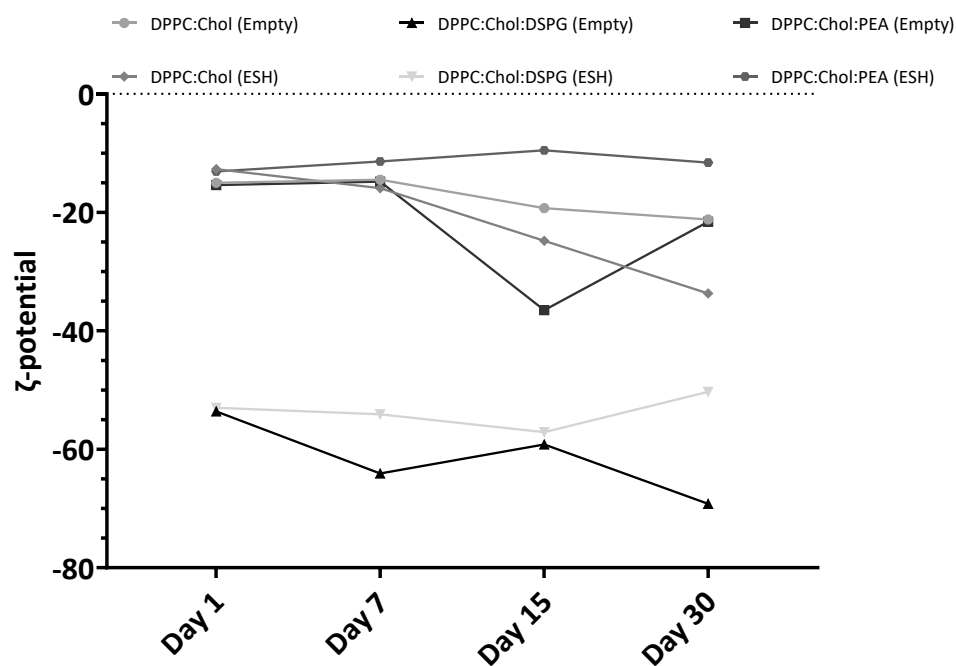


Figure 17.3. ζ-potential from extruded liposome formulation of cholesterol and different neutral or charged phospholipid in distilled H₂O.

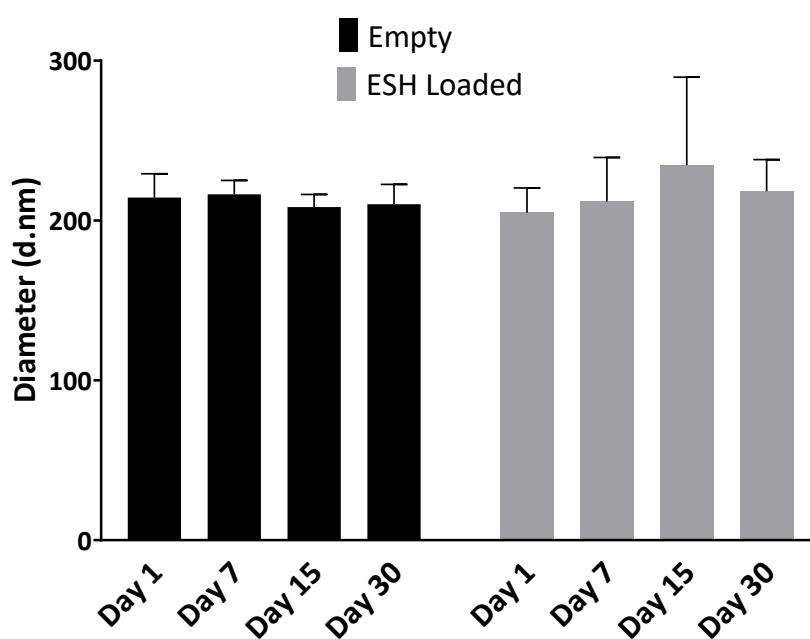


Figure 18.1. Median diameter of final extruded liposome formulation (DPPC:Chol:DSPG 7:2:1) in distilled H₂O.

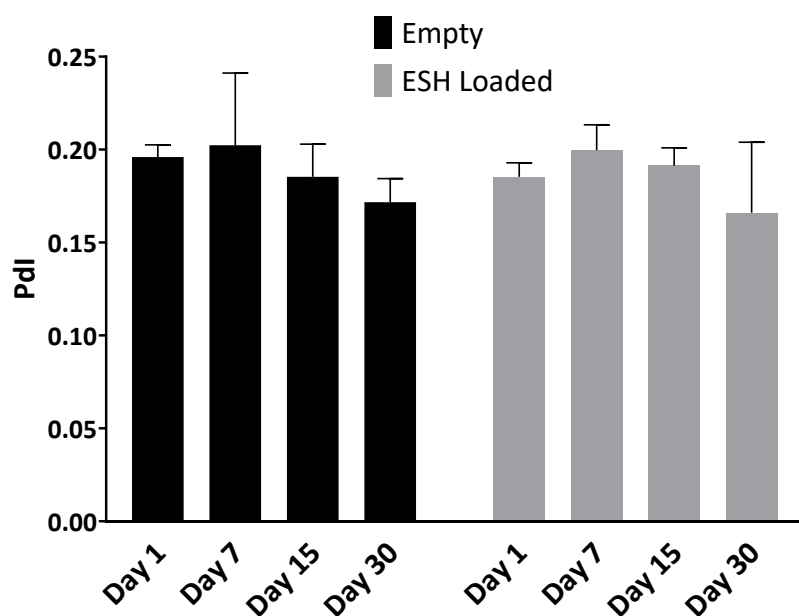


Figure 18.2. Polydispersity index (Pdl) of final extruded liposome formulation (DPPC:Chol:DSPG 7:2:1) in distilled H₂O.

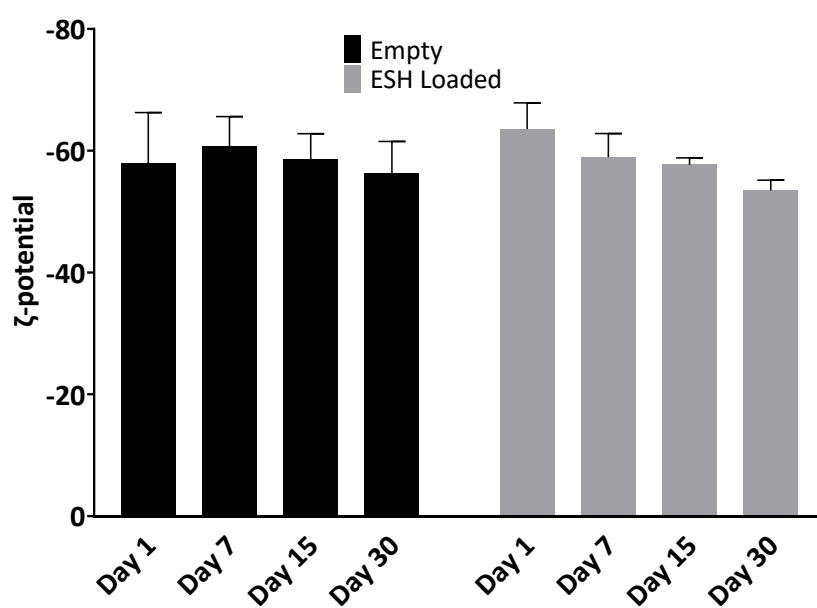


Figure 18.3. Zeta-potential of final extruded liposome formulation (DPPC:Chol:DSPG 7:2:1) in distilled H₂O.

5. DISCUSSION

In the context of this diploma thesis, an adequate dose of ergothioneine in aerosolized form as liposomes was effectively delivered *in vitro*. Not only a suitable liposomal suspension is necessary, but also one that could withstand the stress of aerosolization and provide ergothioneine an effective encapsulation. For the purpose to quantify ergothioneine, an HPLC method was developed (Liu *et al.*, 2016; Zhou *et al.*, 2014). This method allows quantification in less than 10 min. This proceed had a good specificity and sensitivity in Triton X-100 and water with an identifiable peak for ergothioneine. Liposomes with different phospholipid components were studied for encapsulation.

The liposome formulation DPPC:Chol:DSPG (7:2:1) showed the best encapsulation ability and had an encapsulation efficiency of $20.5 \pm 0.5\%$. Although the Zetasizer measurements showed a low liposome median diameter of 161 nm, giving the smallest size, which could be due to the charged nature of both ergothioneine and DSPG. The other two liposome formulations DPPC:Chol (66.7:33.3) and DPPC:Chol:PEA (7:2:1) had a higher median diameter of 203.6 nm and 244.6 nm, respectively, but a lower encapsulation efficiency by a factor of 3 to 15 than the DPPC:Chol:DSPG liposomes. The study by Gasper *et al.* is comparable to the liposome formulations studied. Here, instead of ergothioneine, calcein was encapsulated and the EE was $20 \pm 2\%$ (Gasper *et al.*, 2010).

The dissolved ergothioneine in distilled water shows a pH of 5.67, which is slightly acidic. This means that the dissolved zwitterionic ergothioneine can change to a protonated form and is more positively charged. This would favour negatively charged liposomes with DSPG components and thus increase passive enrichment in liposome vesicles. The fact of favourable charged interactions is confirmed by ζ -potential of DSPG formulation. The DSPG formulations are very negatively charged compared to the others and for both the empty and loaded liposomes (Table 6 and Figure 17.3). In the *in vitro* study by Sahebkar *et al.* the effect of different nanoliposomes was tested. One of the highest uptake rate by macrophages and best lipid modifying activity shows the anionic phospholipid DSPG (Sahebkar *et al.*, 2014).

Regarding stability, there was no significant change in diameter for any of the three liposome formulations. Whereas the ESH loaded liposome DPPC:Chol showed instability surface charge and destabilized the heterogeneity of DPPC:Chol:DSPG and DPPC:Chol (Gaspar *et al.*, 2008). The liposome component DPPC:Chol:PEA indicated that it was most stable in refrigerated storage at 4-6°C for one month. Given the relatively good stability and strong encapsulation ability of the liposome DSPG formulation, further experiments were performed only for this liposome component.

To improve the encapsulation, the extrusion protocols were changed to produce liposomes whose diameter comes in the range of ± 200 nm. Unfortunately, there was not much effect on encapsulation efficiency by changing the more heterogeneous and larger liposome formulations. The study by Zaru *et al.* reported better retention and encapsulation of the drug ripampicin with rigid liposomes compared with liquid liposomes after nebulization (Zaru *et al.*, 2007).

The comparison of three new liposomes DSPG suspensions with larger size did not indicate any improvement over the original liposome samples, thereby it also showed no correlation between mean diameter and encapsulation efficiency. However, in the study of Swaminathan *et al.*, the salmon calcitonin was lyophilized to obtain an increased encapsulation efficiency (Swaminathan *et al.*, 2014). Moreover, a large fluctuation between samples was noticed. However, the 5% difference in encapsulation efficiency, analysis of phospholipid and ergothioneine concentrations for samples two and three yielded similar ergothioneine concentration values. The measured phospholipid concentrations showed a large difference and could be attributed that it was due to the variation in phospholipid complexation.

Lack of improvement and variation could also be due to the leaky extruder and the amount and grade of extrusion used. Significant improvement is achieved with the stability tests, implemented on these new DSPG samples, as well with the ESH-loaded liposomes. For over one month remained stable in all physicochemical characteristics. Empty liposomes showed physicochemical stability compared to ergothioneine-loaded liposomes, which may be due to interactions. This complete instability is uncharacteristic and likely due to the presence of ergothioneine (Gaspar *et al.*, 2008). According to the Stewart study, the biggest impact on the phospholipid concentrations reading could be the alterations in the process of complexation of the phospholipids in the assay (Stewart, 1980).

In general, the liposome composition (DPPC:Chol:DPSG 7:2:1), presented a good stability with encapsulation efficiency of ergothioneine and an average 5% higher aerosolization retention. Despite all, the presence of ergothioneine increases the physicochemical instability of the aerosol (Gaspar *et al.*, 2010). Basically, this indicates a high potential as oral inhalation formulation for the treatment of COPD and various chronic lung diseases. For this usage furthermore studies are required to determine the effect of ergothioneine on resistance to nebulization exposure since the variations in encapsulation and size can lead to changes in *in vivo* profiles, accompanied with changes of the therapeutic effect.

The last step would have been to insert the liposomes *in vitro* into the cell line NCI-H441. Unfortunately, the time was too short to make further attempts (Salomon *et al.*, 2014).

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