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Up-scaling and further development of matrix liposomes

Diploma Thesis

Heidelberg & Hradec Králové 2017 Veronika Skalická

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, 22. 8. 2017 Veronika Skalická

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Contents

ABSTRACT

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Supervisor: Dr. Georgios Paraskevopoulos, Ph.D.; Michael Binnefeld

Title of Diploma thesis: Up-scaling and further development of matrix liposomes

The thesis evaluates novel matrix liposomes formed by dual asymmetric centrifugation technology. Particles' parameters (size, size distribution and ζ-potential) were analyzed by dynamic light scattering method using ZetaSizer device. Encapsulation efficacy was determined by size exclusion chromatography using carboxyfluorescein as fluorescent encapsulation marker. The overall results support that the most beneficial formulation parameters are 15 min of speed mixing process with ceramic beads of diameter 1,0-1,2 mm. Up-scaling of the procedure didn´t drastically affect liposomal parameters up to a lipid batch load of 700 mg. Furthermore, the usage of different types of gelatin or glycerinated gelatin didn´t significantly influenced particles' characteristics. It is noteworthy that the matrix composed of 50% glycerinated gel sustained the ability to form liposomes by dispersion of vesicular phospholipid gels in phosphate buffer saline even after 31 days. Other matrices showed a damage of liposomes as confirmed by size and size distribution. In conclusion, the obtained data could contribute to the transition from lab scale to industrial scale of the manufacturing procedure. Furthermore, matrix formed from 50 % glycerinated gelatin showed advanced parameters after prolonged storage (31 days) and thus offers a possibility in order to extend the shelf life of obtained liposomes.

ABSTRAKT

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Školitel: Dr. Georgios Paraskevopoulos, Ph.D.; Michael Binnefeld

Název diplomové práce: Up-scaling a další vývoj matricových liposomů

Diplomová práce se zabývá hodnocením liposomů připravených metodou duální asymetrické centrifugace. Parametry částic (velikost částic, distribuce částic, zeta potenciál) byly analyzovány za využití dynamického rozptylu světla v přístroji ZetaSizer. Účinnost enkapsulace byla stanovena metodou gelové chromatografie za využití karboxyfluoresceinu jako fluorescenčního markeru. Z výsledků vyplývá, že nejvýhodnější parametry formulace jsou 15 min centrifugace za využití keramických kuliček o průměru 1,0-1,2 mm. Up-scaling procesu neovlivnil vlastnosti liposomů až do navážky 700 mg. Ani použití různých typů želatiny nebo glycerolgelu želatiny nevedlo k signifikantně rozdílným výsledkům. Stojí za povšimnutí, že matrice vytvořená z 50 % glycerolgelu želatiny umožnila redisperzi vesikulárních fosfolipidových gelů v fosfátovém pufru a vytvoření liposomů po 31 dnech skladování. Další matrice vykazovaly známky poškození, které bylo potvrzeno analýzou dle jejich velikosti a distribuce. Závěrem lze říci, že získaná data mohou přispět k převedení výrobního procesu z laboratorního do průmyslového měřítka. Kromě toho, matrice vytvořená z 50 % glycerolgelu želatiny vykazovala vhodné parametry liposomů po měsíční době uchovávání a tím potencionálně umožňuje prodloužit jejich skladovatelnost.

1. Aim of the study

The aims of the study were to:

A. Up-scale and optimize speed mixing technology for the formulation of matrix liposomes by using new dual asymmetric centrifugation device
B. Develop new possible matrices for the entrapment of matrix liposomes
C. Analyze the stability of liposomes during short length of storage

and thus contribute to the evolvement of new solid matrix liposomal formulations for peroral drug delivery.

2. List of Abbreviations

CF	mixture of 5- and-6-Carboxyfluorescein
Chol	Cholesterol
DAC	Dual Assymetric Centrifugation
DLS	Dynamic Light Scattering
EE	Encapsulation Efficiency
EPC	Egg-phosphatidylcholine
GIT	Gastro-Intestinal Tract
GUV	Giant Unilamellar Vesicles
HPH	High-Pressure Homogenization
LUV	Large Unilamellar Vesicles
MLV	Multilamellar Vesicles
OLV	Oligolamellar Vesicles
PALS	Phase Analysis Light Scattering
PBS	Phosphate-buffered saline
PCS	Photon Correlation Spectroscopy
PdI	Polydispersity Index
SEC	Size Exclusion Chromatography
VPG	Vesicular Phospholipid Gel
Z-ave	Z-average

3. Introduction

Liposomes have been widely investigated as effective drug delivery systems. *(Panahi Y. et al 2017)* Since their discovery in the 60's *(Bangham A. D. et al. 1965)*, scientists are facing many challenges while trying to further develop their characteristics. On this regard, essential parameters like the increase of their bioavailability after peroral administration, the improvement and simplification of the manufacturing process as well as the avoidance of microbial contamination, in order to extend their shelf life, are under investigation. *(Bozzuto G. and Molinari A. 2015)*

Matrix liposomes are small spheroid vesicles whose lipid bilayer is composed of egg-phosphatidylcholine (EPC) and cholesterol (Chol) while the entire particles of liposomes are embedded in a gelatin matrix. *(Pantze S. F. et al. 2014)* Regarding its ability to form a gel state and solidify, the final formulation showed solid character at room temperature. In addition, the presence of gelatin afforded liposomes with a higher stability under gastric and intestinal conditions in the gastrointestinal tract (GIT) compared to standard liposome compositions. Therefore, matrix liposomes were suggested as a prospective formulation for peroral drug delivery.

For the needs of this thesis, speed mixing technology was used in order to prepare matrix liposomes. This method is based on a combination of two contra rotating movements which generate shear forces and thus lead to more efficient homogenization of lipid blend. *(Massing U. et al. 2008)* Due to the development of a new speed mixing device, we were able to omit the step of forming dried lipid film which is otherwise needed. Besides time saving, this technology enables to decrease the consumption of organic solvents and make the entire process more friendly for the environment. To conclude, the new speed mixing device was used to up-scale and optimize the formulation of matrix liposomes by dual asymmetric centrifugation (DAC) and develop new, potentially more stable, matrix agents with beneficial properties.

4. Theoretical Background

4.1. Characteristics of Liposomes

Liposomes were firstly described by Baghham and his coworkers in 1965. *(Bangham A. D. et al. 1965)* They are small spheroid vesicles that consist of a lipid bilayer around an aqueous core. This formation allows the entire system to be thermodynamically stable thanks to hydrogen bonding, van der Waals forces and other electrostatic interactions. Due to their amphiphilic character, they are able to encapsulate both hydrophilic and lipophilic drugs. Lipophilic substances are entrapped in lipid bilayer whereas hydrophilic ones are embedded in the aqueous core. *(Pattni B. S. et al. 2015)*

4.1.1 Classification

In general, liposomes are classified either by their size (diameter length) or their lamellarity (number of bilayers). *(Bozzuto G. and Molinari A. 2015, Jesorka A. and Orwar O. 2008)* According to their size, they can be classified as small (<0.1μm), large (~0.1μm to ~1μm) or giant (>1μm) and according to their lamellarity, they can be classified as unilamellar, oligolamellar or multilamellar. The combination of the two afformentioned types of classification can result combinatory characterisations like MLV (multilamellar vesicles), SUV (small unilamellar vesicles), LUV (large unilamellar vesicles) or GUV (giant unilamellar vesicles) and have been used in this thesis (Figure 1).

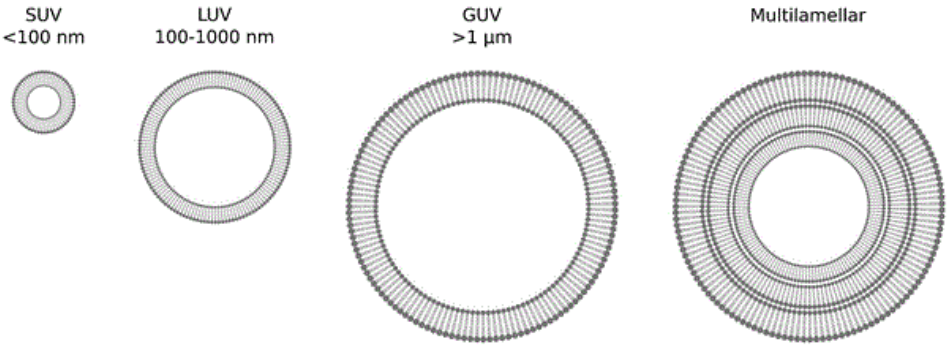

Fig. 1 Scheme of liposomes´classification. (Van Swaay D. and deMello A. 2013)

4.1.2 Stability

The stability of liposomes is determined by their physicochemical characteristics. Their chemical structure is prone to oxidation, due to the presence of unsaturated aliphatic acid chains or hydrolysis due to the presence of ester groups, depending on pH and temperature. In addition, physical stability could be evaluated regarding the potential leakage of encapsulated substance, phase separation of membrane lipids or particle aggregation. *(Crommelin D. J. A. 1994)* In a first sufficient attempt to ensure long term stability, cholesterol (Chol) or α-tocopherol were implemented to the liposomal membrane. *(Hernández-Caselles T. et. al. 1990)* Furthermore, it was proved that the addition of charged compounds to the liposomal membrane lead to electrostatic repulsion preventing particles to aggregate. *(Pattni B. S. et al. 2015)*

4.1.3 Further Types of Liposomal Formulation

Vesicular Phospholipid Gels

Vesicular Phospholipid Gels (VPGs) are described as highly dense, semisolid liposomal dispersions. Their semisolid character is a result of the steric interactions between the neighboring, tightly pressed, vesicles. *(Brandl M. 2007)* These gels enable

chemotherapeutics as well as protein drugs to be released in a controlled manner. *(Tian W. et al. 2010)* One additional advantage is that there is no aqueous buffer surrounding the vesicles. This way the leakage of active substances is prevented since there is no concentration gradient between the core and the surrounding water phase. *(Brandl M. 2007)* High-pressure homogenization (HPH) and DAC are the methods usually used for the formulation of VPGs. *(Elnaggar Y. S. R. et al. 2014)*

Lipotubes (Lipid-Membrane Nanotubes)

Thanks to the fluidity of liposomal membrane, nanotubes can be created. In general, their diameter ranges about 100 nm and their length varies up to hundreds of micrometers. *(Jesorka A. and Orwar O. 2008)* They can be utilized as a transport system for electrophoresis inside the cells in molecular biology. *(Tokarz M. et al. 2005)*

4.1.4 Drug Loading in Liposomes

Liposomes enable entrapment of both hydrophilic and lipophilic drugs. *(Pattni B. S. et al. 2015)* Drug loading is related to the encapsulation process of active substance inside the liposomes and it can be passive or active. Passive loading is called the loading which is accomplished during the formulation of the vesicles. In contrast, active loading includes gradient diffusion which is taking place after the preparation procedure. *(Labajová A. 2014)* The latest is characterized by higher encapsulation efficiency and is commonly employed in the industrial production of liposomes.

4.2 Shelf Life of Liposomes

Efficacious long term stability is an essential premise of appropriate liposomal formulations. Different storage forms of liposomic formulations, like aqueous

dispersions or solid formations, have been previously used and presented herein in more details.

4.2.1 Storage in the Form of Aqueous Dispersions

The first essential possibility for liposome storage is storage in the form of aqueous dispersion described by Van Bommel and coworkers in the 80's. *(Van Bommel E. M. G. et. al. 1984)* The use of liposomes stored as dispersions is limited due to the possible fusion of vesicles and the leakage of active substance. *(Porfire A. et al. 2017)* Furthermore, other involved drawbacks are higher risk of degradation caused by hydrolysis, physical instability and microbial contamination. *(Morais A. R. et al. 2016)*

4.2.2 Storage in the Form of Solid Substance

The ability of liposomes' storage in solid form plays an important role in the extension of their shelf life. Solidification of liposomes' formulations increases their stability by protection against hydrolysis and physical degradation. In general, the most frequently used solidifying method of liposomes is freeze-drying. Another option of obtaining liposomal solid dosage forms is the concept of „proliposomes".

Freeze-Drying.

Freeze-drying deals with the problems of repeated hydration whereat bilayer membrane could be disrupted and thus vesicle aggregation or leakage of active substances could be occurred. To overhelm this process, lyoprotectants or cryoprotectants (trehalose, sucrose) are employed. (*Wilkhu J. S. et al*. 2017) The addition of various cryoprotectants also enables the extension of liposomal shelf life. *(Stark B. et al. 2010)* Furthermore, decreased loss of encapsulated substance was reached by freezing the liposomes with surrounding media containing the

concentration of embedded substance. Based on this evidence, it was allowed to prolong the storage of liposomes up to 8 weeks. *(Van Bommel E. M. G. et. al. 1984)* Regarding other studies further option for increasing stability is an addition of gelatin or calcium alginate to liposomes before freeze drying procedure. *(Guan P. et al. 2015)* Gelatin or calcium alginate incorporated inside the formulation lead to the persistence of size and polydispersity index of the particles. *(Wang L. et al. 2015)*

Proliposomes.

To overwhelm problems connected with storage of liposomal dispersion, the concept of proliposomes has been developed. Proliposomes were firstly defined in 1986 by Payne and his coworkers as dry lipid powder that in contact with aqueous buffer gives a rise to liposomal dispersion. *(Payne N. I. et al. 1986)* The advantage of granulate is a possibility to the subsequent formation of capsules or tablets and thus better suitability for up – scaling. *(Hiremath R. et al. 2016)*

4.3 Methods involved in the Formulation of Liposomes

Liposomes can be formulated by various methods. The choice of the most convenient procedure is a matter of factors like:

1. the physicochemical characteristics of the liposomes and desired entrapped drug (including its toxicity and/or concentration)
2. the character of the solution in which the particles are dispersed;
3. the additional aims connected with the application/delivery of the liposomes;
4. the diameter and the half-life of desired liposomes formulation
5. the financial intensity, reproducibility, and applicability regarding large-scale production for clinical purpose and good manufacturing practice *(Bozzuto G. and Molinari A. 2015, Labajová A. 2014)*

In the following text, different preparation methods are described. Initially, procedures connected with formulation of normal liposomes will be depicted, followed by

methods used for the formation of MLVs. Finally, methodologies of purification after drug encapsulation to liposomes will be briefly discussed. *(Cortesi R. 1999)*

4.3.1 Preparation of Liposomes

Thin-Film Hydration.

The oldest method of liposomes' preparation, first described by Bangham in the 60s , is based on the hydration of dry lipid film. *(Bangham A. D. et al. 1965)* The required membrane phospholipids are dissolved in organic solvent, mixed in a proper ratio and, subsequently, redundant organic solvent is evaporated using nitrogen or argon steam. Alternatively, if working with bigger batch sizes, the organic solvent is evaporated by using rotary evaporator. In addition, the vial or flask can be further dried under high vacuum for complete removal of residual solvents. Next step is the hydration or swelling. The dried film is exposed to an excess of an aqueous medium. The osmotic pressure of contained salts leads to detachment of phospholipid blend and forms the bilayer of membrane vesicles. The observed yields are influenced by the degree of bilayer separation which depends on several factors like temperature and ionic and/or lipid composition of surrounding media. In spite of long and often use the method, it encounters low encapsulation efficiency and high organic solvent consumption. In general, liposomes obtained using this method are MLVs. *(Jesorka A. and Orwar O. 2008)*

Detergent Dialysis.

Detergent dialysis method is used during the formulation of liposomes from detergent solutions at their critical micelle concentration. *(Dua J. S. et al. 2012)* Slow removal of detergent by dialysis results increased phospholipid concentration and the liposomes are formed. The procedure is characterized by high reproducibility and preparation of uniform-sized vesicles. However, it is facing some drawbacks, mainly with detergent residues.

Reverse Evaporation.

During reverse evaporation method, an aqueous buffer is injected to the solution of organic solvent and phospholipids. Subsequently, organic solvents are evaporated under reduced pressure. *(Szoka F. and Papahadjopoulos D. 1978)* This method provides LUVs and GUVs. The great advantage of this procedure is high encapsulation efficiency (up to 65%) of substances dissolved in aqueous buffer (eg. drugs as well as proteins and nucleic acids).

Solvent Injection.

This method involves a rapid injection of lipid solution in water-miscible organic solvent or a water-miscible solvent mixture into water. *(Schubert M. A. and Müller-Goymann C. C. 2003)* The method is generating SUVs in one-step procedure while high reproducibility is routinely ensured. Handling with organic solvents represents a negative aspect of this type of liposomes' formulation. The process is subsequently followed by evaporation, freeze drying or ultrafiltration to remove the potential residue of organic solvents. *(Jesorka A. and Orwar O. 2008)*

Supercritical Fluid Technology.

Supercritical state is described as the state of the substance when its temperature and pressure have overpassed the critical point but its pressure is bellow the one which is required to condense it into a solid. (*Knez Ž. et al 2015*) Thanks to these conditions, fluids gain specific properties as gas-like viscosity and liquid-like density. It is hardly distinguished whether the substance should be considered as gas or fluid. The most widely employed substance for the formation of liposomes is supercritical carbon dioxide due to its low price, chemical inertness, non-toxicity and relatively low critical parameters (critical temperature 31 °C and critical pressure 74 bar). Supercritical fluids are able to replace organic solvents in common liposomes' preparation techniques. During a typical example of reverse phase evaporation, phospholipids are dissolved in supercritical carbon dioxide. An aqueous medium is added and, subsequently, the

pressure of CO_2 is slowly decreased in order to generate liposomes *(Huang Z. et al. 2014)*

Dual Asymmetric Centrifugation (DAC)

DAC method takes advantage the addition of a second contra-rotating movement to a conventional centrifugation. The lipid blend in the vial is at the same time rotated clockwise around its vertical axis and counterclockwise around the vertical axis of the DAC device (Figure 2). The combination of two contra-rotating movements results in the occurrence of shear forces and thus to the efficient homogenization of the blend. Glass or ceramic beads could be added in the blend as agitation aid. After the speed mixing procedure, VPGs are formed and subsequent dispersion in aqueous medium gives rise to liposomes. Entrapment efficiency of model compound calcein ranges about 60 %. *(Massing U. et al. 2008)* An additional type of formulation prepared by DAC is parenteral fat emulsions. *(Tenambergen F. et al. 2013)* The technique is profitable on grounds of good reproducibility within small particles preparation, decreased organic solvent consumption and satisfying encapsulation of hydrophilic substances. *(Huang Z. et al. 2014)*

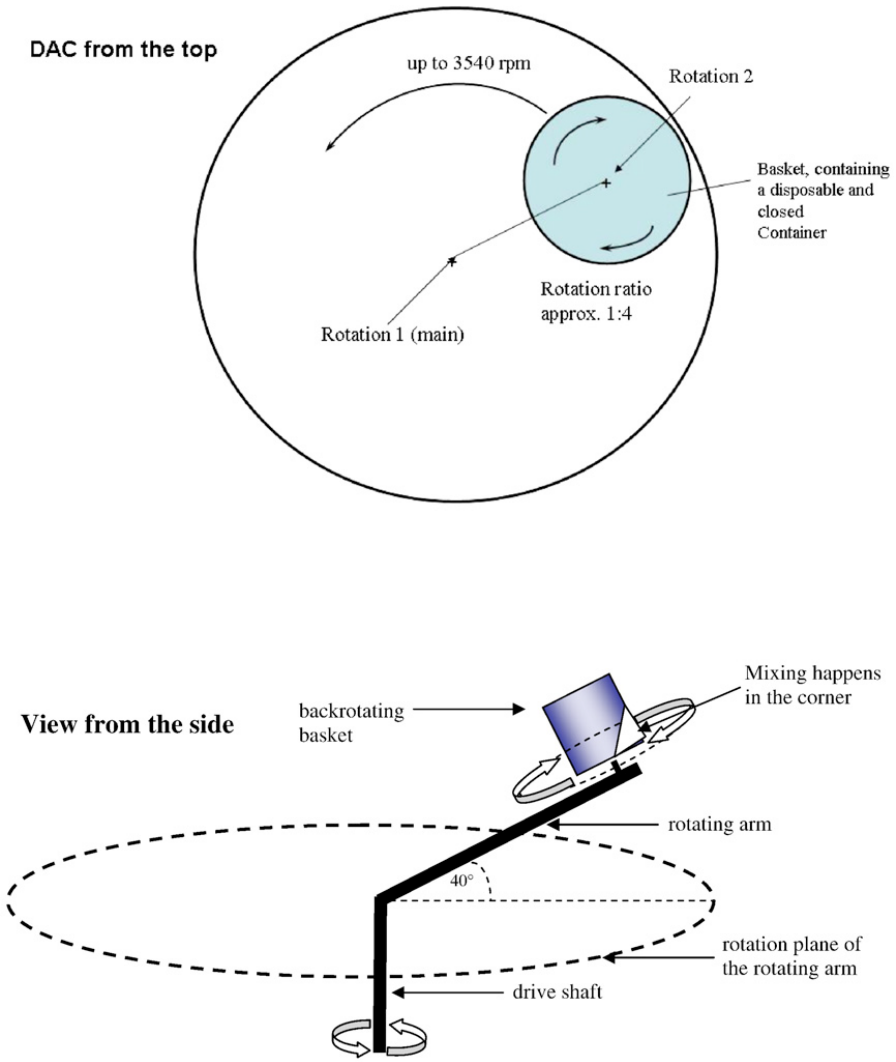

Fig. 2 Description of the DAC method (Massing U. et al. 2008)

Spray Drying

Spray drying is a single step method which is used for proliposomes' preparation. *(Pattni B. S. et al. 2015)* Lipids and desired encapsulated substance are dissolved in organic solvent and dried with the use of mannitol cores at controlled temperature and air flow rate. *(Skalko-Basnet N. et al. 2000)*

Membrane Contactor Technology

Membrane contactor technology allows liposomes' preparation while pressurized ethanol and dissolved lipids are passing through a membrane of defined pore size. On the other side of the membrane, there´s a column that contains a tangentially flowing

aqueous phase which dilutes ethanol and liposomes are self-assembled. *(Pattni B. S. et al. 2015)*

4.3.2 Modification of Liposomes

Sonication

Sonication method is a technology during which the size of liposomes is reduced and is mostly used for the preparation of SUVs from MLVs. *(Jesorka A. and Orwar O. 2008)* There are two possible methods involved in sonication procedure (*Akbarzadeh A. et al. 2013)*. Either a liposomal dispersion is sonicated in a bath sonicator or the MLVs are sonicated with a titanium probe for several minutes. The first kind of sonication enables to preserve the dispersion sterile or under an inert atmosphere while a filtration through 0,45 um filter is needed to prevent liposomal dispersion from contamination during the second one.

Extrusion

Extrusion is the technique which gives rise of unilamellar vesicles of well-defined size by passing multilamellar vesicles through a narrow-pore membrane. *(Ong S. G. M. et al. 2016, Jesorka A. and Orwar O. 2008)* Depending on the desired characteristics of the final liposomes (polydispersity, size distribution), various parameters of the procedure (applied pressure, pore size and number of cycles) should be controlled. Extrusion of MLVs has to be performed in the presence of solution containing the final load concentration because encapsulated content leaks out through filtration.

High-Pressure Homogenization

HPH enables SUVs' formation from MLVs either by volume reduce of original particles or disunion of liposomal aggregates. *(Corrias F. and Lai F. 2011)* Repetition of cycling can lead to finer blend homogenization and thus to the preparation of uniform SUVs. HPH could be followed by an extrusion method. *(Pupo E. et al. 2005)*

Freeze-Thaw Cycling

Multiple freeze and thaw of extruded MLVs' suspension lead to reduced lamellarity and formation of unilamellar vesicles. *(Castile J. D. and Taylor K. M. G. 1999)* In addition, internal volume of the particles was increased owing to repeated membrane disruption caused by water crystallization. Poloxamers could be added in order to inhibit aggregation after membrane damage following preparation of smaller vesicles.

Electroformation

Implementation of electric field within hydration of thin-lipid film affects particles' lamellarity. Depending on desired liposomal parameters, medium parameters (ionic strength, temperature, osmolarity), duration of treatment, type of electric field and thickness of dried lipid film layer are determined. *(Angelova M. and Dimitrov D. S. 1988)* Fluctuations in the bilayer membranes caused by electric field are believed to reason the separation of lamellae and forming liposomes. Electroformation is the most effective method to create GUVs. *(Jesorka A. and Orwar O. 2008)*

4.3.3 Purification of Liposomes

Size Exclusion Chromatography

Size exclusion chromatography (SEC), also known as gel filtration (GF), is a method using differences in size of particles for separation. The liposomal suspension is applied onto a chromatography column filled with one of various loading materials (eg Sephadex G-50, Sepharose 2B or Sephacryl) and perfused by a hydrophilic solvent. Small particles (eg. free drug) are entrapped in the tiny cavities of material and thus are washed up later than the bigger particles (eq. liposomes or loaded liposomes). The whole procedure results separation of two phases – free substance and loaded liposomes. *(Akbarzadeh A. et al. 2013)*

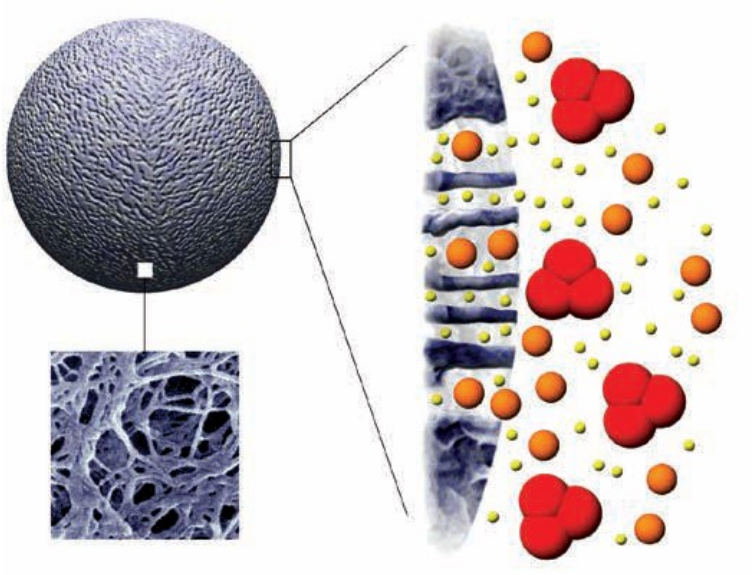

Fig. 3 SEC scheme. Figure shows granule of Sephadex material. The capability of smaller-sized substances´entrapment is outlined. (GE Healthcare Life Sciences 2000–2014)

Dialysis

Dialysis of the non-entrapped drug is performed by a similar manner as detergent dialysis method for liposomes preparation. Free drug is separated by passing through a membrane to a less concentrated aqueous buffer. *(Caddeo C. et al. 2013)*

4.4 Liposomes in Oral Drug Delivery

Oral administration of drugs and drug formulations represents a great facilitation for patients as well as for the healthcare system. It is less expensive for patients' hospitalization when compared with parenteral drug use and increases patients´compliance. Liposomes are prospective oral drug delivery systems for peptide or proteins. Their absorption is limited due to disturbance by acidic stomach media, pancreatic enzymes and potential conjugation with bile salts. *(Fong S. Y. K. et al. 2015)*

Strategies of overwhelming harsh GIT conditions differ from each other. On this regard, the study of calcitonin oral administration shows an application of polymer-protease inhibitor conjugated with chitosan-aprotinin contained in bilayer membrane for increasing bioavailability. *(Werle M. and Takeuchi H. 2009)* Another approach exploits enhancement of intestinal absorption by utilization of bioenhancer as cetylpyridinium chloride, stearyl amine or d-α-tocopheryl polyethylene glycol 1000 succinate. *(Parmentier J. et al. 2014)* In addition, an increase of bioavailability can be reached by intestinal mucoadhesion that is ensured by the formulation of pectin-liposome nano complexes. *(Fricker G. et al. 2010)*

4.5 Types of Matrices for Entrapment of Liposomes

Liposomes' entrapment in matrices can be beneficial in various manners. (*Grijalvo S. et al. 2016)* It enables controlled drug release and improved bioavailability while minimizes the risk of side effects. In addition, it allows to form solid drug formulations and thus to extend the shelf life. Various natural, as well as artificial polymers, were investigated to embed the liposomes.

Chitosan

Chitosan is a biocompatible polymer that composed of β(1 → 4)-linked D-glucosamine and N-acetyl D-glucosamine. Various drugs like doxorubicin, ofloxacin, mupirocin have been reported to be encapsulated in liposomes by using this matrix. *(Grijalvo S. et al. 2016)* Furthermore, it possesses an advantage of delayed drug release and side-specific drug delivery. *(Ruel-Gariepy E. et al. 2002)*

Dextran

Dextran is a polysaccharide composed of α-linked D-glucopyranosyl repeating units with a molecular weight above 1000 Daltons. Liposomes embedded in dextran matrix are utilized as nonviral carriers of proteins or in analytics for the detection of small molecules. *(Grijalvo S. et al. 2016)*

Hyaluronic Acid

Hyaluronic acid is a natural polysaccharide containing linearly bound β-D-(1→3) glucuronic acid and β-D-(1→4)-N-acetylglucosamine. It plays a key role in cellular adhesion, protein delivery and water management. Furthermore, its anti-inflammatory properties are beneficial in wound healing. *(Grijalvo S. et al. 2016)* Due to its essential content in a human eye, liposomes entrapped in HA matrix are convenient drug formulation for ocular delivery. *(Lajavardi L. et al. 2009)*

Alginate

Alginate is a salt of alginic acid with molecular weight around 500 – 2000 kDa. It consists of β-D-mannuronate and α-L-glucuronate. *(Grijalvo S.et al. 2016)* Alginate matrix liposomes are one of the prospective formulations for oral drug delivery. Alginate matrix facilitates an overcoming of acidic pH in GIT and on this account rises the bioavailability of encapsulated drug. *(Aikawa T.et al. 2015)*

Carrageenan

Carrageenan is a sulfated polysaccharide containing galactose and 3,6-anhydrogalactose. *(Grijalvo S. et al. 2016)* Application of loaded liposomes in carrageenan- hydrogel leads to increased protection of the drug loading and sustained release in comparison with free active substance simply embedded in carrageenan hydrogel. *(Kulkarni C. V. et al. 2015)*

Gelatin

According to The United States Pharmacopeia, gelatin can be defined as *„ purified protein obtained from collagen of animals (including fish and poultry) by partial alkaline and/or acid hydrolysis, by enzymatic hydrolysis, or by thermal hydrolysis”*. *(The USP 2015)* Gelatin is able to create a thermoreversible gel. First, an aqueous solution is prepared and, after cooling to 35-40°C, the viscosity is increased while the gel is formed. Gel strength is very important property and is influenced by a lot of factors (pH, temperature, presence of any additives, structure and molecular mass). Gelatin is obtained mostly from cattle bones (lime bone – LB gel), cattle hides, pork skin (pig skin – PS gel) or alternatively poultry and fish.

4.6 Matrix Liposomes

The term *„Matrix liposomes“*, within the meaning involved in this thesis, was defined by Pantze and coworkers as egg-phosphatidyl choline/cholesterol membrane particles embedded in the gelatin matrix. (*Pantze S. F. et al. 2014*) Gelatin plays the role of stabilizing agent in the formulation. Furthermore, the gel is employed as a thickening agent in order to protect from harsh conditions (acidic stomach media, bile salts, and phospholipases) of the GIT and thereby works as a prospective peptide drug delivery component. The gel exists inside the core of vehicles and it forms the surrounding media. Concentration and bloom value of incorporated gel influence the dissolution profile of the liposomes in intestinal-like media. Although 10 % of gel content shows almost first order kinetics, liposomes embed in 20 % gel were released slowly following linear drug release curve. This fact could be explained by the progressive disintegration of the formulation. The most advantageous dissolution rate occurred in the concentration of 15 % whereas the liposomes were released in a controlled manner. Matrix liposomes were prepared by DAC method. This method provides mostly oligolamellar vesicles (OLVs) or MLVs. *(Pantze S. F. et al. 2014)*

5. Materials and methods

5.1 Materials

5.1.1 Chemicals

Reagent	Company	Purity
Cholesterol	Cholesterol Sigma-Aldrich Chemie GmbH	≥ 99 %
Dubbeco's Phospate Buffered Saline (DPBS)	Gibco® by life technologies	n.a.
Gelatine 275 PS 30	Rousselot, Gent, Belgium	n.a.
Gelatine 225 PS 30	Rousselot, Frankfurt am Main, Germany	n.a.
Gelatine 160 LB 30 Pharma	Rousselot, L´Isle sur Sorgue, France	n.a.
Gelatine 220 LB 8 Pharma	Rousselot S.A.S, L´Isle sur Sorgue, France	n.a.
Gelatine 250 LB 8	Rousselot S.A.S, L´Isle sur Sorgue, France	n.a.
Glycerol	Gerbu Biotechnik GmbH	99,5%
5-(and-6)-carboxyfluorescein /mixed isomers/	Molecular Probes, Eugene, Oregon USA, Leiden, The Netherlands	n.a.
Lipoid E 80	Lipoid GmbH, Ludwigshafen, Germany	~80 %
Sephadex G-50 Fine	GE Healthcare GmbH	n.a.
Triton 1% (pure Triton X-100)	Roth GmbH & Co KG, Karlsruhe, Germany	Extra pure

5.1.2 Equipment

Name	Details	Producer
96 - well plates	Costar Assay plate 96 well (black, clear bottom)	Corning GmbH HQ, Wiesbaden, Germany
Analytical balance	ABS-NM/ABS-N	Kern & Sohn GmbH,Germany
Centrifuge	MSE Mistral 3000i	SANYO Gallenkamp PLC, Leicestershire, United Kingdom
Disposable cuvettes	PS	Sarsted AG & Co., Nümbrecht, Germany
Disposable folded capillary cells		Malvern Zeta Sizer Nano Series, Worcestershire UK
Drying chamber		Heraeus® Instruments, Hanau, Germany
Fluorescence reader	Infinite® F200 Pro	Tecan Group Ltd., Männedorf, Switzerland
ISO - Injektionsflaschen	PP, 10 ml	Zscheile & Klinger GmbH, Hamburg, Germany
Particle Analyser	Zetasizer Nano ZS	Malvern Instruments Ltd
Peristaltic pump	IP-4	Ismatec®, Zurich, Switzerland
pH analyser	766 calimatic	Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany
Polyethylen frits	ø 0,5 & 2 cm	Sigma-Aldrich Chemie GmbH

Name	Details	Producer
SiLibeads® Ceramic Beads	Type ZY-E; 1,0-1,2 mm „Premium“	Sigmund Lindner GmbH, Warmensteinach, Germany
SiLibeads® Ceramic Beads	Type ZY-E; 1,4-1,6 mm „Premium“	Sigmund Lindner GmbH, Warmensteinach, Germany
ThermoMixer	Model 5436	Eppendorf®
Ultrasonic cleaning unit	Elmasonic S 300 H	Elma Electronic GmbH, Pforzheim, Germany
Zentrimix R 380	prototype	Hettich Zentrifugen

5.2 Methods

5.2.1 Liquid Phase Preparation

Gelatin

Aqueous solutions of gelatin (5%, 10%, 15% and 20% w/v) were prepared in 15mL Falcon tubes. The samples were sonicated in ultrasonic water bath at 75°C for 1 hour. The prepared solutions were kept at 45°C in order to prevent solidification.

Glycerinated Gelatin

Aqueous solutions of glycerol (30% and 20% w/w) were prepared in 15mL Falcon tubes. Gelatin (PS 275) was added (15% w/v) and the samples were sonicated for 1 hour at 70°C at ultrasonic water bath. The prepared solutions were kept at 45°C in order to prevent solidification.

Carboxyfluoresceined Solutions

Aqueous solutions of carboxyfluorescein (CF) prepared by mixing CF with water and dissolve it with the use of aqueous solution of NaOH (0,1 M) until pH was adjusted to 7.4. The final concentration of CF was 50mM. The solutions were used for the preparation of gelatin and glycerinated gelatin solutions as previously described.

5.2.2 Liposome Formulation

EPC and Chol were mixed in a ratio of 60:40. Ceramic beads were added, followed by the additon of aqueous phase in a ratio of 3:2 regarding the lipid phase. Shortly after the addition of aqueous phase, the samples were inserted in Zentrimix device for the required time and VPG was formed. After their formulation, VPGs were kept at 45 °C in order to prevent solidification. The samples were filtered throw polyamide monofilter to separate ceramic beads from VPGs and VPGs were solved in PBS to

form liposomes. VPGs were stored for further experiments at 4°C. Liposomes were agitated at 37 °C in thermomixer.

5.2.3 Liposome Analyses

Size and Size Distribution

Liposomes were diluted in PBS in a ratio of 1:100 in plastic disposable cuvettes. The dispersions were evaluated by Photon Correlation Spectroscopy (PCS) using Zetasizer Nano ZS with a 633 nm red laser. Z-Average and polydispersity index (PdI) for each sample were determined in automatic mode from the average of three runs.

ζ-Potential Measurement

ζ-Potential of liposomes was analysed by Zetasizer Nano ZS using the Phase Analysis Light Scattering (M3-PALS) technic. The liposomal dispersions were diluted in phosphate-buffered saline (PBS, pH 7,4), in a ratio of 1:5, in disposable folded capillary cell. Mean values of each sample were calculated from three runs.

5.2.4. Encapsulation Efficiency

Encapsulation efficiency (EE) of liposomes was measured by size exclusion chromatography (SEC) method. This method (also known as gel filtration) separates particles according to their different size. CF was used as a model compound due to its fluorescence character.

Column Packing. 1,5 g of Sephadex G-50 Fine powder were weighted into 50 ml Falcon tube. 15 ml of PBS were added and the formed slurry swelled overnight. Next day the slurry was transfered to plastic SEC column and sonicated in ultrasonic water bath at 70 °C for 3 hours. Finally, the suspension degassed under vacuum for 1 hour.

CF solution was prepared as described at 5.2.1. Gelatin or glycerinated gelatin was added and the samples were sonicated for 1 hour at 70 °C to form a gel. Sample was stored at 45°C to avoid solidification. 300 mg of lipids (EPC : Chol in a ratio of 60 : 40) were weighted into plastic vials, 1800 mg of ceramic beads and 450 ml of CF solution were added. The samples were speed mixed by DAC method in Zentrimix for the desired time. Subsequently, the ceramic beads were separated from the prepared VPGs by filtration (polyamide monofilter). 50 mg of VPGs were dispersed in 1,5 ml PBS to form liposomes while agitating and heating to 37°C. Afterwards, 500 µl of liposomal dispersion were loaded onto the SEC column. Column was eluted by PBS using peristaltic pump at flow rate 0,9 mL/min. Two separated layers were visible on column. The former containing CF encapsulated in liposomes and the latter containing free CF. (Figure 4) Afterwards 100 µl of liquid of each layer was put in 96 - well plate (black, transparent bottom). 100 µl 1% Triton in PBS was added to disrupt liposomes and plate was kept at 45°C in drying chamber for 1 hour. Fluorescence was analysed at Infinite® F200 Pro fluorescence reader at an excitation wavelength of 485 and an emission wavelength of 520 nm. Encapsulation efficiency was calculated after correction of dilution according to following formula.

$$EE = \frac{FE\ encapsulated}{FE\ free + FE\ encapsulated} \times 100\%$$

Wherein *FE encapsulated* means fluorescence of CF encapsulated in liposomes and *FE free* means fluorescence of free CF.

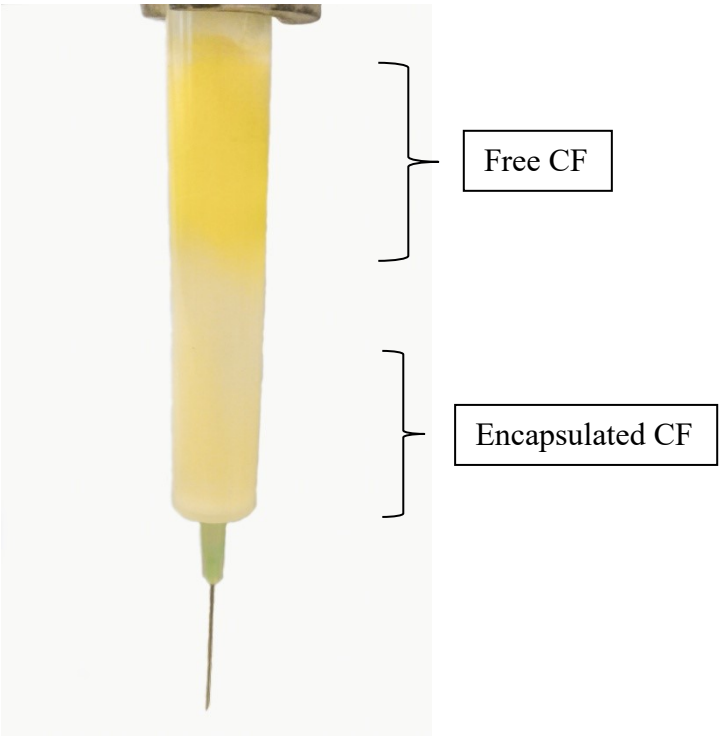

Fig. 4 SEC column with ongoing process of EE analysis. Lower layer indicates layer of CF encapsulated in liposomes, upper means layer of free CF.

6. Results

This thesis contains results which are under the terms of existing signed contract between the University of Heidelberg and a Pharmaceutical company.

Thus, Results section is listed in Appendix 1 and will be excluded from publication.

7. Discussion

This thesis contains results which are under the terms of existing signed contract between the University of Heidelberg and a Pharmaceutical company.

Thus, Discussion section is listed in Appendix 2 and will be excluded from publication.

8. Conclusions

This thesis contains results which are under the terms of existing signed contract between the University of Heidelberg and a Pharmaceutical company.

Thus, Conclusions section is listed in Appendix 3 and will be excluded from publication.

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