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FREEZE-DRYING OF LIPOSOMES FOR ORAL DRUG DELIVERY

LYOFILIZACE LIPOZOMŮ PRO PERORÁLNÍ APLIKACI LÉČIV

Diploma Thesis

Heidelberg & Hradec Králové, 2009 Michaela Vacková

Fair declaration

I declare that I made this diploma thesis oneself and mentioned every used sources and used literature.

ACKNOWLEDGEMENTS

For the most part, I would like to thank Johannes Parmentier, my german supervisor, who assisted and guided me throughout the whole project and helping me throughout my whole stay, mainly in the beginning - for accepting me and thus enabling my participation on the project as a part of his workgroup.

Further words of thanks belong to my family for their support and for enabling my stay in Heidelberg.

Last but not least, I would like to thank PharmDr. Zdeňka Šklubalová, Ph.D., my czech supervisor, for her valuable remarks and advice to this work.

Thank you all

Michaela

Content

1 Introduction and the aim of study

The oral delivery is by far the most convenient route for drug delivery. But, peptides and proteins are poorly bioavailable when administrated orally, mainly due to their low mucosal permeability and lack of stability in the gastrointestinal environment, resulting in degradation of the compound prior to absorption. Therefore it is desirable to develop oral delivery systems, which can protect proteins from the harsh conditions in the gastrointestinal tract (GIT) and thus also improve their absorption. Liposomal formulations offer the posibility of protection and absorption enhancement of poorly bioavailable drugs. Unfortunately, liposomes themselves are often unstable in GIT. In presence of acid, lipase and bile salts. It seems desirable to develop more stable liposomal formulations. In this work is vitamin E used to stabilise the liposomal membrane. Furthermore other lipo- or amphiphilic substances, as cholylsarcosine and stearoylamine, are included into the formulations to enhance the drug uptake through the intestine. However, major disadvantages of liposomes for the oral drug delivery are their short shelf live and the inconvenience of a liquid dosage form. To overcome those problems the liposomal formulations can be freeze-dried. Thus they can be stored over several months and be enclosed in capsules to provide a convenient way of application.

The aim of this work is to develop freeze – dried liposomal formulations based on convencional lipid as egg phosphatidylcholine and other components like vitamin E, cholylsarcosine and sterylamine. It is crucial, in this work to find methods and formulations that provide stable and easy to redisperse liposomes with a low polydispersity.

The techniques used for this project are:

- Producing liposomes by the film method
- Forming liposomes by the film method
- Photon correlation spectroscopy
- Freeze-drying

2 List of abbreviations

10% Suc	10% Sucrose
Chol	Cholesterol
CS	Cholylsarcosine
DPPC	Dipalmitoyl phosphatidylcholine
DSPC	Distearoyl phosphatidylcholine
EPC	Egg phosphatidylcholine
GIT	Gastrointestinal tract
GUV	Giant unilamellar vesicles
LUV	Large unilamellar vesicles
MLV	Multilamellar large vesicles
MVV	Multivesicular vesicles
OLV	Oligolamellar vesicles
PBS	Phosphate buffer saline
PCS	Photon Correlation Spectroscopy
PEG	Polyethylenglycol
PI	Polydispersity
PVP	Plasdone K – 29/32 Povidone
SA	Stearoylamine
Suc/NaCl	2% Succrose/0,7% NaCl
SUV	Small unilamellar vesicles
TPGS	D–alpha tocopherylpolyethylene glycol 1000succinat – Vitamin E
Treh/NaCl	6,8%trehalose/0,3%NaCl

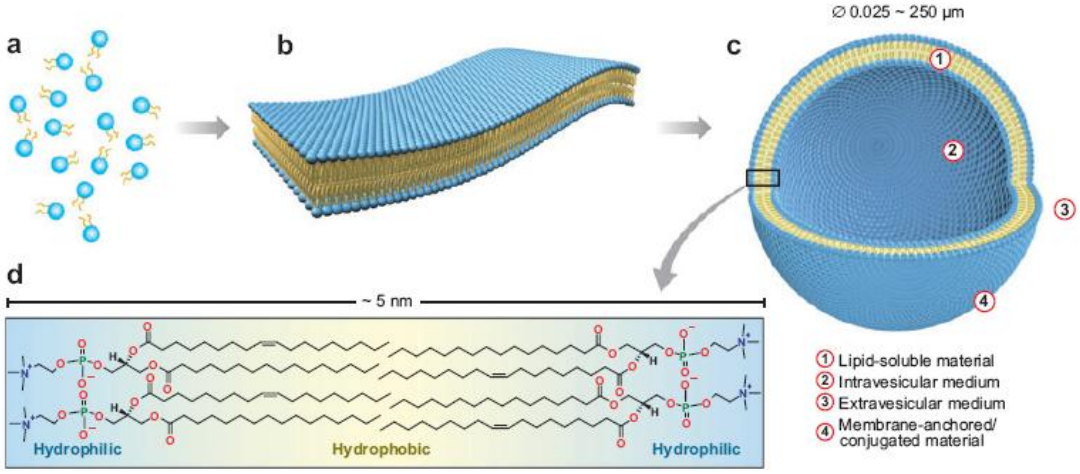
3 Theoretical part

3.1 Liposomes

The field of liposome research has expanded considerably at the end of the 1960s, when liposomes were discovered, and has continued up to the present day. Liposomes help solve the problem of application of drugs of protein character into the body by other than parenteral routes. It is now possible to prepare a wide range of liposomes varying in size, phospholipid composition and with various surface characteristics, making it possible to satisfy different requirements on a peroral application or targeted administration of medicines. The surfaces of liposomes can be modified by the choice of bilayer lipids as well as by the incorporation and covalent linkage of glycoproteins and synthetic polymers. In comparison with other drug carriers, liposomes have some advantages, like biological degradability and relative toxicological and immunological safety.[1]

Liposomes are spherical soft-matter particles consisting of one or more bilayer membranes, and are most commonly composed of phospholipids encapsulating a volume of aqueous medium. The aqueous medium is typically the same as that in which the liposomes are suspended. Liposomes as analogs of natural membranes are generally assembled by spontaneous self-organization from pure lipids or lipid mixtures. They are formed by interaction of amphiphilic lipids suspended in aqueous phase Fig.1.[2] Hydrophilic molecule drugs may be encapsulated into the interior space vesicles, lipophilic molecule drugs may be incorporated into the lipid bilayer, which form in to walls liposomes. [3]

Fig. 1 Schematic illustration of the fundamental self-assembly process.[2] a) individual phospholipid molecule b) bilayer membrane c) liposom



3.1.1 Classification of Liposomes

Liposomes may be classified based on various characteristics, according to size, lamellarity, lipid composition and applications, to name a few. **Tab. 1** summarises the common types of liposomes.[1]

Tab. 1 Classification of Liposomes[1]

According to size
Small unilamellar vesicles
Large unilamellar vesicles
Large multilamellar liposomes
According to circulation *in vivo*
Classical or convention liposomes
Sterically stabilised liposomes
According to lamellarity
Unilamellar
Multilamellar
According to surface charge
Cationic/ DNA vector
Anionic
Neutral

Based on size and lamellarity

Liposomes are, from a morphological perspective, most frequently classified by their size and number of membrane bilayers Fig.2. Liposomes are readily prepared in the laboratory by various methods Fig 3.[2] Unilamellar vesicles are of special interest in research, mostly due to their good characterized membrane properties and easy preparation. They are divided into three size types: small, large, giant. These three groups are the most for analytical application. [2]

The size of small unilamellar vesicles (SUV) is in the range of 0.02 μm – 0.2 μm. They have only one lipid bilayer. SUV are prepared by sonication or extrusion and they size depend on the lipids used, the preparation temperature and the length of sonication. [4]

The size of large unilamellar vesicles (LUV) is in the range of 0.2 μm – 1.0 μm. They are prepared by calcium-induced fusion, sonication of SUV or by extrusion through defined pore size filters. The internal capacity of these liposomes is quite high.[2, 4]

The size of giant unilamellar vesicles (GUV) is > 1μm.[4]

Oligolamellar vesicles (OLV) - This are the vesicles, whose membranes are made up of several layers, where the release of the drug slowed down due to a stronger layer of lipids. They arise as a by-product in the preparation of LUV.[3]

Multilamellar vesicles often show physical properties and behavior that are very different from the unilamellar species, and they are commonly used for industrial applications such as drug delivery and cosmetic. The size of multilamellar large vesicles – (MLV) is very different (0.5 μm – 10.0 μm). They have different lamellarity and different internal capacity. MLV with a given size can be prepared by a careful extrusion through defined pore – size filters with soft pressure.[2]

Multivesicular vesicles (MVV) - In a larger vesicles are encapsulated a few smaller vesicles. They arise as a by-product in the preparation of MLV.[3]

Fig. 2 Schematic representation of the classification scheme for liposomes.[2]

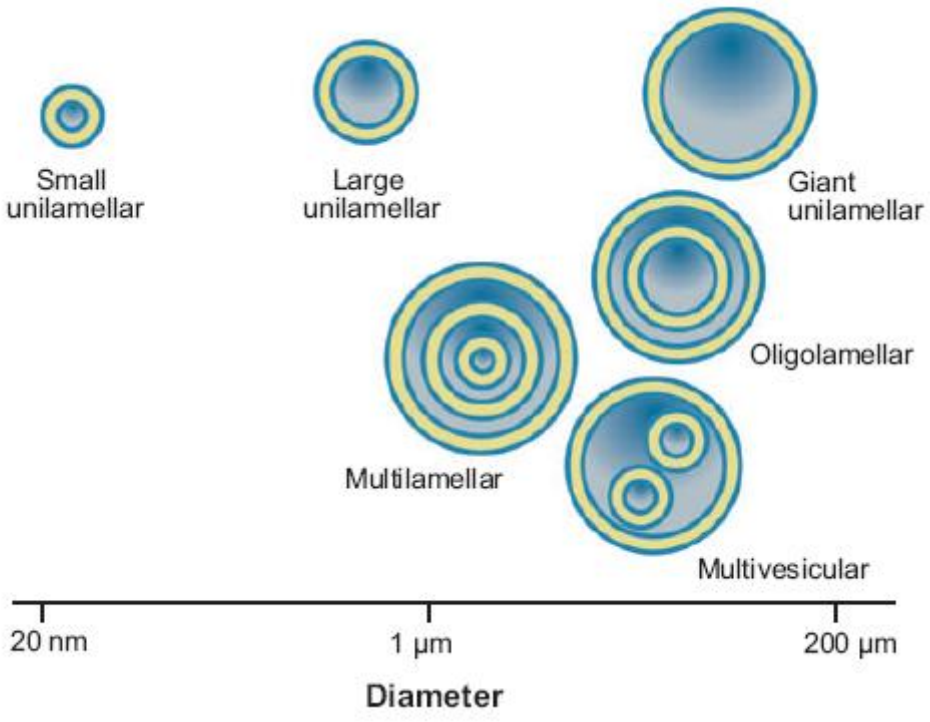
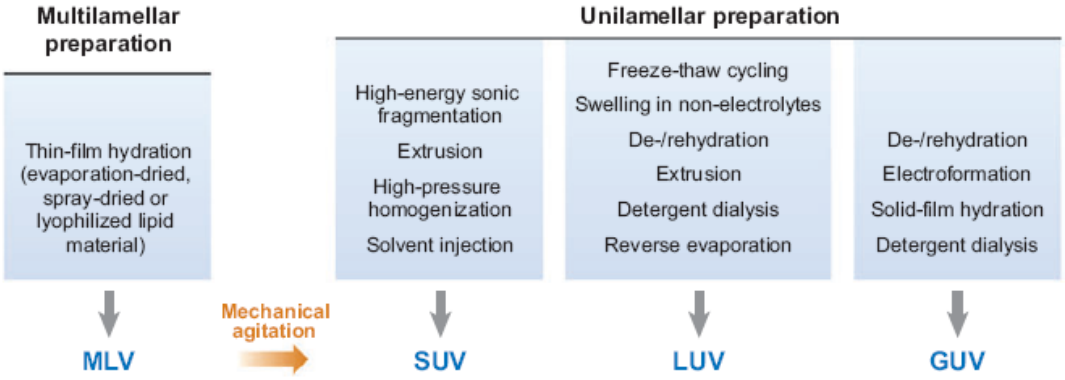


Fig. 3 Common preparation techniques for different types of liposomes. [2]



The size of liposomes plays an important role in the clearance of liposomes—the extent of its importance differs for classical and sterically stabilised liposomes. There is a strong size dependence in the clearance of classical liposomes following intravenous administration, as the liposome diameter increases the rate at which the liposomes are removed from blood into the reticuloendothelial system will also increase.[5] The ability to produce liposomes of controlled size distribution was initially obtained by sonication and by extrusion.[1] Sterically stabilised liposomes

with average size around 250.0 nm are filtered out slowly by the Kupffer cells of the liver. With liposomes sizing 300.0 nm the filtering by the spleen cells is increased, presumably by a passive filtration mechanism. [2]

Based on circulation time in vivo

Conventional liposomes have a very simple structure. Their main subpart is egg phosphatidylcholin (EPC), then cholesterol in various amounts and a small percentage of acidic phospholipids. Cholesterol is added for the increase of liposome stability in the presence of plasma, the negative charge is important for aggregation blocking. These liposomes are recognized by phagocytic cell of the reticuloendothelial system, they are eliminated from the circulation quite quickly, their half – life falls with the growing radius, negative surface charge and fluidity.[6]

Sterically stabilised liposomes

By the addition of specific mixtures to the classical liposomes it is possible to gain liposomes with greater stability in biological fluids and lesser reactivity to plasma proteins and cell surface receptors. First surveys were made with the use of GM1 gangliosid, phosphatidyl inositol, dipalmitoyl phosphatidylcholine (DPPC) or distearoyl phosphatidylcholine (DSPC).

From the 1990s, bipolar or bolaamphiphile ether lipids are used for the purpose of liposomes stabilization; they have unique qualities and are obtained from the cell walls of Archaebacteria. These liposomes are then called archeosomes, which are usually more resistant to low pH in the stomach.[7]

Based on surface charge

Liposomes may have either a positive or a negative charge that is supplied mainly by admixtures to normal lipids, because the charge of these classic liposomes is in essence neutral. Liposome can be prepared with a substantial negative or positive charge. From a practical point of view, the positively charged liposomes are mainly of a big importance. Liposomes with a positive charge are sometimes called liposomal DNA delivery vectors, as they can be used for a transport of DNA right in cancer cells. The liposomes with the surface charge are removed faster than the

classical ones. The negative charged are trapped mainly by the splenic uptake, which is about twice faster than for the positively charged liposomes.[1]

3.1.2 Composition of liposomes

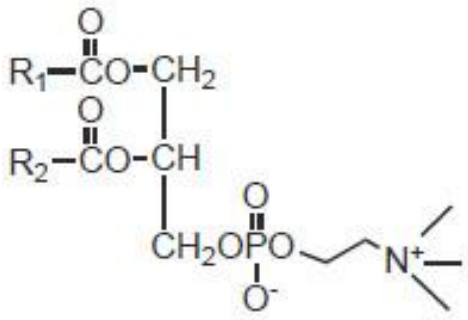
Liposomes should be prepared from constituents that are safe for use in humans. A general pattern is now developing concerning the phospholipids that are tolerated. Phospholipids are essentials components of all cell membranes. Phosphatidylcholines and phosphatidylglycerols from natural sources, semisynthetically or fully synthetically produced, and cholesterol and PEG-ylated phosphatidylethanolamine, are frequently encountered in liposomes designed as drug carriers for parenteral administration.

The most utilized lipids are phospholipids. The neutraly charged phosphatidylcholine and negatively charged phosphatidic acid, phosphatidylglycerol, phosphatidylserine, and phosphatidylethanolamine, each of which has a different combination of fatty acid chains in the hydrophobic region of the molelecule. If it is necessary to reduce the permeability of fluid crystalline state bilayers, cholesterol is added to the bilayer structure. The phospholipids may be from natural sources, semisynthetic, fully synthetic.[4]

Egg phosphatidylcholine (EPC)

Egg phospatidylcholine is one of the phospholipids, which are part of lecithin. Phospholipids are amphiphatic molecule. In the molecule of EPC the non-polar (hydrophobic) part consists of two fatty acids. The polar (hydrophilic) part of the molecule is made up of esters of choline and phosphate, which is in position 3 of glycerol Fig.4. The EPC is capable to create spontaneously in water at a certain concentration and temperature spontaneously create lamella, micelles or liposomes. For pharmacy, it is particularly advantageous, because it nearly untoxic, it shows no contraindications or interactions with medications, is not mutagenic, it is not to overdose. [8]

Fig. 4 Structure of EPC

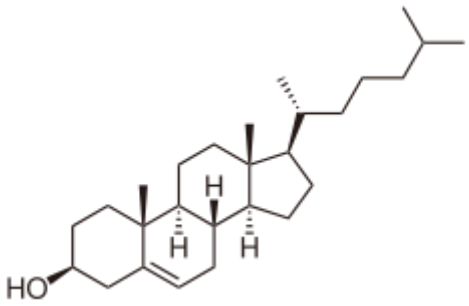


Cholesterol (Chol)

Incorporation of cholesterol in liposome bilayer can bring about big changes in the preparation of these membranes. It does not form by itself bilayer membrane structure, but can be incorporated into phospholipids membrane in very high concentration up to 1:1 or 2:1 molar ratios of cholesterol to phospatidylcholine.

Being an amphipathic molecule, cholesterol Fig 5. inserts into the membrane with its hydroxyl group of cholesterol oriented towards the aqueous surface and aliphatic chain aligned parallel to the acyl chains in the center of the bilayers and also it increases the separation between choline head groups and eliminates the normal electrostatic and hydrogen bonding interaction. Cholesterol increases the fluidity of liposomal membrane.[9]

Fig. 5 Structure of cholesterol



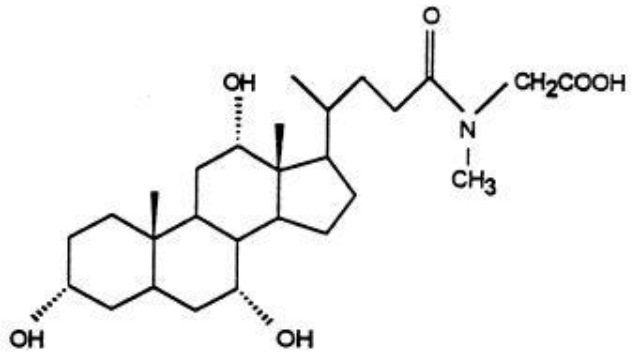
Cholylsarcosine (CS)

It can be described as N - methylglycine conjugated with cholic acid Fig. 6. It is a non-toxic bile salt derivate, and is comparable to conventional bile acids, but which have the potential carcinogenic risk. CS can be used to increase the absorption of peptide drugs) after oral administration (insulin and calcitonin), because it complexes

with calcium cations,and causes therefore an increased leakage of tight junctions, leading to increased permeation and absorption of peptides.[10]

From the small or large intestine only a negligible quantity is absorbed, so that even long-term administration does not change lipid composition or billiards in liver function. In solution it is able to create micelles.[11] In liposomes can create lipophilic ion - pair complexes with various organic cations, which also increases the permeability of cations through biological membranes.[12] The medicine can be used in bile salt replecement therapy of short bowel syndrome.[13]

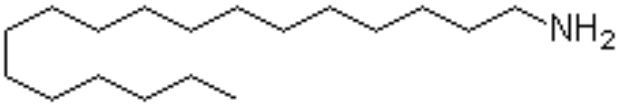
Fig. 6 Molecule of Cholylsarcosine



Stearylamine (SA)

Stearylamine is 1-aminooctadecane Fig. 7. It is a fatty amine and cationic surfactant. In preparing liposomes it is used quite often, due to it is, because the causes of their positive charge. Cationic liposomes are important as vectors for DNA. Moreover, they are not so fast uptake from the circulation as anionic liposomes. SA increases the stability in slightly acidic or alkaline environment.[14] It was demonstrated that SA effectively reduces leakage of cationic drugs from liposomes. Therefore, is SA suitable for the preparation of liposomal drug formulation.[15]

Fig. 7 Structure of stearylamine

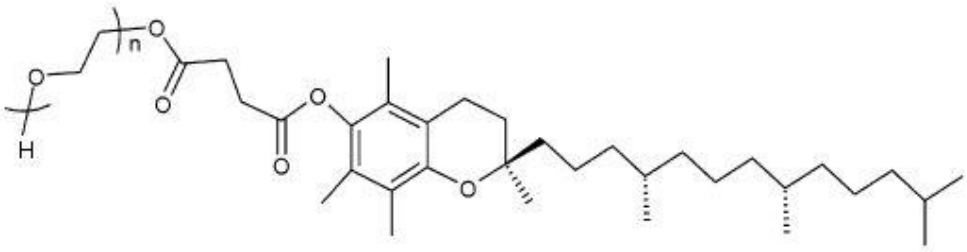


Vitamin E

Systematic nomenclature is referred to as d-α-Tocopheryl polyethylene glycol 1000 succinate TPGS Fig. 8. It is a water-soluble derivative of vitamin E. Structural, it is

an amphiphilic substance and contains a polar head, which comprises a polyethylene glycol and lipophilic tail consisting of tocopherol succinate. This amphiphilic character leads to that TPGS creates micelles. TPGS is surface active, so it can be used as emulsifier, solubilizer, absorption enhancer or water - soluble source of vitamin E. TPGS is among nonioinic surfactants. Providing greater capacity to dissolve poorly soluble drugs and being less toxic to biological membranes. When used in the liposomes TPGS improves their stability, because the polyethylene glycol reduces agregation of liposomes.[16]

Fig. 8 Structure of TPGS



3.1.3 Pharmaceutical application of liposomes

Liposomes have application in the pharmaceutical, cosmetic and food industries. In the pharmacy are liposomes used as drugs carriers, in the biopharmacy and biophysic as membrane models. In the dermatological industry they are used for their ability to bring drugs into the deeper layers of the skin.[3]

Topical application

The topical application of liposomes has a big potential mainly in dermatology. In this area, the main asset of liposomes lies in the fact that they open a possibility to transport hydrophilic substances into the deep layers of the skin, which was earlier impossible. The liposomes are absorbed to the skin relatively quickly. Furthermore, liposomes are trapped selectively by hair follicles. This quality allows transporting melanin, proteins, genes or little molecules directly into the hair follicle. This is laso

the opportunity for genetic therapy that may restore the hair growth, the old hair pigment or to slow the untimely loss of hair.[17]

Systemic application

Liposomes in the systemic application of medications are put into effect in treatment of many diseases and an intensive research is in motion in many other areas. The main advantage of liposomes is the decrease in the systemic toxicity of drugs that are transported directly into the effective place by the liposomes. The liposomes protect the drug itself from the aggressive influences of the environment into which it is applied; they conceal the medication from the cells of the immune system.

Here is a wide range of liposomes use that is also permanently increasing. Liposomes are used in the treatment of cancer already for many years mainly because of their ability to decrease the systemic toxicity of these medications and to increase their circulation time and effectiveness in the place of desired action. For example, doxorubicin closed into polyethlylen glycol coated liposomes has a better pharmacokinetic than doxorubicin itself. This formulation is used as an alternative to the standard treatment of Karposis sarcoma.[18]

Liposomes are used also for the treatment of asthma. The medications closed into the liposomes – that are applied in the aerosol form – are more effective during the relieving bronchial constriction than the medications applied without the liposomes.

Liposomes find their use also for the treatment of arthritis. The absorption and distribution of prednisolon applied by injection in liposomes and of the free steroid in mice have been compared. Liposomal prednisolon was found to be retained by the injected tissue for longer periods of time.[19]

Great hopes with reference to liposomes are rested on the treatment of diabetes by using perorally applied insulin. The insulin in the form of spray dried insulin applied in an aerosol form was already launched in the market, but, unfortunately, the results in the clinical praxes did not live up to the expectations.[1]

3.2 Methods for preparing of liposomes

3.2.1 Preparing of liposomes by film method

- Mechanism of Vesicle Formation

Liposomes are formed when thin lipid films are hydrated and stacks of liquid crystalline bilayers become fluid and swell. The hydrated lipid sheets detach during agitation and self – close to form large, multilamellar vesicles (LMV) which prevents interaction of water with the hydrocarbon core of the bilayer at the edges. Once these particles have formed, reducing the size of the particle requires energy input in the form of mechanical energy.

Properties of lipid formulations can vary depending on the composition (cationic, anionic, neutral lipid species). However, the same preparation method can be used for all lipid vesicles regardless of composition.[20]

3.2.2 Preparing of lipid for hydration

When preparing liposomes with mixed lipid composition, the lipids must first be dissolved and mixed in an organic solvent to assure a homogeneous mixture of lipids. This procces is carried out using chloroform: methanol mixture. The intent is to obtain a clear lipid solution for the complete mixing of lipids.

Typical lipid solutions are prepared at 10 – 20 mg lipid /ml organic solvent, although higher concentrations may be used if the lipid solubility and mixing are acceptable. Once the lipids are thoroughly mixed in the organic solvent, the solvent is removed to yield a lipid film. Vials and flasks are placed in a water bath. For small volumes of organic solvent (<1ml), the solvent may be evaporated using a dry nitrogen stream in fume hood. For larger volumes, the organic can be solvent was removed by rotary evaporation yielding a thin lipid film on the sides of a round bottom flask.

The lipid film is thoroughly dried to remove residual organic solvent by placing the vial or flask for 1 hour in the exsiccator under vacuum. Dry lipid films can be removed from the exsiccator and are ready for hydration.[20]

3.2.3 Hydration of lipid film

Hydration of the dry lipid film is accomplished simply by adding an aqueous medium, magnetic stirrer and glass beads into vials or flask with a dry lipid film. Hydration time may differ slightly among lipid species and structure, however, a hydration time of 30 minutes with vigorous shaking, mixing or stirring is highly recommended. The hydration medium is generally determined by the application of the lipid vesicles. Suitable hydration media include distilled water, buffer solution, saline and nonelectrolytes such as sugar solution.

The product of hydration is a large, multilamellar vesicle analogous in structure to an onion, with each lipid bilayer separated by a water layer. The spacing between lipid layers is dictated by composition with poly-hydrating layers being closer together than highly charged layers which separate based on electrostatic repulsion. Once a stable, hydrated LMV suspension has been produced, the particles can be down sized by the method of extrusion.[20]

3.2.4 Preparing of liposomes by extrusion

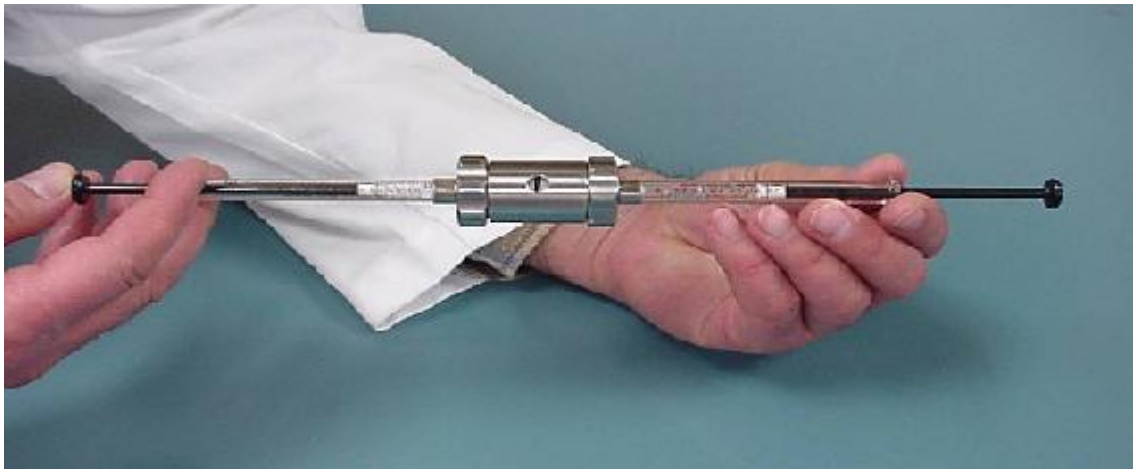
Liposome extrusion is a widely used process in which liposomes are forced under pressure through filters with defined pore sizes to generate a homogenous population of smaller vesicles with a mean diameter that reflects that of the filter pore.[21] This technique has grown in popularity and has become the most common method of reducing multilamellar liosomes (MLV), to large unilamellar liposomes (LUV) for model membrane and drug delivery research.[22]

Extrusion can be performed with a hand - held syringe fitted with a standard sterilization filter holder or purpose - built hand - held units, such as those supplied by Avanti Lipid and Avestin. These devices **Fig. 9** are suitable only for small volume applications (<1ml) one example consist of two Hamilton syringes conned by a filter holder, allowing for back and forth passage of the sample.[23] Using this technique a dilute suspension of liposomes (composed of liquid crystalline lipid) can be passed through the filters to reduce vesicle size. This method, however, is limited by the

back pressure that can be applied manually. Generally, phospholipid concentrations must be less than 30mM in order to comfortably extrude liposomes manually.[22]

Extrusion can be applied to a wide variety of lipid species and mixtures. The sample is extruded in an unpaired number. The final extrusion should fill the alternace syringe. This is to reduce the chances of contamination with larger particles or foreign material.[20]

Fig. 9 LiposoFast – Basic extruder[24]



3.3 Method for checking the quality of the product

3.3.1 Photon Correlation Spectroscopy (PCS)

The size of liposomes is one of their most essential characteristics, as its knowledge helps us to estimate the fate of the liposome in the organism: how long the liposome will probably be in circulation until it will be caught by the cells of the RES. The knowledge of the liposomes size also offers the possibility to estimate its inner volume. The fast measuring of the liposomes size offers a control whether or not a fault was committed during their production, which is usually the extrusion through defined pore-size filters, or whether the membrane was intact.

There are two methods how the size of liposomes may be found, PCS and the electron microscopy. PCS is used massively.

Photon Correlation Spectroscopy (sometimes referred as Dynamic Light Scattering or Quasi – Elastic Light Scattering) is a technique for measuring the size of particles typically in the sub micron region. Therefore, it is useful for the determination of liposomes size.

PCS is a very fast method: the sample measurement itself lasts about 10 minutes. If we add the time needful for the preparation of the sample in the case of the electron microscopy, we get the time in the order of hours.

In the case of PCS, liposomes are measured in their natural state while during electron microscopy, it is necessary to dehydrate the sample in a complicated way or to stain it and then to use a difficult freeze fracture technique. While PCS measures a distribution of tens up to thousands of particles, the electron microscopy needs hundreds of photos for a comparable analysis.[25]

3.3.2 Principle of PCS

PCS measures the Brownian motion of particles and correlates it with their size. The Brownian motion is a random movement of particles caused by the collisions with the molecules of the solvent they are surrounded by. The big particles move slower, while the small ones faster. The dimension of the Brownian motion is measured by

the diffusion of the ray of light that passes through the suspended sample. This dimension is expressed as so called translational diffusion coefficient (usually given the symbol D). The diameter of the particles is then calculated by the Stokes-Einstein equation:

$$d(H) = \frac{k \cdot T}{3 \pi \cdot \eta \cdot D} \quad (1)$$

Where: d (H) = hydrodynamic diameter [m]

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

k = Boltzmann's constant [$1{,}38 \cdot 10^{-23}$ $J \cdot K^{-1}$]

T = absolute temperature [K]

η = viscosity of solution [$N \cdot s \cdot m^{-2}$]

The hydrodynamic diameter that is gained from this method corresponds to the diameter of a sphere with the same translational coefficient. During the measuring, it is necessary to keep the same temperature, as the viscosity of the liquid is dependent right on the temperature.

The translational diffusion coefficient is not dependent only on the size of the particle "core", but also on the surface structure of the particle and on the concentration and type of ions in medium.[26]

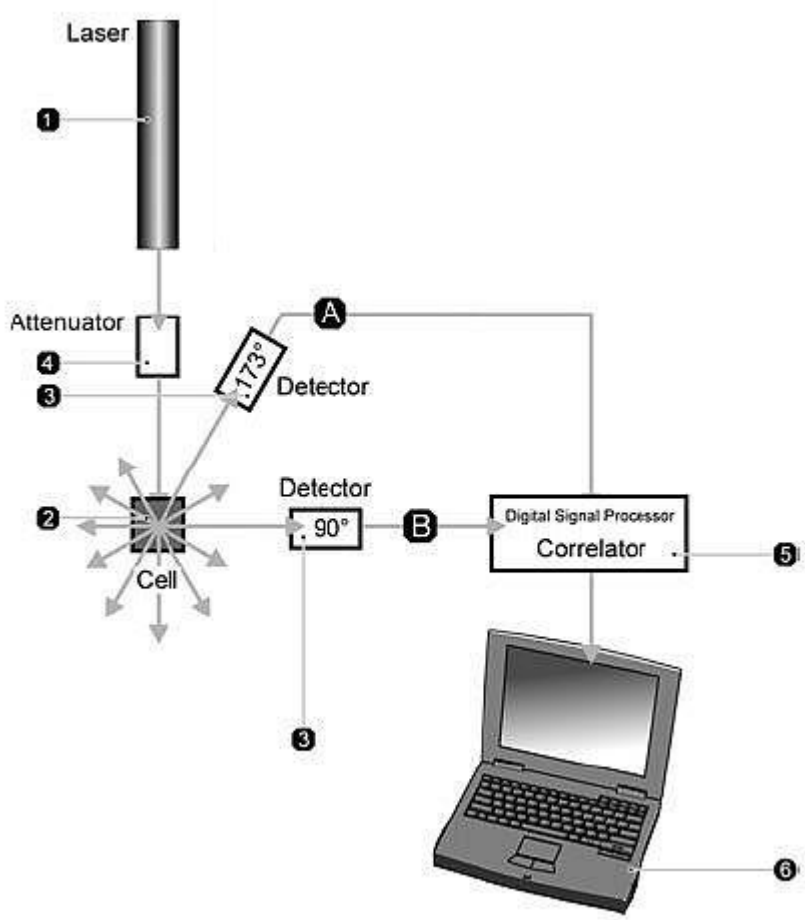
3.3.3 Optical Configuration of the PCS Instrument

A typical PCS system consists of six parts Fig.10. In our case, we worked with a Zetasizer 3000®. The Zetasizer 3000 was used for size determination in the range from 10 nm up to 5μm. Typical particle population for this technique are liposomes, nanoparticles, membrane vesicles and proteins. A routine size measurement needs time period between 5 and 30 minutes.

Fig. 10 Optical Configuration of PCS Instrument.[27]

The laser 1 is the light source used to illuminate the sample enclosed in the measurement cell 2. The scattered light is then captured by the detector 3, and rotated against the incoming laser beam by 90 or 173 degrees or specific angle (according to different instrument types). The intensity of the scattered light must fall into a certain

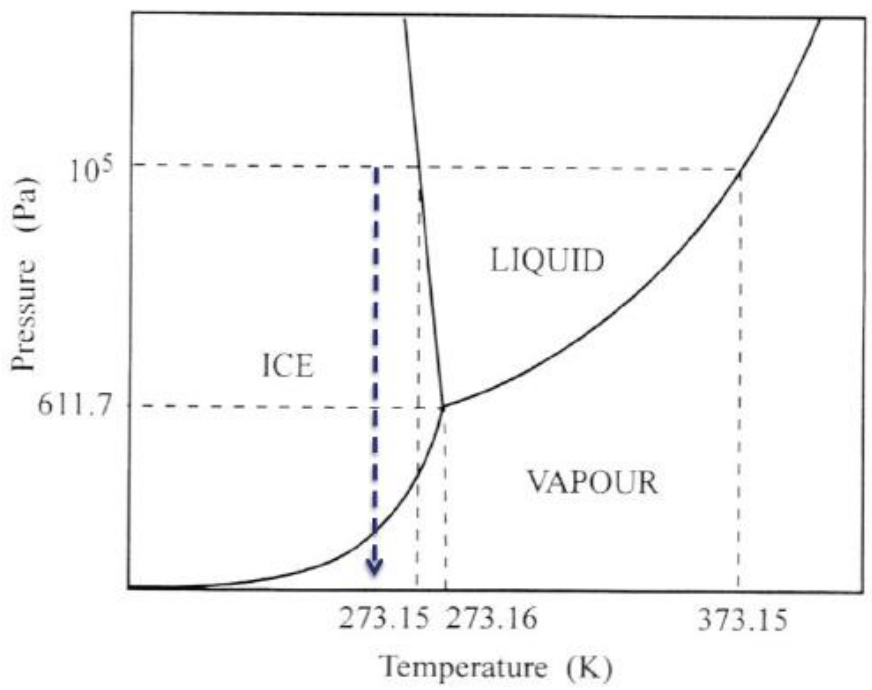
range in order for the detector to measure it correctly. Should the intensity be too high, the detector would be saturated and the results very inaccurate. The laser source, and consequently the dispersion of light, is regulated by the attenuator 4. The scattering intensity of the signal is then relayed to the correlator 5. The correlator compares the scattering intensity at successive time intervals to derive its rate of variation. Data from the correlator are passed on to the computer 6 to be analysed by special software that calculates on their basis the size of the particles in the sample.[28]



3.4 Freeze-drying

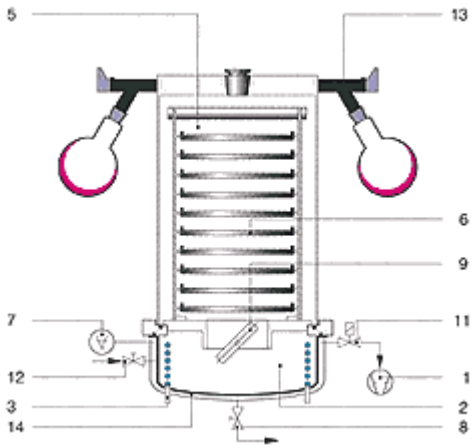
Freeze drying process dates from the time during World War II. Freeze-drying, also known as lyophilization or cryodesiccation, is a dehydration process typically used to preserve a perishable material. Freeze-drying works by freezing the material and then reducing the surrounding pressure and adding enough heat to allow the frozen water in the material to sublime directly from the solid phase to gas Fig. 11.[29]

Fig. 11 The phase diagram of water it shows that at low temperature, (solid) ice is the stable phase. At moderate temperatures and high pressure, (liquid) water is the stable phase, and at high temperature and low pressure, (gas) vapor is the stable phase. Lines separate these phases.



Freeze-drying is a energy and time intensive process. This process has a three phases: freezing, primary drying, and secondary drying. Each phase will be discussed in charter freeze drying of liposomes. This process occurs in the freeze – dryer Fig. 12.

Fig. 12 Chart of freeze-dryer: 1 vacuum pump, 2 ice condenser chamber, 3 condenser coil, 4 acrylic cover, 5 drying chamber, 6 heatable shelf, 7 vacuum-gauge, 8 drain valve, 9 engine driven intermediate valve, 10 stoppering device, 11 pressure control valve, 12 aeration valve, 13 rubber valve, 14 thermal insulation



The stability, during freeze drying and storage, and the duration of the cycle are the two major considerations for freeze-drying process optimization. Given a freeze-drying process yelding a stable product, the shorter freeze-drying cycle has the adventage of higher throughput for a given dryer. At non-optimum conditions, the freeze-drying process may compromise drug stability, take longer, and cost more than is necessary.[30]

For the quality control of the products, there are a various methods: differential scanning calorimetry, Karl-Fisher titration, photon correlation spectroscopy (measurement of size and polydispersity), and functionality assays. [31]

The formulation of a FD a product needs so called lyo – and cryoprotectants. Cryoprotectants protecte the protuct during freezing and reduce crystallization of ice near the product. Lyoprotectants protecte the product in the dry state.

3.4.1 Lyoprotectants and other excipients

Lyoprotectants protect liposomes by preventing fusion of liposomes, preventing the rupture of bilayers by ice crystals, and maintaining the integrity of the bilayers in the absence of water. To do so, the lyoprotectants must form an amorphous, glassy matrix in and around the liposomes. Interaction between lyoprotectant and the phospholipid head groups is considered especially important for preventing leakage during rehydration of liposomes that have a liquid-crystalline bilayer in the hydrated state at ambient temperature.[32]

More recently, a different mechanism was proposed by which sugars may reduce the stress on bilayers caused by drying.[32] Bilayers dried without lyoprotectant approach each other closely and the suction of water by the phospholipid causes a lateral comprehensive stress. The mere presence of a glass between adjacent bilayers during drying provides spacing between bilayers, and in combination with the attraction of water by the sugars the compressive stress on the bilayers is reduced.

The preffered lyoprotectants are disaccharides such a sucrose, maltose, trehalose, lactose, etc. The use of excipicients with a low molecular weight, such as sodium chloride, glucose, and amino acids, should be minimised. If such excipients remain amorphous in the sugar matrix. They will lower the $T_{g'}$,glass transition temperature of the freeze concentrate and the $T_{g,}$ glass transition temperature of the freeze-dried cake. these quantities will be discussed in more detail in charter freeze-drying of liposomes. A low $T_{g''}$ requires freeze-drying at a low product temperature, which is an inefficience process. [33]

3.4.2 Application of freeze - drying

The products of lyophilisation process have a various application in pharmaceutical, biotechnology, food and technological industries. Freeze-drying is widely used for pharmaceuticals to improve the stability and longterm storage stability of labile drugs, especially protein drugs.[30] The freeze-drying is used to increase the shelf life of products, such as vaccines and other injectables. By removing the water from the material and sealing the material in a vial, the material can be easily stored, shipped,

and later reconstituted to its original form. Freeze-drying is also used to preserve food and make it very lightweight. The process has been popularized in the forms of freeze-dried ice cream, an example of astronaut food.

3.4.3 Freeze – drying of liposomes

Freeze-drying of liposomes can prevent hydrolysis of the phospholipids and physical degradation of the vesicles during storage. In addition, it may help stabilize the substance that is incorporated in the liposomes. Freeze – drying of a liposome formulation results in an elegant dry cake, which can be reconstituted within seconds to obtain the original dispersion, that is, if the appropriate excipients are used and if suitable freeze-drying conditions are applied.[33]

Freezing phase

In the freezing phase, cooling of the sample results in the formation of ice crystals. As a result, both the remaining solutes and the liposomes become more concentrated. At this stage, the lyoprotectants form an amorphous matrix in and around the liposomes that prevents fusion process and protects the liposomes against rupture due to the growth of ice crystals. As the temperature of the freeze – concentrate is lowered and its water content is reduced as a reset of the progression of ice formation, the viscosity of the freeze-concentrate increases, until it forms a glass with low molecular mobility. The temperature at which this occurs is called T_g, the glass transition temperature of the freeze concentrated solution, or, alternatively, the collapse temperature (T_c). The T_g is a key parameter for the primary drying process. Generally, the product is cooled to about 5°C below $T_{g'}$ before the start of the drying phase. The $T_{g'}$ depends on the composition of the amorphous freeze-concentrate, which in most cases contains a lyoprotectant and minimal amount of buffer components. The $T_{g'}$ values of lyoprotectants are listed in **Tab. 2**.

Tab. 2 Glass transitive and collapse temperatures of lyoprotectants

Component		$T_{g'}$/°C	T_c/°C	T_g/°C
Sugars	Sucrose	-32	-31	75
	Trehalose	-29	-28.5	118
Polymers	PVP	-20.5	-24	180

Liposomes do not contribute to the value of T_g. The presence of small molecules such as sodium chloride in the amorphous matrix can strongly decrease the T_g. The cooling rate of the freezing phase is also an important factor. Slow cooling is preferred (e.g., 0.5°/min). Once freezing occurs, the whole sample freezes nearly instantaneously, resulting in similar crystal sizes throughout the product. Such pores reduce the resistance of the cake to water vapor during sublimation. Moreover, slow cooling has been shown to minimase the leakage of contents from liposomes after freeze drying and rehydration, depending on the liposome type.[33]

Primary drying

During primary drying the ice crystals are sublimated, resulting in the porous cake structure of the freeze - concentrated matrix. As long as the product temperature or more precisely, the temperature at the sublimation front (T_{front}), is maintained at about 4° below T_g, degradation of the liposomes is minimized and ice crystals can be removed by sublimation without collapse of the cake structure. However, if T_{front} rises above T_g, collapse of the cake occurs, which results in a reduced surface area of the dried matrix. As a consequence, the final residual water content after drying may increase, as well as the time required for reconstitution, and the liposomes may fuse and lose part of their contents on rehydration.

The product temperature at the sublimation front is determined by local pressure, according to the ice vapor pressure temperature curve Tab 3. For this reason, the pressure in the freeze-drying chamber should be controlled well. The set point of the pressure is chosen at a value that corresponds to an ice temperature of about 4°C below T_g. The shelf temperature should be higher than the ice temperature in order to transfer the heat to the sublimation front that is required for drying.

Tab. 3 Ice temperature as function of vapor pressure.

Ice Temperature (°C)	Pressure (Pa)
-20	86
-25	59
-30	37
-35	22
-40	12
-45	07
-50	04

The driving force of the drying process is the difference in temperature and, therefore, difference in water vapor pressure, between the condenser of the freeze-dryer and the sublimation front. The condenser temperatures of freeze-dryers reach -54°C. A limiting factor in the drying process can be the heat transfer from the shelf to the sublimation front, which occurs via conduction by glass and ice, as well as by gas molecules. Therefore, more heat is available for sublimation at the ice front at a higher chamber pressure.[33]

Secondary drying

In the secondary drying phase, the residual water content of the amorphous matrix is further decreased. After all the ice has disappeared and the product temperature has approached the value of the shelf temperature, the secondary drying phase can be initiated. For this reason, temperature probes provide useful information during the freeze-dry run. In the secondary drying phase, the pressure is reduced to a minimal value and the shelf temperature can be gradually increased to. Reduction of the water content of the cake also occurs during primary drying, but lower values (<1%) can be achieved by adjusting the conditions. Reduction of the water content increases the glass temperature T_g of the cake, and this allows a gradual increase in the shelf temperature.

The shelf temperature is a key parameter for obtaining low residual water content. Generally, a freeze dried product is likely to be stable at 20 to 30°C below T_g and, therefore, reduction of the residual water content as of utmost importance. It is worthwhile to mention that the T_g of the amorphous cake is not the only parameter

that affects the stability of the freeze-dried liposomes. The physical processes that can occur in a cake of freeze-dried liposomes are rather complex, low water contents are generally expected to enhance the stability of the dried product. Vials can be closed under vacuum or after releasing the vacuum with nitrogen gas or argon.[33] An overview of physical characterizations is in Tab. 4.

Tab. 4 Physical properties of freeze-drying

Freeze drying stages	Physical properties/in process transitions
Freezing	Super cooling, ice crysallization
	Annealing
	morphology of ice crystals
	Volume expansion due to crystallization
Primary drying	Glass transition temperature Tg'/Eutectic temp.
	Collapse temperature
	Mobility in frozen state
	Ice sublimation
Secondary Drying	Water desorption
	Desorption rate
	Glass transition temperature

4 Experimental part

4.1 Material and Methods

4.1.1 Material

Cuvette – Einmal – Küvetten aus Polystyrol; 4,5 ml; 4 klare Seiten (Carl Roth GmbH, Germany)

Eppendorf pipettes (Eppendorf, Hamburg, Germany)

Eppendorf tubes 1.5ml, 2ml (Eppendorf, Hamburg, Germany)

Membrane filter - Rotilabo® - Spritzenfilter, steril, 0,22 µm (Carl Roth GmbH, Germany)

Pipette tips (Brand, Wertheim, Germany)

Polycarbonate membrane, diam. = 19mm, pore diam. = 200 nm (Avestin Europe GmbH, Germany)

Syringe 1ml, 5ml (Becton Dickinson Labware, USA)

4.1.2 Instruments

Analytical balance (Sartorius AG Götingren, Germany)

Christ – Delta 1-20 KD (M. ChristGefriertrocknungsanlage GmbH, Germany)

Extrudor – LiposoFast Basic (Avestin Europe GmbH, Germany)

Software – PCS, ver. 1.61 (Malvern Instruments GmbH, Germany)

Waterbath (Eppendorf, Hamburg, Germany)

Zetasizer 3000 HS (MalvernInstruments GmbH, Germany)

4.1.3 Chemicals

d –alpha tocopherylpolyethylene glycol 1000succinat – Vitamin E TPGS NF (Eastman, UK)

D (+) Trehalose (Sigma Aldrich, Germany)

D (+) Sucrose - (Acros Organics, USA)

Distilled water (IPMB, Germany)

Egg phosphatidylcholine (EPC) (Lipoid, Germany)

Hydrochloric acid (HCl) (J. T. Baker, Holland)

Chloroform ($CHCl_3$) (VWR Prolabo, EC)

Cholesterol (Chol) (Synopharm GmbH, Germany)

Cholylsarcosine (CS) (Prodotti Chimici e Alimenteri S.p.A., Italy)

Methanole – HiPerSolv Chromanorm (CH_3OH) (VWR Prolabo, EC)

Plasdone K – 29/32 Povidone (ISP Technologies, USA)

Potassium chloride (KCl) (AppliChem, Germany)

Potassium phosphate monobasic (KH_2PO_4) (J. T. Baker, Holland)

Sodium chloride (NaCl) (Wassex House, UK)

Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2\ H_2O$) (J. T. Baker, Holland)

Stearoylamine (SA) (Sigma Aldrich, Germany)

4.2 Preparing of aqueous medium

I prepared next aqueous solutions by addition of appropriate amount of substance to distilled water:

- 6.8% trehalose/0.3% NaCl (Treh/NaCl)
- 10% Succrose (10% Suc)
- 2% Succrose/0.7% NaCl (Suc/NaCl)
- 20% PVP

Phosphate buffer saline (PBS)

I weight 8.0 g sodium chloride, 0.2 g potassium chloride, 1.44 g sodium phosphate dibasic dihydrate, 0.2 g potassium phosphate monobasic and added to 100.0 ml distilled water. This 100.0 ml I added to 850.0 ml distilled water and adjusted the pH to 7.4 with hydrochloric acid. I filled up this solution to 1000.0 ml with distilled water and filtrated the buffer through a filter of 0.22 μm pore size.

4.3 Preparing of liposomes by film method

4.3.1 Film Formation

It is necessary to dissolve lipids for preparing liposomes. I separately dissolved each lipid in a organic solvent mixture of chloroform: methanol (9:1) to achieve the concentration of 10 μmol/ml. In the 5 ml glass vial, I prepared the mixtures of two or three lipids according to the proportion of individual lipids Tab 5. The final concentration of the solution mixture of lipids was 10 μmol/ml again, and the volume in the vial was 1 ml.

The glass vials were positioned into the water bath at 50° C. The solvent was evaporated with a nitrogen stream or under vacuum. The tip of the nitrogen tube was positioned near the liquid level. In the range of 5 to 10 minutes, the solvent was vaporized. After evaporation of the solvent a lipid film created on the wall. The lipid film I maintained under vacuum for one hour in a desiccator to remove solvent traces.

The next mixtures of lipids were used:

Egg phosphatidylcholine: cholesterol 1:1 (EPC:Chol 1:1)

Egg phosphatidylcholine: cholesterol 2:1 (EPC:Chol 2:1)

Egg phosphatidylcholine: stearoylamine: cholesterol 5:1:4 (EPC: SA: Chol 5:1:4)

Egg phosphatidylcholine: stearoylamine: cholesterol 2:1:1 (EPC: SA: Chol 2:1:1)

Egg phosphatidylcholine: cholylsarcosine: cholesterol 5:1:4 (EPC:CS:Chol 5:1:4)

Egg phosphatidylcholine: cholylsarcosin: cholesterol 2:1:1 (EPC:CS:Chol 2:1:1)

Egg phosphatidylcholine: vitamin E: cholesterol 5:1:4 (EPC: TPGS: Chol 5:1:4)

Egg phosphatidylcholine: vitamin E: cholesterol 10:1:9 (EPC: TPGS: Chol 10:1:9)

Tab. 5 Mixtures of lipids in various proportions.

mixtures of lipids	Aqueous media (1ml)				FD
	Treh	Suc	PBS	Suc /NaCl + PVP	48
EPC:Chol 1:1	•				•
		•			•
			•		•
				•	•
EPC:Chol 2:1	•				•
		•			•
			•		•
				•	•
EPC:SA:Chol 5:1:4	•				•
		•			•
EPC:SA:Chol 2:1:1	•				•
		•			•
EPC:CS:Chol 5:1:4	•				•
		•			•
EPC:CS:Chol 2:1:1	•				•
		•			•
EPC:TPGS:Chol 5:1:4	•				•
		•			•
EPC:TPGS:Chol 10:1:9	•				•
		•			•

4.3.2 Hydration

I took the vial from desiccator and I added the magnetic stirrer and glass beads to the dry sample. Then I added 1 ml of aqueous medium: 6.8% trehalose with 0.3% NaCl, 10% succrose, PBS, and 2% sucrose with 0.7% NaCl, respectively. In order to dissolve the film of lipids, the sample was stirred for 30 minutes in the glass vial. I left the samples of 30 minutes at room temperature at rest.

4.3.3 Extrusion

I took the sample after hydration. Between the cylinders of the Liposofast extruder I have put a membrane with a pore size of 200 nm. I rinsed the extruder one time with aqueous medium and extruded the sample in an unpaired number, i.e. odd number of pushing through the membrane, to reduce contamination of the sample, in my case 41 times. I injected the sample from the syringe into a clean Eppendorf cup and stored at 4° C in the fridge. I prepared all samples three times.

4.4 Freeze drying (FD)

I weight a lyo-vial with a stopper on an analytical balance. Into the vials I injected a dispersion of the extruded LUV and weight the vial with the sample again. In case of suspension (Suc/NaCl), I added to samples 20% Plasdone K – 29/32 Povidon (PVP) in quantities of 20% of the original sample. I put vial in the freeze – dryer. After placing the sample in the drying chamber I closed the lid and the process was started.
I engaged freeze drying of liposome under following conditions:

freezing -30° C, 8 hours

primary drying –10° C, 32 hours, 37 Pa,

secondary drying 10° C, 8 hours

The Delta 1-20 KD freeze-dryer announced the end of the process with a beeping signal. To avoid air contact of the sample I flooded the drying chamber with argon. I closed the lyo-vial directly in the chamber under the argon atmosphere. I left the lyo-vials at room temperature and for 20 minutes to let them equilibrate.
I noted the visual properties of the product and I reconstituted the dry cake with distilled water. The quantity of distilled water is equal to the quantity of the sample prior to freeze drying. I observed the solubility of the product carefully.
In the last step I measured PCS for control of quality product.

4.5 Control of liposomes quality

The measurement of liposome size and polydisperzity due to PCS.

I used a Zetasizer for measururing the size of the liposomes and their polydisperzity that way that I diluted the sample in relevant aqueous media in the range 1:200 – 1:600 after extrusion. I used the original aqueous media of the sample. I added 2 ml of aqueous media and 10 µl of the sample and 2 ml of aqueous media and 5 µl of the sample, etc, into a 4.5 ml plastic cuvette. I added the aqueous media by the syringe with a sterile filter with pore size 0.22 µm. I placed the plastic cuvette into cell on the backside of the measuring chamber. Properly, I closed a lid.

In the first step I checked the concentration of the sample. The kilo counts per second (kCps - the photons per second on the multiplier divided by thousand) should be between 100 – 200 kCps (50 – 250 is still tolerable). The measurements were done at the temperature of 30° C, intensity of the scattered light of 90°, and wavelength of 633 nm. Refractive index by trehalose with NaCl was 1,3423 and 10% sucrose 1,3462. In a routine measurement, the Zetasizer measure 3 times 10 single runs (10 – 30 min).

5 Results and discussion

All mixtures of liposomes I prepared for each experiment 3 times. For each sample I conduct a quality of control by measuring of PCS and observed visual characteristics. In tables and graphs, the average values are presented.

Each sample was measured by PCS to verify size and polydispersity (PI) of the liposomes before and after the freeze drying. The polydispersity is a parameter which indicates the width of the distribution. For strongly monodisperse populations, it is under 0.1 a polydispersity under 0.2 still indicates a monodisperse distribution.

During a single measuring each sample was measured 30 times. These 30 measuring were divided into three tens. For each of the three tens, the software calculated values of size and PI and then calculated the final value of size and PI. In tables, the values of PI and size are presented with arithmetical mean value (∅) and standard deviation (in brackets).

After freeze-drying, the visual characteristics of the product (the appearance) and solubility in the water was observed. The appearance was evaluated according to the color and shape of each sample after freeze drying. The sample should be in the shape of a cake. The solubility was observed by adding an appropriate amount of distilled water. The sample after freeze drying should be fast and well-soluble.

5.1 The selecting of appropriate media

Two lipids dispersions EPC:Chol 1:1 and EPC:Chol 2:1 in various aqueous media: 6.8% trehalose with 0.3% NaCl, 10% succrose, PBS and 2% sucrose/ 0.7% NaCl wit PVP were prepared. All samples were extruded through the membrane with pore size 200 nm. The results of experimental measurements are summarized in Tab. 6 -16.

It was evaluated: appearance–white (w), transparent (t), cake, network or net cake; solubility in distilled water–fast and good solubility (+) and slow solubility (-); size and polydispersity was determined by PCS.

Tab. 6 The measured values of liposome size and polydispersity in 6.8% trehalose/0.3%NaCl before freeze-drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:Chol 1:1	276.90	289.53 (10.98)	0.11	0.09 (0.02)
	294.90		0.08	
	296.80		0.07	
EPC:Chol 2:1	285.20	277.90 (6.68)	0.07	0.10 (0.05)
	276.40		0.08	
	272.10		0.15	

Tab.7 The measured values of liposome size and polydispersity in 6.8%trehalose/0.3%NaCl after freeze drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:Chol 1:1	290.20	322.93 (28.64)	0.11	0.12 (0.03)
	343.40		0.14	
	335.20		0,09	
EPC:Chol 2:1	299.40	295.97 (3.73)	0.13	0.13 (0.02)
	292.00		0.14	
	296.50		0.11	

Tab.8 The properties of freeze-dryied samples in 6.8%trehalose/ 0.3%NaCl.

Mixtures of lipids	Appearance	Solubility in H_2O	Weight of sample [g]	Weight of sample FD [g]
EPC:Chol 1:1	w cake	+	0.90	0.06
	w cake	+	0.83	0.06
	w cake	+	0.65	0.05
EPC:Chol 2:1	w cake	+	0.89	0.06
	w cake	+	0.79	0.06
	w cake	+	0.62	0.05

Tab.9 The measured values of liposome size and polydispersity in 2% sucrose/0.7% NaCl with PVP before freeze-drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:Chol 1:1	269.70	265.73 (21.13)	0.06	0.09 (0.03)
	284.60		0.08	
	242.90		0.11	
EPC:Chol 2:1	310.00	284.63 (22.87)	0.14	0.13 (0.01)
	265.60		0.12	
	278.30		0.13	

The samples after freeze drying were impossible to measuring due to PCS.

Tab.10 The properties of freeze-dryied samples in 2%sucrose/ 0.7%NaCl with PVP.

Mixtures of lipids	Appearance	Solubility in H_2O	Weight of sample [g]	Weight of sample FD [g]
EPC:Chol 1:1	w network	-	0.92	0.07
	w network	-	0.86	0.06
	w network	-	0.89	0.26
EPC:Chol 2:1	w network	-	0.89	0.06
	w network	-	0.96	0.07
	w network	-	0.99	0.07

Tab.11 The measured values of liposome size and polydispersity in 10% sucrose before freeze-drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:Chol 1:1	301.50	307.50 (22.02)	0.15	0.15 (0.04)
	289.10		0.18	
	331.90		0.10	
EPC:Chol 2:1	274.40	282.83 (7.32)	0.06	0.09 (0.02)
	286.60		0.10	
	287.50		0.09	

Tab.12 The measured values of liposome size and polydispersity in 10% sucrose after freeze-drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:Chol 1:1	237.10	232.10 (7.00)	0.07	0.09 (0.04)
	224.10		0.07	
	235.10		0.14	
EPC:Chol 2:1	214.70	226.47 (10.21)	0.13	0.14 (0.00)
	231.70		0.14	
	233.00		0.14	

Tab.13 The properties of freeze-dryied samples in 10% sucrose.

Mixtures of lipids	Appearance	Solubility in H_2O	Weight of sample [g]	Weight of sample FD [g]
EPC:Chol 1:1	w cake	+	0.84	0.09
	w cake	+	0.93	0.10
	w cake	+	0.93	0.10
EPC:Chol 2:1	w cake	+	0.81	0.08
	w cake	+	0.65	0.07
	w cake	+	0.73	0.08

Tab.14 The values of liposome size and polydispersity in phosphate buffer saline before freeze-drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:Chol 1:1	266.80	266.73 (0.40)	0.06	0.05 (0.01)
	267.10		0.04	
	266.30		0.04	
EPC:Chol 2:1	255.30	261.70 (5.74)	0.03	0.09 (0.05)
	263.40		0.13	
	266.40		0.10	

The samples were impossible to measured due to PCS after freeze drying

Tab. 15 The properties of freeze-dryied samples in phosphate buffer saline.

Mixtures of lipids	Appearance	Solubility in H_2O	Weight of sample [g]	Weight of sample FD [g]
EPC:Chol 1:1	w network	-	0.74	0.01
	w network	-	0.78	0.02
	w network	-	0.77	0.02
EPC:Chol 2:1	w network	-	0.75	0.02
	w network	-	0.70	0.01
	w network	-	0.74	0.01

Tab.16 The overview of average of liposome size and polydispersity and properties of the samples.

Mixtures of lipids	Aqueous media	Ø Size [nm]	Ø PI	Ø Size [nm] FD	Ø PI FD	Appearance	Solubility in H_2O
EPC:Chol 1:1	Treh/NaCl	289.50	0.09	322.90	0.12	w cake	+
	Suc/NaCl with PVP	265.70	0.09	0.00	0.00	w network	-
	10% Suc	307.50	0.09	232.10	0.09	w cake	+
	PBS	266.70	0.09	0.00	0.00	w network	-
EPC:Chol 2:1	Treh/NaCl	277.90	0.10	295.90	0.13	w cake	+
	Suc/NaCl with PVP	284.60	0.13	0.00	0.00	w network	-
	10% Suc	282.80	0.09	226.40	0.14	w cake	+
	PBS	261.70	0.09	0.00	0.00	w network	-

Liposomes composed of (EPC: Chol 1:1) were much larger in size than the pore diameter membrane. This is due to the high proportion of cholesterol. Liposomes composed of (EPC: Chol 2:1) were smaller. From this (Tab.16) it is clear, that the appropriate aqueous media for freeze drying were trehalose/NaCl and sucrose. Sucrose is a very common medium and trehalose leads to a molar ratio of sugar to lipid from 20:1 for a liposomal dispersion of 10 µmol/ml lipid. NaCl was added to achieve an isotonicity.

In these mediums are products of freeze drying fast and good soluble and have the shape of cake with white color. In PBS and 2% sucrose/NaCl with PVP was prepared samples impossible to measuring. Usually PVP is used to reduce fusion of the liposomes during the drying.[34] The PVP formed aggregates with the liposomes and so the samples are impossible to measured. In the case of PBS no lyo- and cryoprotectant has been used so the samples could not be protected.

There are several reasons why the liposome size can change after reconstitution. An increase in size can be caused by the fusion of liposomes. This also leads to an increase of the polydispersity. Liposomes can undergo phase transition during the reconstitution with water. They are often in the glassy state when they are dried and will be again in the fluid crystalline state after reconstitution. During this phase transition the liposomes are very unstable and leakage but also fusion and fragmentation can occur. The ice crystals during the freezing can damage the liposomal membranes and thus induce fragmentation and later reorganisation of the liposomes. This can reduce the size and the polydispersity. Finally the liposomes might be not totally rehydrated directly after the reconstitution with water and are therefore smaller.

In comparison, trehalose and sucrose seem to be preferable lyoprotectants for biomolecules. The roles of sugars, trehalose and sucrose, include the stabilization of membrane by lowering the lipid phase transition temperature and the prevention of liposome aggregation and fusion.[35] Because they stabilise the liposomes during the freezing and drying, they have a stabilising influence on the liposome size and the polydispersity. Other properties of trehalose are also considered to be advantageous, which include, less hygroscopicity, an absence of internal hydrogen bonds, which allows more flexible formation of hydrogen bonds with proteins, and very low reactivity.[36]

The sucrose could be equally effective in protecting biomolecules. In reality, the relative stabilization effect of these two sugars seems to be depended on both the protein and sugar concentration. Based on the results of the liposome size and

polydispesity was chosen sucrose and trehalose to study properties of liposomes consisted of three lipids.

5.2 Freeze drying of liposomes

The mixtures of three lipids were prepared in different proportions in two aqueous media: 6.8% trehalose/0.3% NaCl and 10% sucrose. All samples were extruded through the membrane with pore size 200nm. The results of experimental measurements are summarized in Tab. 17-23.

Tab.17 The measured values of liposome size and polydispersity in 6.8% trehalose/0.3%NaCl before freeze drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:SA:Chol 5:1:4	275.30	279.70 (7.19)	0.09	0.09 (0.01)
	275.80		0.08	
	288.00		0.07	
EPC:SA:Chol 2:1:1	267.20	277.60 (1.53)	0.12	0.15 (0.03)
	290.00		0.17	
	275.60		0.1	
EPC:CS:Chol 5:1:4	240.90	230.63 (1.54)	0.08	0.11 (0.03)
	242.90		0.14	
	208.10		0.10	
EPC:CS:Chol 2:1:1	188.60	192.47 (3.42)	0.07	0.10 (0.04)
	195.10		0.08	
	193.70		0.14	
EPC:TPGS:Chol 5:1:4	187.90	183.37 (1.77)	0.10	0.10 (0.02)
	192.20		0.11	
	170.00		0.07	
EPC:TPGS:Chol 10:1:9	203.20	200.90 (7.62)	0.10	0.12 (0.02)
	192.40		0.14	
	207.10		0.11	

Tab. 18 The measured values of liposome size and polydispersity in 6.8% trehalose/0.3%NaCl after freeze drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:SA:Chol 5:1:4	262.10	264.27 (8.75)	0.12	0.15 (0.02)
	256.80		0.16	
	273.90		0.15	
EPC:SA:Chol 2:1:1	273.20	278.13 (8.81)	0.11	0.11 (0.01)
	288.30		0.12	
	272.90		0.10	
EPC:CS:Chol 5:1:4	182.50	209.63 (4.88)	0.09	0.14 (0.04)
	267.20		0.18	
	179.20		0.13	
EPC:CS:Chol 2:1:1	165.70	167.30 (1.44)	0.21	0.17 (0.05)
	167.70		0.18	
	168.50		0.11	
EPC:TPGS:Chol 5:1:4	200.60	207.13 (5.66)	0.13	0.18 (0.04)
	210.40		0.21	
	210.40		0.18	
EPC:TPGS:Chol 10:1:9	195.20	199.17 (4.59)	0.10	0.15 (0.04)
	204.20		0.16	
	198.10		0.18	

Tab.19 The properties of freeze-dryied samples in 6.8%trehalose/ 0.3%NaCl.

Mixtures of lipids	Appearance	Solubility in H_2O	Weight of sample [g]	Weight of sample FD [g]
EPC:SA:Chol 5:1:4	w net-cake	+	0.86	0.06
	w cake	+	0.83	0.06
	w cake	+	0.83	0.06
EPC:SA:Chol 2:1:1	w cake	+	0.81	0.06
	w cake	+	0.83	0.06
	w cake	+	0.77	0.05
EPC:CS:Chol 5:1:4	w net-cake	+	0.79	0.06
	w net-cake	-	0.88	0.07
	w cake	+	0.67	0.05
EPC:CS:Chol 2:1:1	w net-cake	-	0.77	0.06
	w cake	-	0.73	0.05
	w cake	-	0.83	0.06
EPC:TPGS:Chol 5:1:4	w net-cake	+	0.79	0.06
	w net-cake	+	0.54	0.04
	w net-cake	+	0.65	0.05
EPC:TPGS:Chol 10:1:9	w cake	+	0.62	0.05
	w net-cake	+	0.68	0.05
	w cake	+	0.58	0.04

Tab. 20 The measured values of liposome size and polydispersity in 10% sucrose before freeze-drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:SA:Chol 5:1:4	251.80	251.53 (4.81)	0.09	0.11 (0.05)
	246.60		0.16	
	256.20		0.06	
EPC:SA:Chol 2:1:1	340.30	350.00 (9.51)	0.22	0.24 (0.07)
	350.40		0.17	
	359.30		0.32	
EPC:CS:Chol 5:1:4	201.30	212.73 (12.57)	0.20	0.13 (0.07)
	210.70		0.07	
	226.20		0.10	
EPC:CS:Chol 2:1:1	214.20	211.83 (2.30)	0.11	0.11 (0.01)
	211.70		0.09	
	209.60		0.11	
EPC:TPGS:Chol 5:1:4	204.50	199.27 (4.79)	0.13	0.12 (0.02)
	198.20		0.13	
	195.10		0.09	
EPC:TPGS:Chol 10:1:9	209.10	210.07 (18.37)	0.10	0.09 (0.02)
	228.90		0.07	
	192.20		0.09	

Tab. 21 The measured values of liposome size and polydispersity in 10% sucrose after freeze-drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:SA:Chol 5:1:4	212.50	254.53 (40.81)	0.12	0.14 (0.02)
	294.00		0.14	
	257.10		0.15	
EPC:SA:Chol 2:1:1	275.90	281.43 (24.77)	0.15	0.17 (0.02)
	308.50		0.19	
	259.90		0.16	
EPC:CS:Chol 5:1:4	175.60	187.47 (10.28)	0.09	0.10 (0.02)
	193.10		0.09	
	193.70		0.12	
EPC:CS:Chol 2:1:1	190.70	195.00 (7.19)	0.07	0.10 (0.06)
	203.30		0.06	
	191.00		0.17	
EPC:TPGS:Chol 5:1:4	184.60	187.13 (4.56)	0.08	0.13 (0.04)
	184.40		0.13	
	192.40		0.17	
EPC:TPGS:Chol 10:1:9	187.40	195.80 (7.57)	0.17	0.11 0.06)
	202.10		0.08	
	197.90		0.06	

Tab. 22 The properties of freeze-dryied samples in 10% sucrose.

Mixtures of lipids	Appearance	Solubility in H_2O	Weight of sample [g]	Weight of sample FD [g]
EPC:SA:Chol 5:1:4	w cake	+	0.62	0.05
	w cake	+	0.68	0.05
	w cake	+	0.58	0.04
EPC:SA:Chol 2:1:1	w cake	+	0.53	0.06
	w cake	+	0.87	0.10
	w cake	+	0.78	0.09
EPC:CS:Chol 5:1:4	w cake	+	0.83	0.09
	w cake	+	0.77	0.08
	w cake	+	0.72	0.07
EPC:CS:Chol 2:1:1	w net-cake	+	0.89	0.09
	w cake	+	0.92	0.09
	w cake	+	0.77	0.08
EPC:TPGS:Chol 5:1:4	w cake	+	0.79	0.09
	w cake	+	0.81	0.08
	w cake	+	0.83	0.09
EPC:TPGS:Chol 10:1:9	w cake	+	0.61	0.06
	w cake	+	0.64	0.07
	w cake	+	0.71	0.07

Tab. 23 Overview of average values in trehalose and sucrose

Mixtures of lipids	Aqueous media	Ø Size [nm]	Ø PI	Ø Size [nm] FD	Ø PI FD	Appearance	Solubility in H_2O
EPC:SA:Chol 5:1:4	Treh/NaCl	279.70	0.09	264.27	0.15	w cake	+
	10 % Suc	251.54	0.11	254.54	0.14	w cake	+
EPC:SA:Chol 2:1:1	Treh/NaCl	277.60	0.15	278.14	0.11	w cake	+
	10 % Suc	350.00	0.24	281.43	0.17	w cake	+
EPC:CS:Chol 5:1:4	Treh/NaCl	230.63	0.11	209.63	0.14	w net-cake	+
	10 % Suc	212.74	0.13	187.14	0.10	w cake	+
EPC:CS:Chol 2:1:1	Treh/NaCl	192.47	0.10	167.30	0.17	w cake	-
	10 % Suc	211.84	0.11	195.00	0.10	w cake	+
EPC:TPGS:Chol 5:1:4	Treh/NaCl	183.37	0.10	207.13	0.18	w net-cake	+
	10 % Suc	199.27	0.12	187.14	0.13	w cake	+
EPC:TPGS:Chol 10:1:9	Treh/NaCl	200.90	0.12	199.17	0.15	w cake	+
	10 % Suc	210.07	0.09	195.80	0.11	w cake	+

Liposomes composed of three lipids are fairly stable, the samples were fast and good soluble, except of the sample (EPC:CS:Chol 2:1:1) which was dissolved slowly. Stability during freeze-drying is influenced by the composition of lipids. Due to the high cholesterol content, liposomal membrane was more fluid and liposomes were larger than the pores of the membrane.[37] It was used EPC:Chol as a basic formulation for liposomes, but this formulation was rather unstable, so it was stabilized with vitamin E (TPGS).

Liposomes composed of EPC:TPGS:Chol, were prepared with size almost equivalent to the pores of the membrane. This confirmed the assumption that incorporation of TGPS to the membrane of the liposomes affects their size, making them usually smaller. To improve the property of the liposomes to act as an absorption enhancer, it was added components like stearoylamine (SA). Cationic lipids are good bioenhancer and SA is furthermore pretty cheap. Cholylsarcosine (CS) acts also as a bioenhancer. SA prevents aggregation of liposomes due to its positive charge. The rather high intermolecular interaction of the stearylgroups can increase the membrane rigidity and therefore reduce the size of the liposomes. One of the factors in determining the extent to which sugars such as trehalose and sucrose can protect

liposomes during dehydratation, is a vesicle size. The size has an impact on the conservation of the content liposomes.[35]

Based on the results of the liposome size, the polydispesity and cholesterol content was chosen the samples EPC:SA:Chol 5:1:4, EPC:CS:Chol 5:1:4 and EPC:TPGS:Chol 5:1:4.

5.3 Study of liposome stability

The mixtures of three lipids were prepared in two aqueous media: 6.8% trehalose/0.3% NaCl and 10% sucrose. All samples were extruded through the membrane with pore size 200nm. After freeze-drying samples were left of 3 weeks at room temperature at rest in the laboratory. The results of experimental measurements are summarized in Tab 24 - 30. Influence of the freeze-drying on the liposome size and polydispersity index are illustrated in Fig. 13 -Fig. 16.

Tab.24 The measured values of liposome size and polydispersity in 6.8% trehalose/0.3%NaCl before freeze drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:Chol 1:1	213.70	225.30 (11.86)	0.08	0.11 (0.07)
	237.40		0.18	
	224.80		0.06	
EPC:SA:Chol 5:1:4	180.50	192.80 (10.66)	0.06	0.06 (0.02)
	199.20		0.09	
	198.70		0.05	
EPC:CS:Chol 5:1:4	176.90	184.07 (19.37)	0.05	0.09 (0.04)
	169.30		0.14	
	206.00		0.09	
EPC:TPGS:Chol 5:1:4	178.60	175.63 (2.61)	0.06	0.09 (0.03)
	174.60		0.09	
	173.70		0.13	

Tab.25 The measured values of liposome size and polydispersity in 6.8%trehalose/ 0.3%NaCl after freeze-drying, after 3 weeks storage at room temperature.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:Chol 1:1	236.60	251.83 (22.93)	0.16	0.20 (0.10)
	278.20		0.31	
	240.70		0.13	
EPC:SA:Chol 5:1:4	206.70	209.40 (5.29)	0.24	0.20 (0.03)
	206.00		0.18	
	215.50		0.19	
EPC:CS:Chol 5:1:4	140.00	150.67 (9.52)	0.13	0.14 (0.06)
	153.70		0.22	
	158.30		0.09	
EPC:TPGS:Chol 5:1:4	260.90	288.93 (70.00)	0.38	0.47 (0.21)
	237.30		0.32	
	368.60		0.72	

Tab.26 The properties of freeze-dryied samples in 6.8%trehalose/ 0.3%NaCl after 3 weeks storage at room temperature.

Mixtures of lipids	Appearance	Solubility in H_2O	Weight of sample [g]	Weight of sample FD [g]
EPC:Chol 1:1	w cake	±	0.59	0.04
	t network	-	0.66	0.05
	t network	-	0.90	0.07
EPC:SA:Chol 5:1:4	w cake	+	0.81	0.05
	w cake	+	0.91	0.06
	w cake	+	0.87	0.06
EPC:CS:Chol 5:1:4	w cake	+	0.61	0.04
	w cake	+	0.74	0.05
	w cake	+	0.85	006
EPC:TPGS:Chol 5:1:4	w net-cake	+	0.86	0.06
	w net-cake	+	0.86	0.06
	w net-cake	+	0.78	0.05

Tab.27 The measured values of liposome size and polydispersity in 10% sucrose before freeze-drying.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:Chol 1:1	218.30	221.57 (2.84)	0.18	0.15 (0.03)
	223.40		0.16	
	223.00		0.12	
EPC:SA:Chol 5:1:4	199.60	201.80 (7.06)	0.13	0.12 (0.02)
	209.70		0.10	
	196.10		0.14	
EPC:CS:Chol 5:1:4	201.40	194.40 (13.71)	0.13	0.15 (0.10)
	203.20		0.27	
	178.60		0.06	
EPC:TPGS:Chol 5:1:4	181.60	197.77 (14.52)	0.25	0.19 (0.06)
	202.00		0.17	
	209.70		0.14	

Tab.28 The values of liposome size and polydispersity in 10% sucrose after freeze-drying, after 3 weeks storage at room temperature.

Mixtures of lipids	Size [nm]	Ø Size [nm]	PI	Ø PI
EPC:Chol 1:1	215.30	217.07 (5.47)	0.18	0.13 (0.04)
	223.20		0.11	
	212.70		0.11	
EPC:SA:Chol 5:1:4	271.20	253.10 (17.11)	0.37	0.40 (0.04)
	250.90		0.44	
	237.20		0.38	
EPC:CS:Chol 5:1:4	179.10	174.57 (5.00)	0.08	0.10 (0.03)
	175.40		0.09	
	169.20		0.14	
EPC:TPGS:Chol 5:1:4	201.80	206.53 (4.70)	0.11	0.12 (0.02)
	206.60		0.14	
	211.20		0.10	

Tab.29 The properties of freeze-dryied samples in 10% sucrose after 3 weeks storage at room temperature.

Mixtures of lipids	Appearance	Solubility in H_2O	Weight of sample [g]	Weight of sample FD [g]
EPC:Chol 1:1	w network	-	0.58	0.06
	w cake	+	0.88	0.09
	w cake	+	0.67	0.07
EPC:SA:Chol 5:1:4	t network	-	0.91	0.10
	t network	-	0.83	0.09
	t network	-	0.84	0.09
EPC:CS:Chol 5:1:4	w cake	+	0.64	0.07
	w cake	+	0.82	0.08
	t network	-	0.77	0.08
EPC:TPGS:Chol 5:1:4	w cake	+	0.79	0.08
	w cake	+	0.87	0.09
	w cake	+	0.75	0.07

Tab. 30 Overview of average values in trehalose and sucrose

Mixtures of lipids	Aqueous media	Ø Size [nm]	Ø PI	Ø Size [nm] FD	Ø PI FD	Appearance	Solubility in H2O
EPC:Chol 1:1	Treh/NaCl	225.30	0.11	251.83	0.20	t net-cake	-
	10 % Suc	221.57	0.15	217.07	0.13	w cake	+
EPC:SA:Chol 5:1:4	Treh/NaCl	192.80	0.06	209.40	0.20	w cake	+
	10 % Suc	201.80	0.12	286.43	0.40	t network	-
EPC:CS:Chol 5:1:4	Treh/NaCl	184.07	0.09	150.67	0.14	w cake	+
	10 % Suc	194.40	0.15	174.57	0.10	w cake	+
EPC:TPGS:Chol 5:1:4	Treh/NaCl	175.63	0.09	288.93	0.47	w net-cake	+
	10 % Suc	197.77	0.19	206.53	0.12	w cake	+

In this Tab. 30, the values of samples after freeze-drying are recorded, measured after three weeks storage. Even in appearance, it is clear that the samples: EPC:Chol 1:1 in Suc, EPC:SA:Chol 5:1:4 in Treh/NaCl, EPC:CS:Chol 5:1:4 in Treh/NaCl and Suc, EPC:TPGS:Chol 5:1:4 in Suc are stable.

Fig. 13 The influence of the freeze-drying in trehalose on liposome size. The change of liposome size before, after freeze – drying and after three weeks storage at room temperature.

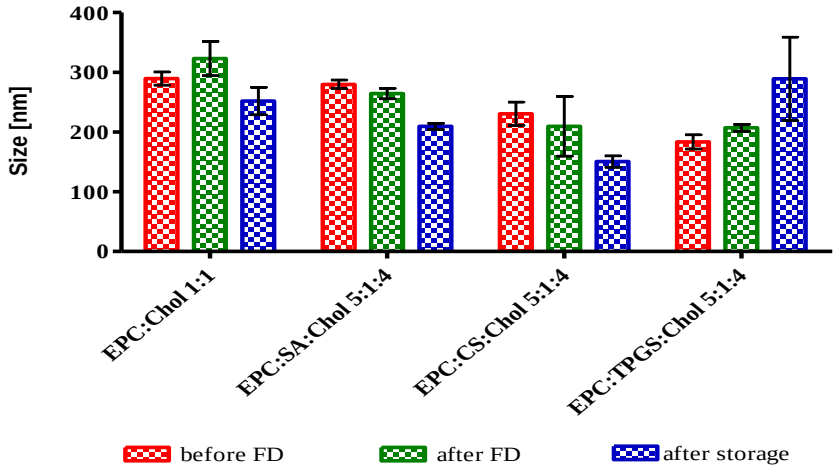
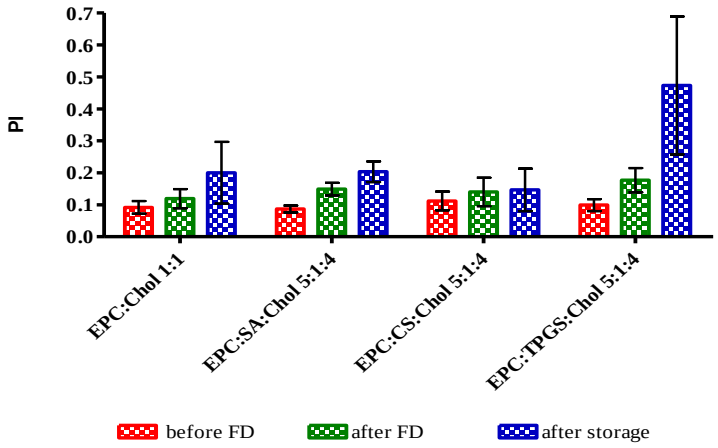


Fig. 14 The influence of the freeze-drying in trehalose on polydispersity index. The change of polydispersity before, after freeze-drying and after 3 weeks storage at room temperature.



Study of liposome stability showed, that the influence of freeze - drying increased the liposome size and polydispersity in the mixture consisted of EPC:TPGS:Chol 5:1:4. The increase in size can be caused by the fusion of liposomes. This also leads to an increase of the polydispersity. The freeze-dryied samples had the shape of white net cake. The appearance and increase of the liposome size after three weeks storage showed, that this mixture is unstable.

Fig. 15 The influence of the freeze-drying in sucrose on liposome size. The change of liposome size before, after freeze-drying and after three weeks storage at room temperature.

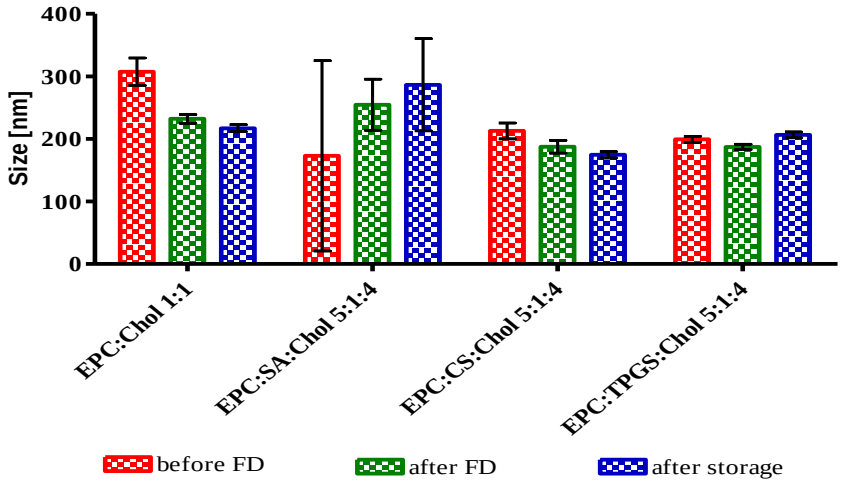
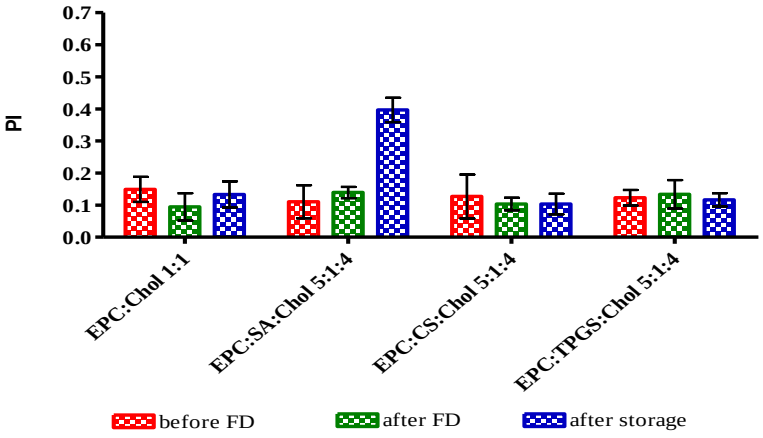


Fig. 16 The influence of the freeze-drying in sucrose on polydispersity index. The change of polydispersity before, after freeze-drying and after 3 weeks storage at room temperature.



In the case of sucrose, stability studies shows, that the storage and freeze – drying did not have a large influence of liposome size and polydispersity, except of the mixture consisted of EPC:SA:Chol 5:1:4. The variability of liposome size could be caused by manual extrusion. The freeze-dryied samples had the shape of white net cake. The appearance and increase of the liposome size after three weeks storage showed, that this mixture is unstable.

6 Conclusion

During the experimental stage of this work, it was achieved by manual extrusion to prepare liposomes of different sizes, which has been shown that the liposome size depends on their composition. The higher cholesterol content increases the fluidity of liposomal membrane, causing the formation of liposomes larger than the membrane pores of extruder. By the liposomes containing stearoylamine and cholylsarcosine were measured size as the membrane pores of extruder. Liposomes containing vitamin E were of smaller size due to the polyethylenglycol chains; these chains increased stability through the prevention of aggregation.

The freeze-drying is crucial for the preparation of stable drugs forms, because freshly prepared liposomes succumb to degradation. It has been shown, that the appropriate media for freeze-drying are trehalose and sucrose. Both these lyo- and cryoprotectants were evaluated as the most, on the basic of appearance, solubility in the water and values of size and polydispersity. Liposomes consisted of three lipids extended the stability after three weeks storage at room temperature. These liposomes did not change the shape of white cake, fast and good solubility in the water. The liposome size did not show major changes and the polydispersity was low except of mixture EPC:TPGS:Chol 5:1:4 in trehalose, the increase in size and polydispersity can be caused by the fusion of liposomes, and EPC:SA:Chol 5:1:4. The variability of liposome size could be caused by manual extrusion.

In studying the stability of liposomes we made only the first step. In the another step will be to verify the water content by method Karl Fisher, determination of the amount of the incorporated drug, they are able to hold inside, and many other studies. The whole research should lead towards administration of peptide drugs (e.g. insulin or calcitonin) by oral application.

7 Summary

The protein or peptide substances are not appropriate for oral application. One of the many obstacles, is an aggressive environment of the gastrointestinal tract. One way to protect these substances is the preparation of liposomes. The aim of this work was preparation of liposomes of the different composition, choice of the optimum aqueous media, and stabilization using freeze-drying. All samples consisted of egg phosphatidylcholine(EPC) and cholesterol (Chol), with the addition of one out of the substances: cholylsarcosine (CS), stearoylamine (SA) and vitamin E (TPGS), respectively, in various ratio. Liposomes were prepared using a film method and manual extrusion through membrane with pore size 200 nm. The liposome size and polydispersity was measured by photon corellation spectroscopy. After the extrusion, the liposomes underwent freeze-drying in the presence of selected appropriate cryoprotectants: sucrose and trehalose, respectively. Stability has been established by evaluating the visual characteristics of the product (the appearance), solubility in the water and measuring of liposome size and polydispersity.

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