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DIPLOMOVÁ PRÁCE

Zkoumání membránových vlastností lipozómů pomocí měření zeta potenciálu

Hradec Králové 2009 Miroslava Kortusová

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DIPLOMA THESIS

Investigating the membrane properties of liposomal formulations by zeta potential measurement

Hradec Králové 2009 Miroslava Kortusová

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a jsou v práci řádně citovány.

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10. května 2009 ……..

Miroslava Kortusová

Ráda bych poděkovala oddělení Farmaceutické technologie institutu Farmacie a Molekulární Biologie při Ruprecht – Karls Universität Heidelberg a hlavně jejímu vedoucímu Prof. Dr. Gertu Frickerovi za umožnění vykonání mé diplomové práce. Velké poděkování patří mému školitelovi z Heidelbergu Johannesovi Parmentierovi za odborné vedení, rady a spolupráci. V neposlední řadě bych ráda poděkovala Prof. RNDr. Petru Solichovi, CSc. za odborné vedení při psaní mé diplomové práce.

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ANOTACE

Perorální podání je nejpohodlnější aplikace léčiv. Bohužel látky zejména bílkovinné nebo peptidické povahy nesnesou podmínky, které se v trávicím traktu vyskytují, navíc díky své velikosti se i velmi málo vstřebávají skrz střevní stěnu, tudíž je nutné je pro perorální podání speciálně upravit a chránit. Vhodnými kandidáty se jeví lipozómy – částice s fosfolipidovou membránou, které nejen že dokážou lipidy chránit před agresivním prostředím v GIT, ale navíc zlepšují jejich absorpci. Lipozómy lze připravit pomocí široké škály různých fosfolipidů, povrchově aktivních látek či solí žlučových kyselin.

Cílem této práce bylo připravit lipozómy s přídavkem v praxi zřídka používaných surovin (konkrétně cholylsarcosinu, stearylaminu a d-α-tokoferol sukcinátu) a zjistit některé jejich povrchové vlastnosti, které jsou rozhodující pro další použití lipozómů. Lipozómy byly připravovány filmovou metodou a následnou hydratací a ruční extruzí přes membránu s definovanou velikostí pórů 200 nm. Jejich průměrná velikost poté byla stanovována pomocí foton – korelační spektroskopie (PCS). Podařilo se potvrdit, že velikost lipozómů je mimo jiné závislá na složení lipidové membrány, přidané substance svým nábojem nebo stavbou molekuly vedly ke zmenšení velikosti lipozómů.

Další zkoumanou povrchovou vlastností byl zeta potenciál lipozómů. Podařilo se prokázat, že zeta potenciál úzce souvisí s nábojem použitých substancí, konkrétně u kladně nabitého stearylaminu byl naměřen kladný zeta potenciál, u záporně nabitého cholylsarkosinu zeta potenciál záporný. S touto problematikou souvisí i vliv pH použitého rozpouštědla na zeta potenciál lipozómů. Byl potvrzen předpoklad, že kyselé pH posouvá zeta potenciál směrem ke kladným hodnotám, pH bazické naopak k hodnotám záporným.

Zkoumané substance tedy dodávají lipozómům podobné povrchové vlastnosti, jako substance již tradičně používané, ale před jejich větším rozšířením do praxe je nutné provést ještě další výzkum nejen jejich vlastností.

SUMMARY

The most comfortable drug administration is the oral one. Unfortunately the protein or peptide substances are not stable in conditions present in the digestive system. Also, thanks to their size and polaritz they are very badly absorbed through the wall of the intestine so it is necessary to modify and specially protect them. Liposomes appear to be suitable candidates – particles with phospholipid membrane not only can protect peptides and proteins against the aggressive environment in the gastrointestinal system they also improve their absorption. Liposomes could be prepared of the wide scale of different phospholipids, surface active substances or bile acid salts.

The aim of this research is to prepare liposomes with addition of rarely used substances in practice and substances (particularly cholylsarcosine, stearylamine and d-α-tocopherol polyethylene glycol succinate) and find out some of their surface properties crucial for the further use in liposomes. Liposomes were prepared by film method followed by hydration and manual extrusion through a membrane with defined pore size of 200nm. The average vesicle size was defined by the method of photon - correlation spectroscopy (PCS). We succeeded in confirmation that the liposome particle size is also dependent on the constitution of the lipid membrane, added substances lead to decrease of the size of liposomes due to their charge or the structure of the molecule.

The next surface property examined was the zeta-potential of liposomes. We succeeded in confirmation that there is a close connection of zeta-potential with the charge of used substances. It was measured that in the liposomes with positively charged stearylamine the zeta-potential was positive and in the liposomes with negatively charged cholylsarcosine the zeta-potential was negative. The presumption of influence of the pH was also confirmed. Acid pH moves the zeta-potential values to positive and basic pH moves the values to the negative scale.

The examined substances give the liposomes similar surface properties as usually used substances but before using them commonly further studies, not only of their properties, are needed.

CONTENT

1 INTRODUCTION

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 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 of gastrointestinal tract (GIT) and thus also improve their absorption. Liposomal formulations offer the possibility of protection and absorption enhancement of poorly bioavailable drugs. Unfortunately, liposomes themselves are often unstable in GIT, e. g. In presence of acid, lipase and bile salts. It seems desirable to develop more stable liposomal formulations. Various lipo – and amphiphilic substances are used to stabilise the liposomal membrane. These compounds modify the properties of the liposomal membranes. To understand the in vivo behaviour of the liposomal formulations it is necessary to know about the interaction of special lipids or enhancers with normal phospholipid bilayers.

The aim of the project was to examine with various methods the behaviour of several bioenhancers and special lipids in phospholipids membrane. So the concentration dependent influence of charged substances on the average and the zeta potential of the liposomes was investigated.

The techniques used for this project were:

- producing liposomes by the film method
- forming liposomes by extrusion
- photon correlation spectroscopy
- measurement of zeta potential

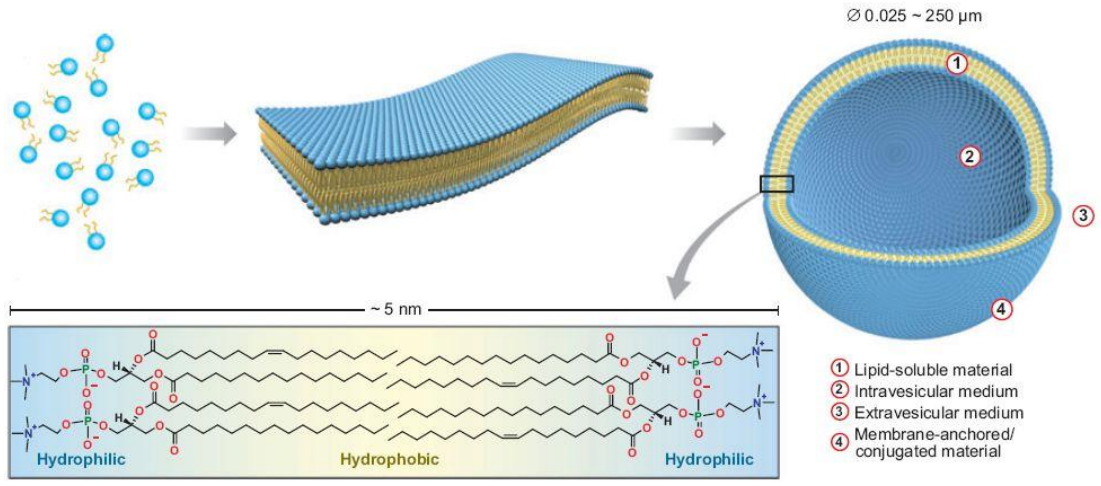
2 THEORY

2.1 What Is a Liposome

An intense study in the liposome field began 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. Thanks to research findings it is possible today to prepare liposomes of various sizes, various lipid composition, and with various surface characteristics, making it possible to satisfy different requirements on a peroral application or a targeted administration of medicines. The surface characteristics are determined by the choice of lipids forming the surface bilayer, and can be changed additionally by the covalent bond or the incorporation of glycoproteins, normal or synthetic polymers, sugars etc. In comparison with other medicinal carriers, liposomes are distinguished by their better biological degradability and relative toxicological and immunological safety [1].

Liposomes are generally spheric lipid bilayers of a 50 to 1000 nm diameter. They arise by the interaction of amphiphilic lipids in aqueous phase and they serve as an osmotically sensitive model membrane system, which is 10^5-times more permeable for anions than for cations [2].

Figure 1 Schematic Illustation of liposome [3]



2.2 Classification of Liposomes

Liposomes can be classified according to various criteria, such as size, lamellarity, lipid composition, application or name. The most frequent types of liposomes are summarised in Table 1

2.2.1 Based on Size

The size of liposomes plays an important role in their clearance. There is a difference in clearance between classical liposomes and sterically stabilised liposomes. With classical liposomes administered intravenously the clearance increases in conjunction with the size of the liposomes, because they are filtered out by the reticuloendothelial system [4].

Sterically stabilised liposomes with average size around 250 nm are filtered out slowly by the Kupffer cells of the liver. With liposomes sizing 300 nm the filtering by the spleen cells is increased, presumably by a passive filtration mechanism. Thus, by the preparation of liposomes with a defined size (by sonication or extrusion) the control of the circulation time of liposomes and an increased extravasation can be reached.

By peroral application the particles are absorbed in the intestinal. Generally, nanoparticles have relatively higher intracellular uptake compared to microparticles and are available to a wider range of biological targets due to their small size and relatively mobility. Many studies regarding size effects on nanoparticle absorption by intestinal epithelia have been performed using polystyrene standard particle suspensions of defined size distributions. Particle with mean diameters of 50 or 100 nm showed a higher uptake in the rat intestine then larger particles [5]. Particles < 100 nm show higher rates of uptake by absorptive enterocytes than particles > 300 nm. The uptake of particles < 100 nm by the follicle – associated epithelia is more efficient than uptake via absorptive enterocytes. Uptake of particles > 500 nm by absorptive enterocytes is an unlikely event and only particles < 500 nm reach the systematic circulation. The uptake of 100 nm particles in the rat intestine was significantly increased compared to larger particles of 1 – 10 µm. Nearly identical uptake rates were observed in Peyer's patch regions and enterocytes for 100 nm particles, while particles

> 100 nm were only observed in the Peyer's patches. As a rule of thumb, size < 500 nm will be required for optimal uptake and bioavailability [6].

2.2.2 Based on Circulation Time *in vivo*

Conventional Liposomes

Liposomes of a very simple structure are labelled conventional or classical liposomes. Their main subpart is egg phosphatidylcholine – EPC and cholesterol in various amounts. Cholesterol is added for the increase of liposome stability in the presence of plasma and for the increase of the fluidity of the membrane, whereby is minimised the drug leakage.The negative charge is important for aggregation blocking. These liposomes are recognized by phagocytic cells 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 [7].

Table 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 application
Diagnostic
Therapeutic
According to surface charge
Cationic/ DNA vector
Anionic

Neutral
Specialised liposomes
Targeted liposome
Immunoliposome
Transferosome
Liposomal DNA vector

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 (PI), dipalmitoyl phosphatidylcholine (DPPC) or distearoyl phosphatidylcholine (DSPC) [8].

Later, polyethylen glycol (PEG) was used and appeared more efficient than gangliosids or PI. Today, PEG is used also in the form of fatty acids ester.

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. Some types of Archaebacteria live in extreme conditions of vulcanic hot springs, salt lakes or places with high basic or acidic pH (e.g. Thermoplasma acidophilum). From the cell walls of these bacteria we can gain lipids from which we can prepare liposomes more resistant to low pH in the stomach (this is important for the peroral application of liposomes).

2.2.3 Based on Lamellarity

Small Unilamellar Vesicles (SUVs)

Small Unilamellar Vesicles have a radius smaller than 50 nm and have only one lipid bilayer. They are prepared by sonication or extrusion. The size of SUVs depends on the

lipids used, the preparation temperature and the length of sonication. Generally these are metastable liposomes, which tend to connect into larger unilamellar vesicles.

Large Unilamellar Vesicles (LUVs)

Large Unilamellar Vesicles have a radius of 100-2000 nm and again only one lipid bilayer. They can be prepared either by calcium-induced fusion, sonication of SUVs, or by extrusion through defined pore-size filters. By this method, LUVs of a given size can be prepared. The internal capacity of these liposomes is quite high, therefore they appear to be the most suitable liposomes for the protection of big protein molecules of pharmaceuticals.

Large Multilamellar Vesicles (LMVs)

Large Multilamellar Vesicles arise spontaneously if the bilayer-forming lipid mixture is hydrated in a large amount of water or buffer. However, liposomes of very different sizes (from 0.5 to 10 μm), with different lamellarity and different internal capacity, are produced in this way. LMVs with a given size can be prepared by a careful extrusion through defined pore-size filters with soft pressure. Nevertheless, even these LMVs differ in size and the number of individual inner compartments. The overall internal capacity of a LMV is quite large, but the space in the individual compartments is too small to accommodate large medicinal proteins. LMVs are therefore used most often as a model membrane system for the monitoring of membrane systems qualities, their susceptibility to hydrolysis by phopholipases, etc. [9].

2.2.4 **Based on Surface Charge**

On their surfaces, liposomes may carry either a positive or a negative charge that is supplied mainly by admixtures to normal lipids (EPC and cholesterol) because the charge of these classic liposomes is in essence neutral. It is not a problem to prepare liposomes with a substantial negative or positive charge. However, 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. Therefore, it is also

possible to include cationic liposomes into the specialized liposomes, as liposomes represent one of the safest and most universal transfer vectors of today's. The cationic lipid is mixed with the membrane – destabilising lipid - dioleoyl phosphatidylethanolamine (DOPE) - in approximately equimolar ratio. DOPE destabilises the membrane of the target cell and the DNA is released into the cytoplasm [10].

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. On the other hand, cationic liposomes are trapped up to four times faster by the brain uptake and lung tissue than the anionic ones [11].

2.2.5 **Specialised Liposomes**

Targeted Liposomes are liposomes that are routed straight to recognition and direct continuation on specific cells *in vivo*. This is achieved by incorporation of a specific ligand into the surface layer of the liposome. The half-life of the ligand should be roughly the same as that of the liposome itself; moreover, the liposome should not decrease it. If the surface ligand is an antibody, the liposomes are called *Immunoliposomes*. The clearence of classical liposomes with surface antibodies is very fast: it endures only a few minutes in circulation, whereas the half-life of *Immunoliposomes* prepared from sterically stabilised liposomes is in orders of hours. It follows that *Immunoliposomes* are prepared mainly from sterically stabilized liposomes [12].

2.2.6 **Clinical Applications**

2.2.6.1 ***Diagnostic***

Big liposomes are trapped quickly by phagocytic cells of the reticuloendothelial system (RES). This fact is used for transport of diagnostic imaging agents into the liver and spleen. The aqueous contrast-enhancing agents are closed into liposomes that are

inserted to the body and, after some time, the differences between healthy and tumorous tissue are displayed by computer tomography [13]. The liposomes are filled not only with substances soluble in water but also with gas. These liposomes are of a big importance for diagnostics by ultrasound waves or by magnetic resonance. In these methods, the principle of the liposomes' use is the varying magnetic susceptibility and the ability to reflect the sound waves. Lipids of these liposomes are enriched mostly with components supporting the stability of liposomes (for example, dipalmitoyl phosphatidylcholine or distearoyl phosphatidylcholine).

2.2.6.2 *Therapeutical Application*

Localised/Regional Use

The topical application of liposomes has a big potential mainly in dermatology and transfers from this field also to the commercial sphere of pharmaceutical companies and the cosmetic industry. 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 (right to Stratum Corneum), which was earlier impossible. Moreover, liposomes are absorbed to the skin relatively quickly thus the duration of the effective substance's exposition on the skin's surface is decreased as well as the danger of their wipe still before their absorption. Furthermore, liposomes are trapped selectively by hair follicles. This quality allows transporting melanin, proteins, genes or little molecules directly into the hair follicle; thereby it is possible to accomplish the local genetic therapy that may restore the hair growth, the old hair pigment or to slow the untimely loss of hair [14].

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 medications that are transported directly into the effective place by the liposomes. Furthermore, 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. Therefore, there 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 Karposi's Sarcoma [15].

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 [16].

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. It would be possible to write many pages about the advantages of peroral application of insulin over that by injection, but what is important may be the fact that the ideal liposomes for peroral application have not yet be found although the research on this problem solving is really intensive.

2.3 Photon Correlation Spectroscopy (PCS)

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. As mentioned above, 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 liposome's size also offers the possibility to estimate its inner volume. Finally yet importantly, the fast measuring of the liposome's 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 etc.

There exist some methods how the size of liposomes may be found. The former is PCS mentioned above; the next are the electron microscopy, field flow fractionation or atom force microscopy. However, it is PCS that is used massively. What are the reasons?

- 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.

- PCS measures a distribution of tens up to thousands of particles. The electron microscopy needs hundreds of photos for a comparable analysis [17].

2.3.1 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:

$$d(H) = \frac{kT}{3\pi\eta D}$$

Where: d(H) = hydrodynamic diameter

D = translational diffusion coefficient

k = Boltzmann's constant

T = absolute temperature

η = viscosity

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 [18].

2.3.2 Factors Affecting Diffusion Speed of Particles

Ionic Strength of Medium

The ions in the medium and total ionic concentration can affect the particle diffusion speed by changing the thickness of the electric double layer called the Debye length (κ^{-1}). Hence even a low conductivity medium can extend the double layer of ions around the particle, decreasing diffusion speed and increasing hydrodynamic diameter. Conversely, higher conductivity media suppress the electric double layer and the measured hydrodynamic diameter.

Surface structure

Some changes of surface structure can induce a change of particle diffusion speed, which however does not correspond to the change of its size. An adsorbed polymer projecting into the medium reduces diffusion speed more than a polymer adsorbed directly on the surface of the particle. Nature of the surface and ionic speed of the medium may also influence polymer conformation, changing the apparent particle size by a few nanometers.

Non – spherical particles

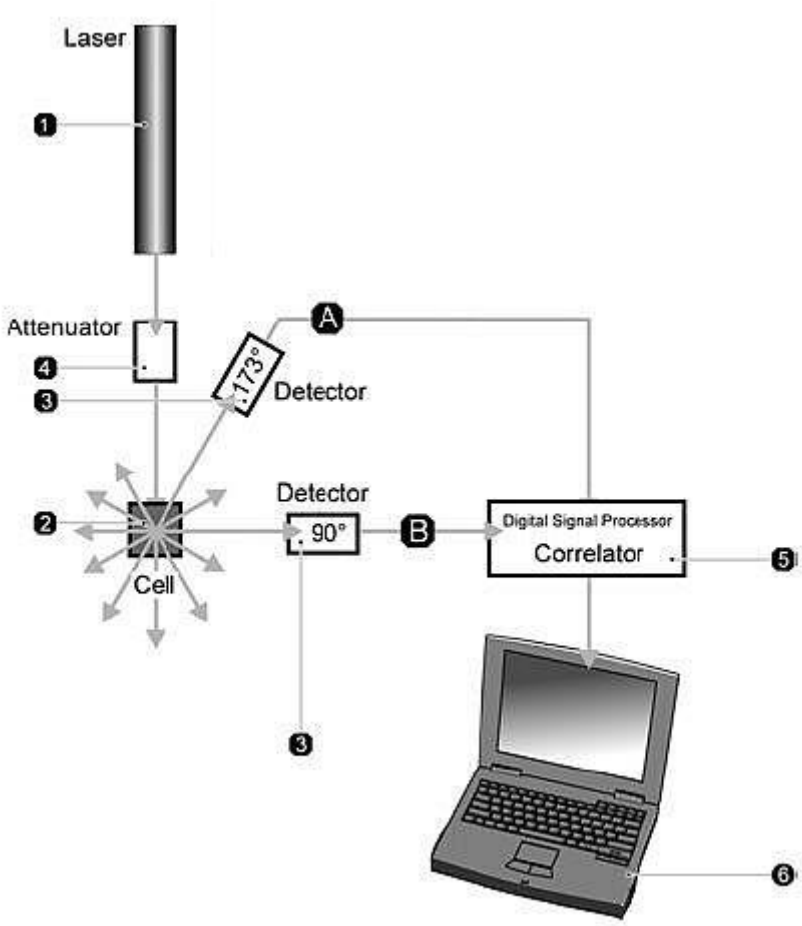
All techniques for establishing the size of a particle encounter a problem – how to describe a non – spherical particle? Different techniques are sensitive to different particle properties – e.g. projected area, density, scattering intensity – and therefore produce different size distributions for any measured sample. It is important to note that none of these results is completely accurate. The measured hydrodynamic diameter of a particle corresponds to the hydrodynamic diameter of a sphere with the same translational diffusion coefficient.

2.3.3 Optical Configuration of the PCS Instrument

A typical PCS system consists of six parts (see Figure 2). 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 a special software that calculates on their basis the size of the particles in the sample [19].

Figure 2 Optical Configuration of PCS Instrument [20]



2.4 Zeta Potential

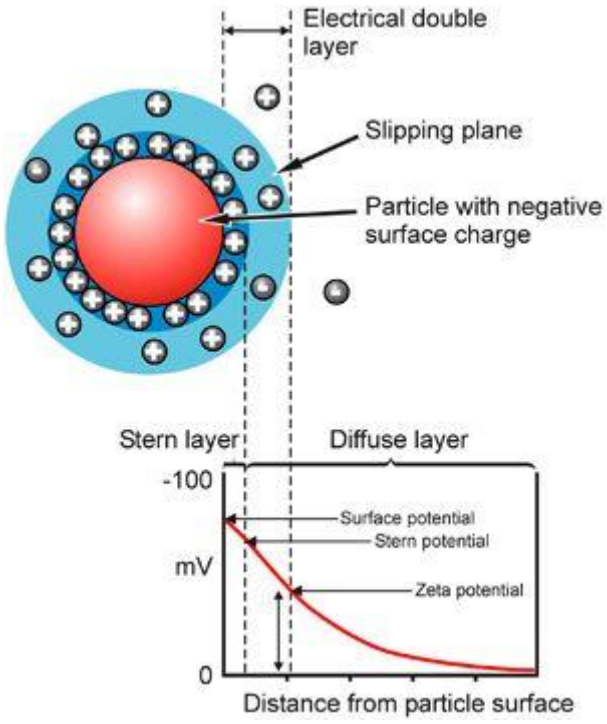
Zeta potential is the charge that a particle (e.g. a liposome) acquires when suspended, usually in a water-type medium. It is however not the charge on the surface of the particle, but rather on the surface of its solvation shell. Knowledge of the zeta potential enables estimating the fate of the liposome in the organism, i.e. how quickly it will be marked by proteins in blood plasma that speed up its capture by the RES. This phenomenon can be partly slowed down by masking the surface of the liposome with a suitable polymer, such as polyethylene glycol (PEG). PEG not only creates a steric

barrier against binding of the plasma proteins on the liposome, but also changes its zeta potential [21].

But how is charge generated on the surface of an apparently neutral particle? The cause is the nature of the particle surface and the properties of the medium in which it is dispersed. One of the mechanisms is the ionisation of surface groups. Dissociation of acidic groups on particle surface leads to a negative surface charge. Conversely, dissociation of basic groups delivers a positive charge. The size of this charge depends on the acidic or basic strengths of the surface groups, and on the pH of the medium. Surface charge can also be generated by adsorption of ions or ionic surfactants on the surface of the particle. Cationic surfactants provide the particle with a positive charge, anionic surfactants with a negative one [22].

The existence of electric charge on the surface of the particle then affects the position of ions in the medium in the immediate neighbourhood of the particle. Ions with the opposite charge converge around the particle and create the electrical double layer surrounding it. This layer of liquid around the particle can be ideally divided into two parts: The inner Stern layer, in which the ions are firmly bound, and the outer diffuse region, where the bond is much weaker. It is possible to find an imaginary boundary in the diffuse region within which the ions and the particle make up a stable entity. When the particle moves, the ions within this boundary move along with it. The potential on this boundary (the slipping plane) can be measured and is called zeta potential [23].

Figure 3 Schematic representation of zeta potential [24]



2.4.1 Factors Affecting Zeta Potential

pH

The pH is one of the most important factors affecting the zeta potential in water solutions. A value of zeta potential without acknowledgement of the pH at which it was measured is a completely meaningless number. The same sample will exhibit different zeta potentials when measured at different pH values. Basic solutions shift the zeta potential of the dispersed particles towards negative values, whereas acidic solutions shift it towards positive values. The pH value corresponding to a zeta potential of zero is called the isoelectric point. It is the least stable state of the colloid system (widely applied in colloid system technologies).

Conductivity

Thickness of the electrical double layer (Debye layer, κ^{-1}) depends on the concentration of ions in the medium, and can be calculated from its ionic strength. The higher the ionic strength, the lower it compresses the electrical double layer of the particle. Inorganic ions can react with the charged surface of the particle principally in two

ways. Either through non – specific ion adsorption, which does not affect the isoelectric point, or through specific ion adsorption, which induces a change of the isoelectric point. Specific ion adsorption on the surface of the particle, even in low concentration, can have a dramatic effect on the zeta potential [22].

2.4.2 The Principle of Measurement of Zeta Potential

Measurement of zeta potential is based on electrophoresis. When we apply an electric field to an electrolyte with suspended charged particles, the particles are attracted towards the electrode with the opposite charge. Viscous forces of the liquid act against this movement. When an equilibrium between these forces is reached, the particles move with a constant velocity. This velocity depends on the strength of the electric field (or voltage gradient), on the dielectric constant of the medium, on the viscosity of the medium, and on the zeta potential. The velocity of a particle in a unit electric field is referred to as its electrophoretic mobility. Its zeta potential is then calculated from its electrophoretic mobility by Henry's law.

$$U_E = \frac{2\varepsilon Z f(\kappa a)}{3\eta}$$

Where: U_E = electrophoretic mobility

Z = zeta potential

ε = dielectric constant

η = viscosity

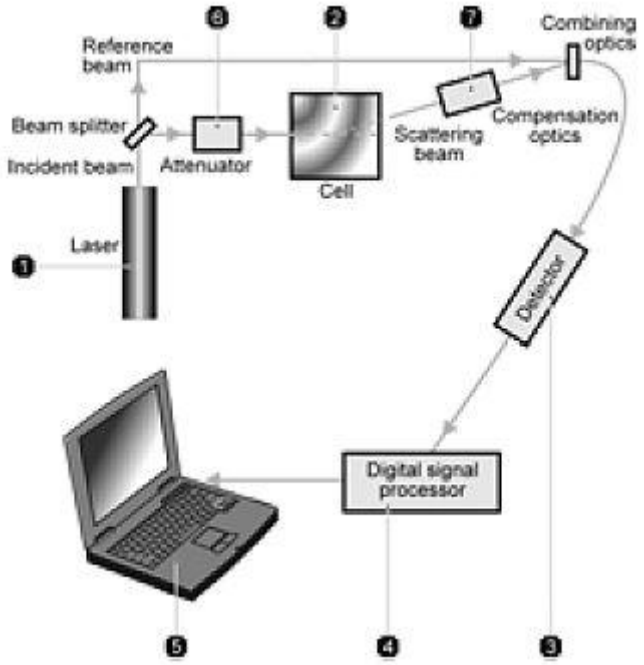
f(κa) = Henry's function [25]

2.4.3 Optical Configuration of the Zeta Potential Instrument

A typical instrument for measurement of zeta potential consists of 6 parts (see Figure 4). The laser 1 is the source of a beam of light. The beam illuminates the sample in the measurement cell 2. Scattered light is detected in the angle of 13 degrees 3. Intensity of the scattered light must be in a certain range for the detector to measure it correctly. This is achieved by the attenuator 6 that regulates the light beam generated

by the laser. Light dispersion is proportional to the velocity of the moving particles. This information is passed on to the digital signal processor 4 and further to the computer 5. A special computer software calculates from the values thus obtained the value of the zeta potential of particles in the sample. Sometimes compensation optics 7 are also included for correction of dispersant refraction and of errors generated by the walls of the measurement cell.

Figure 4 Optical Configuration of Zeta Potential Instrument



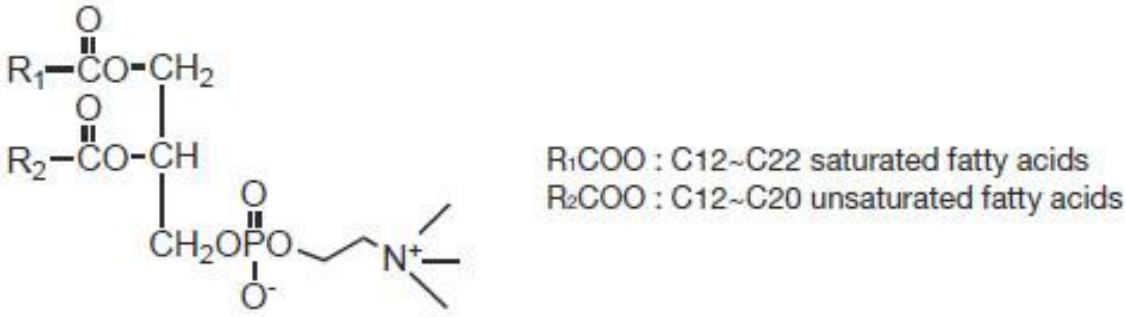
2.5 Materials for Liposomes

2.5.1 Eggs phosphatidylcholine (EPC)

Eggs phosphatidylcholine belongs to the group of phospholipids that is part of lecithin. Phospholipids are the essentials components of all cell membranes. The molecules of phospholipids are amphiphilic that means they have both polar and non-polar parts. The non-polar (hydrophobic) part of the EPC molecule is formed by two chains of fatty acids. These form esters in the first and the second position of the glycerol backbone. The polar (hydrophilic) part of the molecule is formed by the ester of choline and phosphate in the third position of the glycerol molecule (see Figure 5). In aqueous solution, at a specific concentration and temperature the EPC is able to form lamellas,

micelles or liposomes spontaneously. The most advantageous for pharmacy is that EPC is essentially non-toxic, it doesn't provide any contraindications or interactions with other drugs, and it is not mutagenous and can't be overdosed.

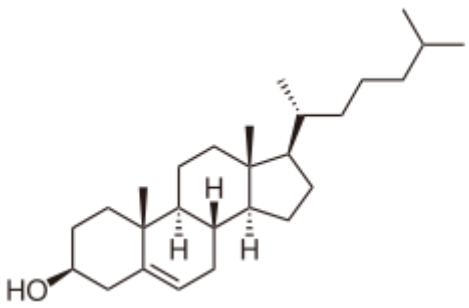
Figure 5 Molecule of EPC [26]



2.5.2 **Cholesterol (Chol)**

Incorporation of cholesterol in liposome bilayer can bring about big changes in the preparation of these membranes. It does not mean that it forms bilayer membrane structure by itself, but can be incorporated into phospholipid membrane in very high concentration up to 1:1 or 2:1 molar ratios of cholesterol to phosphatidylcholine. Being an amphiphilic molecule, cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface and aliphatic chain aligned parallel to the acyl chains in the centre of the bilayers and also it increases the separation between choline head groups and eliminates the normal electrostatic and hydrogen bonding interactions. Cholesterol increases the fluidity of liposomal membrane [27].

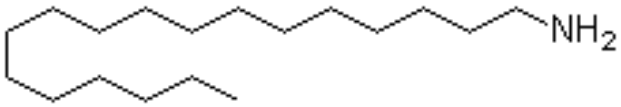
Figure 6 Molecule of cholesterol



2.5.3 **Stearylamine (SA)**

In systematic nomenclature is SA called 1-aminooctadecane. It is a fatty amine and cationic surfactant. SA is used in liposome preparation because it causes the liposomal surface to be positively charged. Cationic liposomes are important as DNA vectors. In addition, these particles are not being removed from the circulation so quickly in comparison to anionic liposomes. SA increases the liposomal stability in mild acidic or basic environment. However these liposomes are not resistant to destruction by bile acids and low pH level present in digestive system, so SA does not solve the problem of oral administration of liposomes [28]. Nevertheless, it was proved that SA decreases effectively the leaking of cationic drugs from liposomes so it is suitable for liposomal drug formulation [29]. The low price within the group of cationic surfactants is its main advantage but on the other hand it is quite toxic. In higher concentrations, it causes skin, eye and respiratory system irritation and destroys mucosa membranes.

Figure 7 Molecule of SA

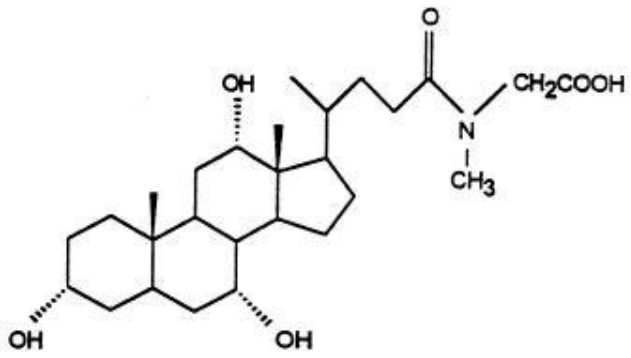


2.5.4 **Cholylsarcosine (CS)**

CS could be described as N – methylglycine conjugated to a cholic acid. It is a non-toxic bile salt derivative that is the absorption enhancer. CS is comparable to conventional bile acids that are potentially carcinogenic. CS could be used to increase absorption of peptide drugs after oral administration (e.g. insulin, calcitonine) because it forms complex compounds with calcium ions. These cause the decrease of tightness of tight junctions that causes the increase of permeation and absorption of peptides [30]. It is absorbed in a negligible amount from the small or large intestine so the long time administration induces chances neither in biliary lipid composition nor liver function. It is able to form micelles in the solutions of critical micellar concentration [31]. CS could form lipophilic ion – pair complexes with different organic cations in liposomes that also increases the permeability of cations through biological membranes [32]. In

human medicine CS could be used in bile salt replacement therapy of short bowel syndrome [33].

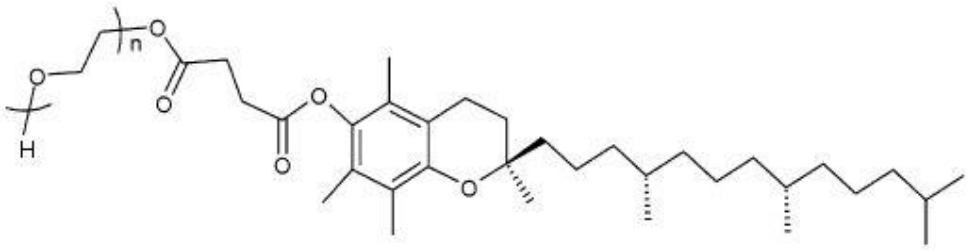
Figure 8 Molecule of CS [31]



2.5.5 Vitamin E TPGS NF (TPGS)

In systematic nomenclature it is called d-α-tocopheryl polyethylene glycol 1000 succinate. It is a derivative of vitamin E soluble in water. By its structure the compound has an amphiphilic character that means it contains a polar head that consist of polyethylene glycol and a lipophilic tail composed by tocopherol succinate. The amphiphilic character of the compound causes that TPGS could form micelles if its concentration in the solution exceeds the critical micellar concentration. TPGS is a surface active compound so it could be used as an emulsifier, a solubiliser, an absorption enhancer or water – soluble source of vitamin E. Sokol a kol. [34] clinically demonstrated that TPGS could increase the absorption of highly lipophilic cyclosporine during the immunosuppressive therapy in patients after the organ transplantation. TPGS belongs to the non-ionic surfactants, which are more hydrophobic than the ionic ones. These provide bigger capacity for solubilisation of poorly soluble drugs and are less toxic to biological membranes. These also influence the cellular drug transport activity especially by the inhibition of efflux pumps (i.e. P – glycoprotein) [35]. When TPGS is used in liposomes the stability is being better because polyethylene glycol decreases liposomal aggregation [36]. The quite low price is a not negligible merit within commonly used phospholipids with PEG chain.

Figure 9 Molecule of TPGS [37]



3 PRACTIC PART

3.1 Material

d – alpha tocopheryl polyethylene glycol 1000 succinat – Vitamin E TPGS NF (TPGS)	Eastman, UK
Distilled water	IPMB, Germany
Egg phosphatidylcholine (EPC)	Lipoid, Germany
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
Potassium chloride (KCl)	AppliChem, Germany
Potassium phosphate (KH_2PO_4)	J. T. Baker, Holland
Sodium chloride (NaCl)	Wassex House, UK
Sodium phosphate dihydrate ($Na_2HPO_4 \cdot 2\ H_2O$)	J. T. Baker, Holland
Stearylamine (SA)	Sigma Aldrich, Germany

3.2 Equipment

Extrusion

Extrudor – LiposoFast Basic	Avestin Europe GmbH, Germany
Membranes – Polycarbonate membrane, diam. = 19mm, pore diam. = 200 nm	Avestin Europe GmbH, Germany

PCS

Zetasizer 3000 HS	Malvern Instruments GmbH, Germany
Software – PCS, ver. 1.61	Malvern Instruments GmbH, Germany
Cuvette – Einmal – Küvetten aus Polystyrol; 4.5 ml; 4 klare Seiten	Carl Roth GmbH, Germany
Membrane filter - Rotilabo® - Spritzenfilter, steril, 0.22 µm	Carl Roth GmbH, Germany

Zeta – potential

Zetasizer 3000 HS	Malvern Instruments GmbH, Germany
Software – PCS, ver. 1.61	Malvern Instruments GmbH, Germany
Membrane filter - Rotilabo® - Spritzenfilter, steril, 0.22 µm	Carl Roth GmbH, Germany

3.3 Preparing of liposomes, film method

3.3.1 Film formation

For the preparation of liposomes whose membrane is composed of diverse lipids, it is necessary to dissolve individual lipids first in an organic solvent. As an organic solvent, a mixture of chloroform and methanol in 9:1 ratio was used. The final lipid concentration was 10 µmol/ml. The lipid solutions were then mixed in a glass vial of 5 ml volume, according to the desired ratio of individual lipids in the prepared liposomes, so that the final total lipid amount was 10 µmol. Then, the organic solution was vaporized in a water bath heated to 50 °C, either by nitrogen stream or under vacuum. After the evaporation of solvent, a film of lipid mixture was

formed on the vial wall. Possible solvent residues were thoroughly evaporated in an exsiccator under vacuum, where the vial was left for one hour.

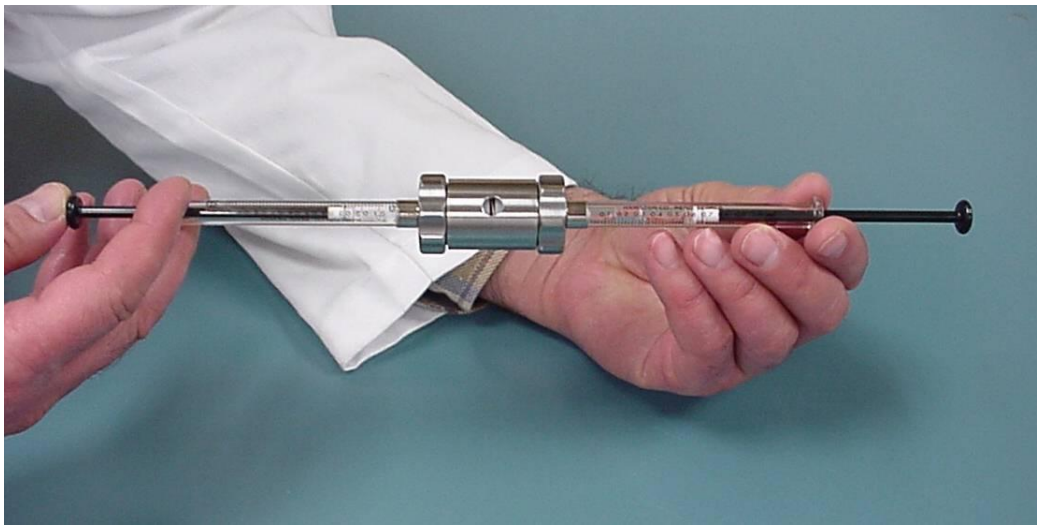
3.3.2 Hydration

A magnetic stirrer, a few glass beads and 1 ml of buffer were added to the dry film in the vial. As buffer, phosphate buffered saline (PBS; pH 7.4; sterile filtrated through a 0.22 µm filter; with the following composition: 8.0 g NaCl + 0.2 g KCl + 1.44 g Na_2HPO_4 x $2H_2O$ + 0.2 g KH_2PO_4 + H_2O ad 1000 ml) was used. The mixture was stirred with a magnetic stirrer for 30 minutes using the maximum velocity, unless the whole of the film was suspended in the buffer. Before the very extrusion, the suspension was left inactive for at least 30 minutes.

3.3.3 Extrusion

The extrusion was performed using the LiposoFast-Basic extruder (see Figure 10). Between the cylinders of the extruder, a membrane with pore size 200 nm is inserted, through which the lipid suspension is extruded. Each sample was extruded 41-times. After the extrusion, the suspension of large unilamellar liposomes was stored in Eppendorf cups. Before performing next tasks, the liposomes had to be left inactive for at least 30 minutes.

Figure 10 LiposoFast-Basic [38]



3.4 Photon correlation spectroscopy

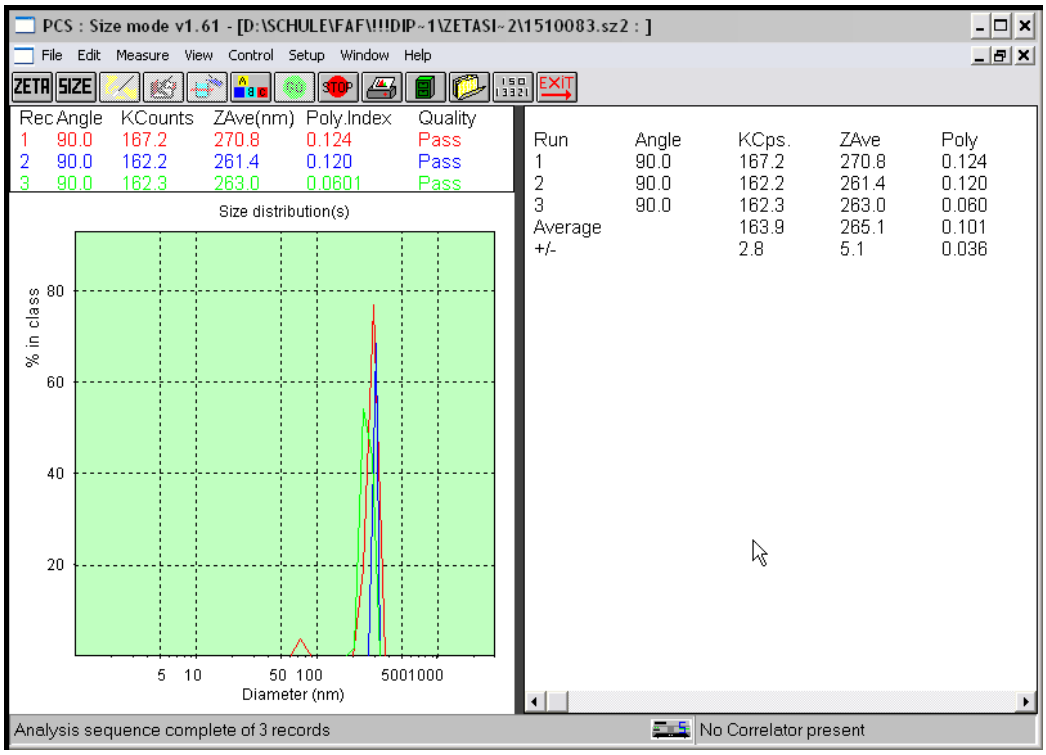
3.4.1 Sample preparation

The size of liposomes and the polydispersity of the sample was measured with a Zetasizer 3000HS. The samples were measured in cuvettes of 4.5 ml volume, made of polystyrene and having 4 pellucid walls. The sample was diluted by PBS, so that the final concentration of the sample counted 100 – 200 kilo counts per second (Kcps = collected photons per second divided by one thousand). This concentration was usually reached with dilution 1:200, or eventually 1:100. Before the buffer was used, it was filtered through a sterile filter with pore size 0.22 µm. The measuring was carried out at 30 °C, the intensity of the scattered light was measured under a 90° angle, the wavelength of the laser beam was 633 nm, and the refractive index of the PBS was 1.3357.

3.4.2 Model results

During a single measuring, each sample was measured 30-times. For each of the three tens, the software provided a single average, that is three averages for the whole measuring. From these three averages, the final average was calculated. As the results of each average, the software provided data about kilo counts per second, z – average and polydispersity (PI). The Z – average is the intensity weighted mean size of the particle population. 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. From the given data, the software projected also the graph of the particle size distribution in the sample (see Figure 11).

Figure 11 PCS software, results of measure



3.5 Zeta potential measurement

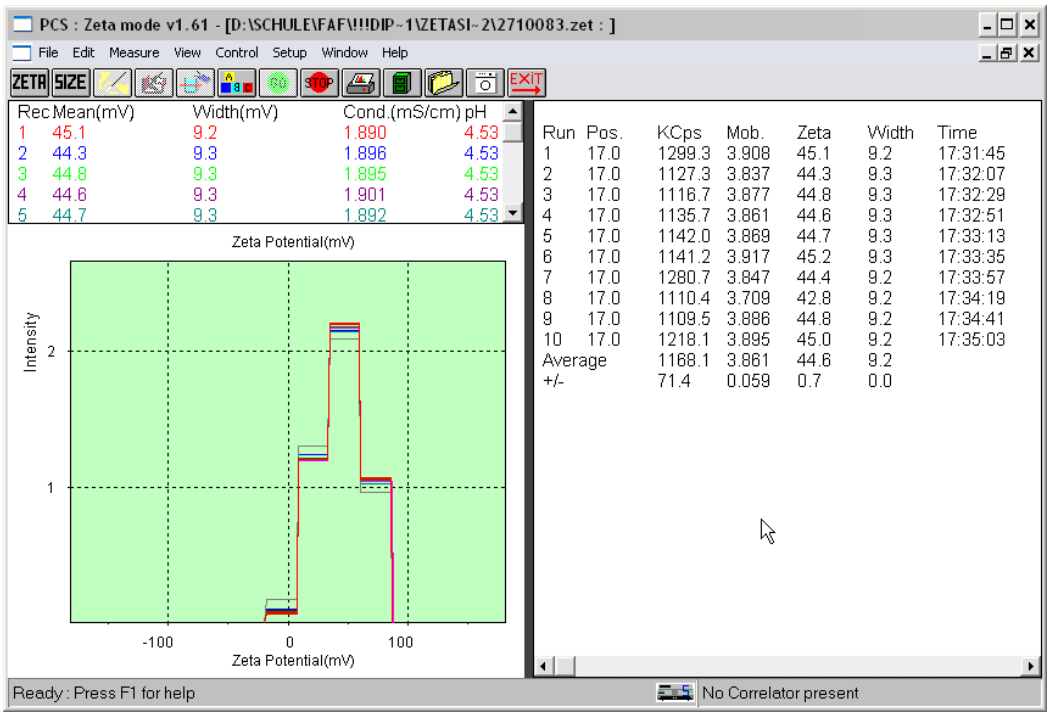
3.5.1 Sample preparation

Zeta potential was measured again with Zetasizer 3000HS. It was necessary to dilute the samples before the measuring, so that the final concentration would count 1000 – 2000 kilo counts per second, which usually matches 1:30 dilution. Before the buffers were used, they were filtered through a sterile filter with pore size 0.22 µm. With all samples, the measuring was made in three buffers – in PBS, pH 7,4 (for the composition see 3.3.2 Hydration), in a phosphate buffer (PTP, pH 7.0; composition: 0.534 g Na_2HPO_4 x $2H_2O$ + 0.272 g KH_2PO_4 + H_2O ad 1000 ml) and in a distilled water. The measuring was carried out under 30 °C in a capillary cell.

3.5.2 Model results

Within a single measuring, each sample was measured 10-times. The software then calculated the average zeta potential of the individual measurings. Most important were the data about kilo counts per second and Zeta in mV. From the data, the software projected a graph of zeta potential distribution in the sample (see Figure 12).

Figure 12 Zeta potential software, results of measurement



4 RESULTS and DISCUSSION

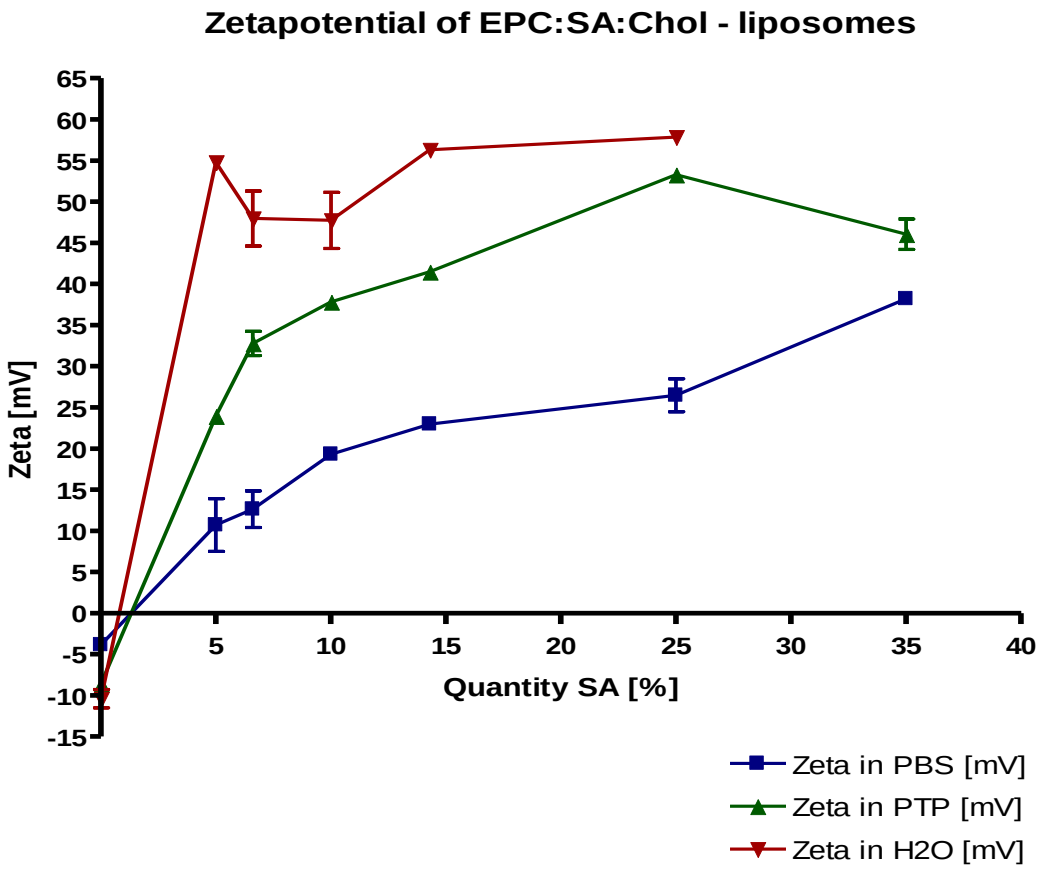
Size of each liposome was measured using PCS and zeta potential. All liposome types were prepared at least three times. The data were then averaged and arranged in tables and charts.

4.1 EPC:SA:Chol - liposomes

Table 2 EPC:SA:Chol - liposomes, results

Liposomes	SA [%]	Z ave [nm]	Poly	Zeta [mV]		
				in PBS	in PTP	in H2O
EPC:Chol 1:1	0.0	274.6	0.117	-3.9	-8.6	-10.4
EPC:SA:Chol 10:1:9	5.0	267.5	0.100	10.7	24.0	54.8
EPC:SA:Chol 15:2:13	6.6	287.7	0.163	12.6	32.8	48.0
EPA:SA:Chol 5:1:4	10.0	255.3	0.069	19.3	37.8	47.7
EPC:SA:Chol 7:2:5	14.3	227.6	0.106	23.0	41.5	56.3
EPC:SA:Chol 2:1:1	25.0	187.3	0.079	28.5	53.3	57.9
EPC:SA:Chol 10:7:3	35.0			38.2	46.1	

Graph 1 EPC:SA:Chol - liposomes, zeta potential



Liposomes prepared by extrusion through a membrane with 200 nm pores containing only EPC and Chol (in 1:1 ratio) had an average size of 274.6 nm. Due to the relatively high Chol content, the liposomal membrane was more fluid and the liposomes were larger than the pores of the membrane. After adding SA to the lipid mixture, extrusion through a membrane with the same pore aperture yielded liposomes of sizes between 267.5 nm (at 5% SA) and 187.3 nm (at 25% SA). The decreasing size can be explained by the falling Chol content, but also by the impact of SA, which prevents aggregation of liposomes due to its positive charge. The high intermolecular interactions of the stearylgroups increase the membrane rigidity and therefore reduce the size of the liposomes.

Measurement of zeta potential revealed that SA lends, due to its positive charge, the liposomes a positive zeta potential. This happens even at neutral pH (the measurements were carried in PBS of pH 7.4 and in PTP with pH 7.0). Water is not suitable as a medium for zeta potential measurements, as it has a low buffer capacity. This seems to have happened during measurements of the zeta potential of water-based liposomes at 35% SA content, which yielded completely meaningless values. Data from zeta potential measurements in the other two buffers were more significant. It was possible to confirm that zeta potential rises with the rising SA content in the liposomes. The zeta potential of SA-free liposomes was –3.9 mV in PBS and –8.6 mV in PTP. Adding SA to the mixture gradually increased the zeta potential, reaching 38.2 mV in PBS (35% SA) and 53.3 mV in PTP (25% SA). It was impossible to produce liposomes with even higher SA ratio through manual extrusion.

Increase of zeta potential upon addition of SA into a mixture of lipids was also observed by Biruss and Valenta [39], who measured the zeta potential of the liposome mixture DPPC[1]:Chol (7:2) 1.42 mV, and of the mixture DPPC:SA:Chol (7:2:2) 55.03 mV. The same study also investigated change of liposome parameters upon addition of progesterone. The above-mentioned liposomes then exhibited zeta potentials of 7.54 mV and 49.97 mV, respectively. Unfortunately, the authors did not mention the pH and buffers of their measurements. Liposomes with negative zeta potentials showed even larger differences after addition of progesterone. This favours the conclusion that

[1] DPPC = 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine

both the composition of the lipidic bilayer and of the interior of the liposome effect the zeta potential, because the substances of the interior can go in the exterior buffer and influence the zeta potential.

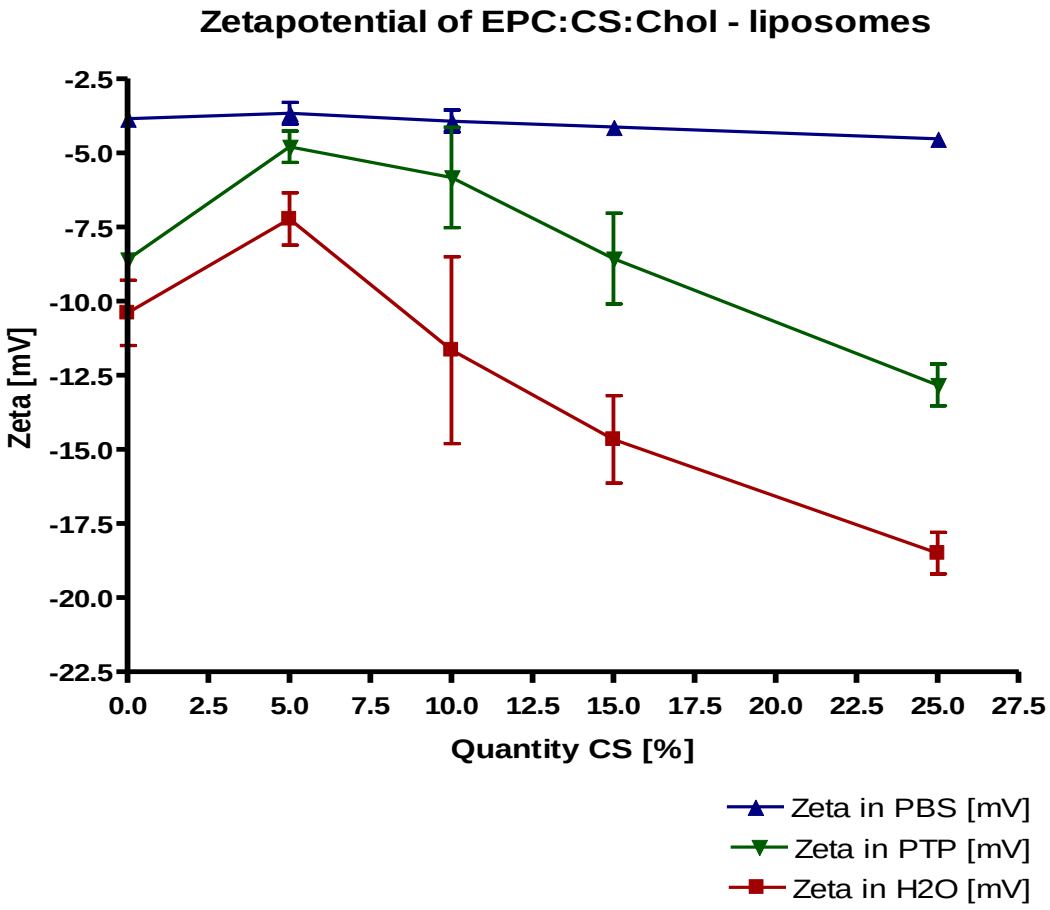
Zschöring and others [40] have shown that in liposomes of the EPC and SA composition, zeta potential increases sharply with the SA content only up to 20mol% of SA. Higher concentrations of SA cause saturation of zeta potential, which doesn't increase substantially afterwards. These measurements were carried out in a buffer with pH 7.4 and of the composition 3 mM HEPES + 0.1 M NaCl + 0.05 mM EDTA.

4.2 EPC:CS:Chol – liposomes

Table 3 EPC:CS:Chol - liposomes, results

Liposomes	CS [%]	Z ave [nm]	Poly	Zeta [mV]		
				in PBS	in PTP	in H2O
EPC:Chol 1:1	0.0	274.6	0.117	-3.9	-8.6	-10.4
EPC:CS:Chol 10:1:9	5.0	196.4	0.065	-3.7	-4.8	-7.2
EPA:CS:Chol 5:1:4	10.0	252.8	0.175	-4.0	-5.8	-13.2
EPC:CS:Chol 10:3:7	15.0	229.2	0.181	-4.1	-8.6	-14.7
EPC:CS:Chol 2:1:1	25.0	185.3	0.103	-4.5	-12.8	-18.5

Graph 2 EPC:CS:Chol - liposomes, zeta potential



Extrusions through the 200 nm-pored membrane produced liposomes of widely varying sizes: 196.4 nm (5% CS), 252.8 nm (10% CS), 229.2 nm (15% CS), and 185.3 nm (25% CS). In this case, no gradual shrinking of liposome sizes can be seen, unlike the liposomes with SA. Theoretically, such shrinking should still be taking place, but subsequent investigations suggested that not all CS is built into the liposomal membrane. Some CS is apparently bound, due to its negative charge, only to the surface of the liposome. This would also explain larger sizes and polydispersity of liposomes with 10 and 15 per cent content of CS. It is very likely that the reduction of size in the formulation with more than 10% CS is caused by the building of mixed micelles, which are in the size around 30 nm.

The measurements of zeta potential confirmed that the negative charge of CS also affects the zeta potential of the liposomes in whose membrane it is caught. The constant value around –4 mV measured in PBS even at rising CS content might be a result of the zeta potential of the liposomes being suppressed by the relatively strong buffer with high ion concentration. In contrast, measurements in both PTP and water confirmed that increasing content of negatively charged CS raises the negative charge of the liposomes up to -12.8 mV in PTP and -18.5 mV in water (both at 25% CS). Again, it was impossible to prepare liposomes with CS content higher than 25% through manual extrusion.

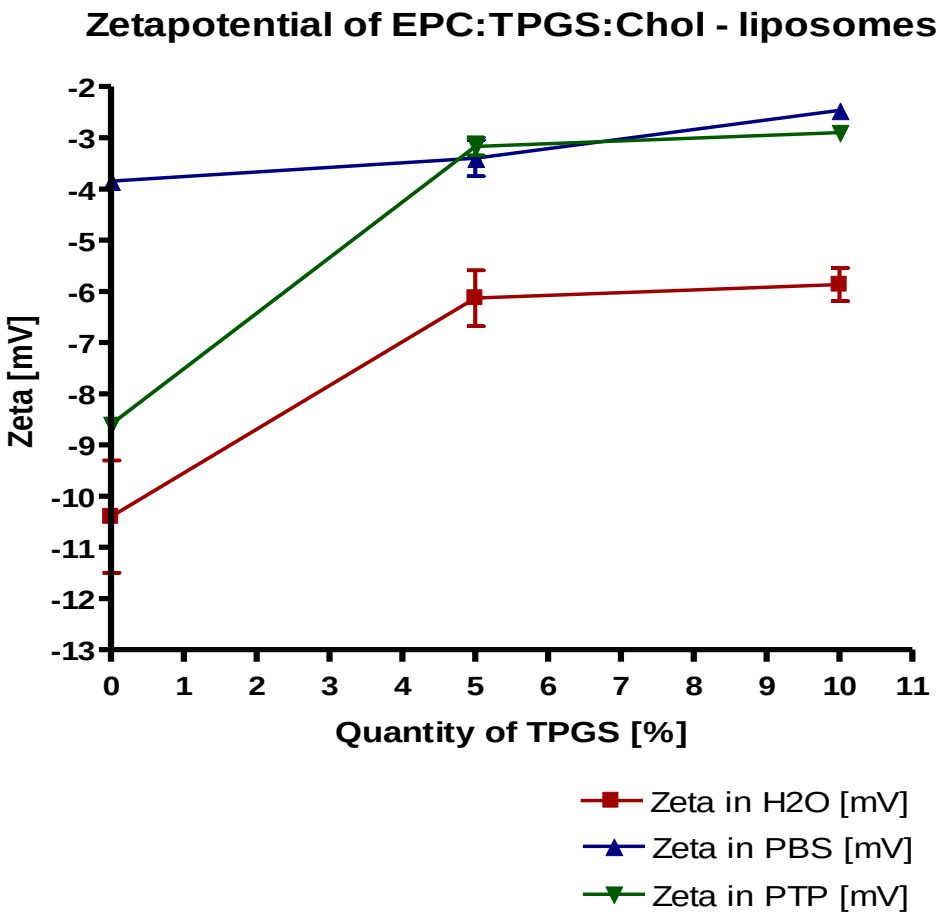
Negative zeta potential of liposomes containing bile acids, in particular the deoxycholic acid (DA), was also shown by Fang and others [41]. For liposomes containing EPC, Chol and 5% of DA, they measured a zeta potential of -18.3 mV. They also confirmed that the zeta potential is to a large extent dependent on the composition of the liposomal membrane. For liposomes containing beside EPC and Chol also 5% diacetyl phosphate (DP), which is negatively charged, they measured the zeta potential -66.0 mV, for liposomes containing EPC, Chol and 5% SA the zeta potential 68.6 mV. However, authors did not indicate the pH and buffer of the measurements. Studies of the zeta potential of liposomes containing CS are unfortunately not available.

4.3 EPC:TPGS:Chol – liposomes

Table 4 EPC:TPGS:Chol - liposomes, results

Liposomes	TPGS [%]	Z ave [nm]	Poly	Zeta [mV]		
				in PBS	in PTP	in H2O
EPC:Chol 1:1	0.0	274.6	0.117	-3.9	-8.6	-10.4
EPC:TPGS:Chol 10:1:9	5.0	190.7	0.079	-3.4	-3.2	-6.1
EPA:TPGS:Chol 5:1:4	10.0	190.9	0.058	-2.5	-2.9	-5.9

Graph 3 EPC:TPGS:Chol - liposomes, zeta potential



Extrusions through the 200 nm-pored membrane produced liposomes with TPGS of the sizes 190.7 nm (5% TPGS) and 190.9 nm (10% TPGS). We thus succeeded in producing liposomes 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 than liposome containing only EPC and Chol. The first probable cause is the decreasing amount of Chol, translating into decreasing fluidity of the liposomal membrane. The second is, that the PEG part of the TPGS is rather big and forms a layer around the liposomal membrane itself. As the refractive index of the PEG is pretty close to that of water, it might be hard to see in the PCS. This is may be another reason, why the TPGS reduces the size of the liposomes. Using TGPS, strongly monodisperse populations of liposomes were successfully prepared, as attested by low values of polydispersity (0.079 and 0.058, respectively).

TPGS is an amphiphilic substance, a nonionic surfactant consisting of a lipophilic chain of tocopherol succinate and a polar chain of polyethylene glycol (PEG). PEG is not built into the liposomal membrane, it stretches into the surrounding space, increasing friction at the liposome surface. This also enlarges the Stern layer of charges firmly bound to the liposome surface, shifting it towards the solution. All this decreases the zeta potential. The values measured in PBS were –3.4 mV (5% TPGS) and –2.5 mV (10% TPGS), those in PTP were –3.2 mV and –2.9 mV, and in water –6.1 mV and –5.9 mV, respectively. All these zeta potential values are lower than the values of TPGS-free liposomes under the same conditions.

TPGS has only been used in liposome preparation for a short time and no remarks about its influence on zeta potential could be found in the literature. On the other hand, Yoshioka [42] proved that PEG bound to a phospholipid or cholesterol affects the value of zeta potential. The longer the chain of the PEG used, the more the liposomal zeta potential dropped.

Different effects of PEG on zeta potential were shown by Arnold and others [43]. If PEG is only contained in the solution and not directly bound to the liposomal membrane, it raises markedly the value of the zeta potential. If, on the other hand, it is bound directly to the liposome surface via a surfactant built into the liposomal membrane, friction at the surface of the liposome increases and the zeta potential falls.

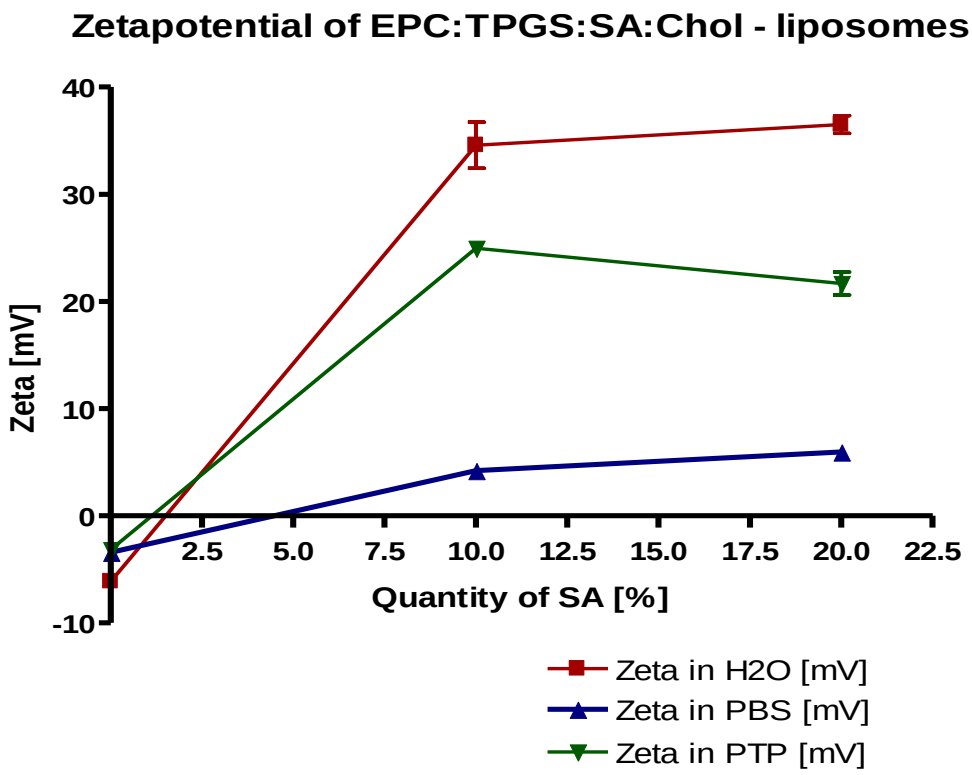
4.4 EPC:TPGS:SA:Chol and EPC:TPGS:CS:Chol – liposomes

In all of these cases, the liposomes prepared contained 5% TPGS.

Table 5 EPC:TPGS:SA:Chol - liposomes, results

Liposomes	SA [%]	Z ave [nm]	Poly	Zeta [mV]		
				in PBS	in PTP	in H2O
EPC:TPGS:Chol 10:1:9	0.0	190.7	0.079	-3.4	-3.2	-6.1
EPC:TPGS:SA:Chol 10:1:2:7	10.0	175.6	0.096	4.2	25.0	34.6
EPA:TPGS:SA:Chol 10:1:4:5	20.0	140.3	0.154	6.0	21.7	36.5

Graph 4 EPC:TPGS:SA:Chol - liposomes, zeta potential



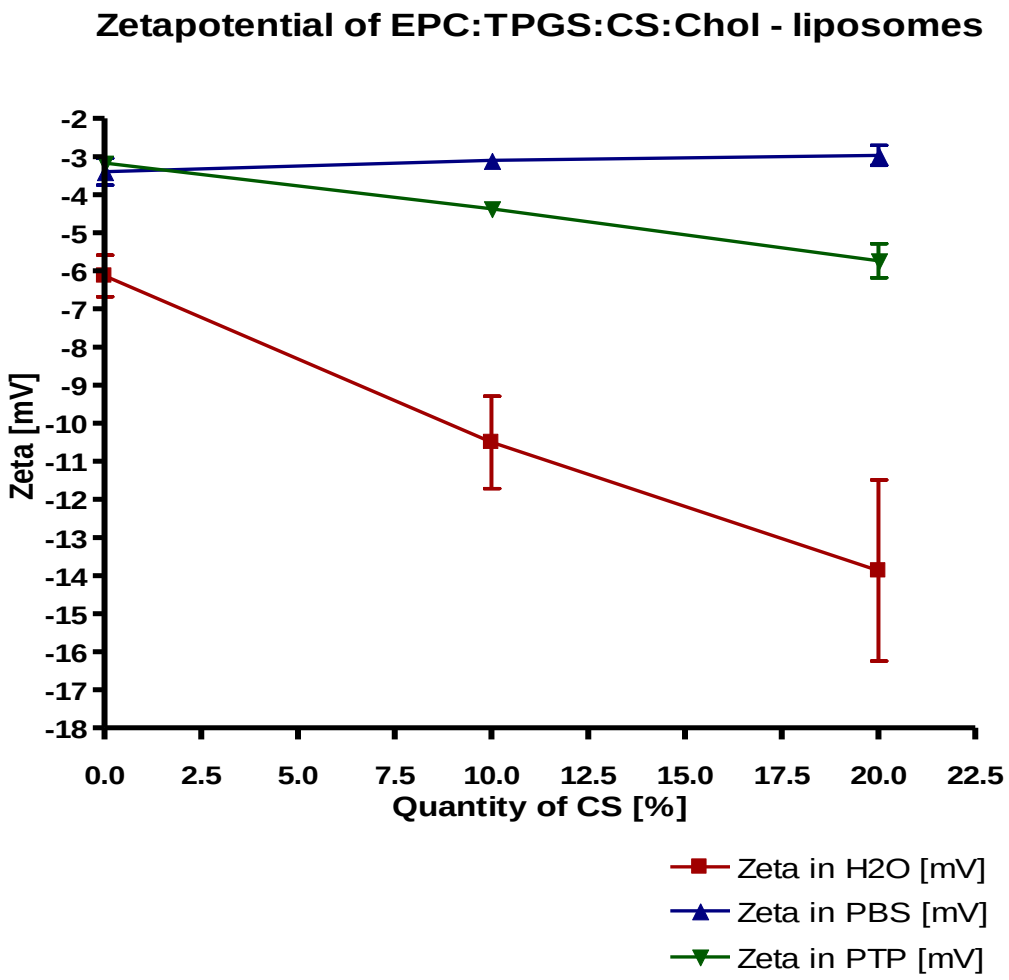
Measurements again confirmed that adding SA to the liposomes reduces their size due to the positive charge of SA. For liposomes containing beside EPC and Chol also 5% of TPGS and 10% or 20% of SA, average sizes were 175.6 nm and 140.3 nm, respectively. The positive charge of SA influenced the zeta potential, which reached positive values in all three buffers, namely 6.0 mV in PBS (20% SA), 25.0 mV in PTP (10% SA), and 36.5 mV in water (20% SA).

Similar results were obtained for liposomes of the EPC:TPGS:CS:Chol formula.

Table 6 EPC:TPGS:CS:Chol - liposomes, results

Liposomes	CS [%]	Z ave [nm]	Poly	Zeta [mV]		
				in PBS	in PTP	in H2O
EPC:TPGS:Chol 10:1:9	0.0	190.7	0.079	-3.4	-3.2	-6.1
EPC:TPGS:CS:Chol 10:1:2:7	10.0	167.1	0.075	-4.4	-4.4	-10.5
EPA:TPGS:CS:Chol 10:1:4:5	20.0	179.7	0.077	-3.0	-5.7	-13.9

Graph 5 EPC:TPGS:CS:Chol - liposomes, zeta potential

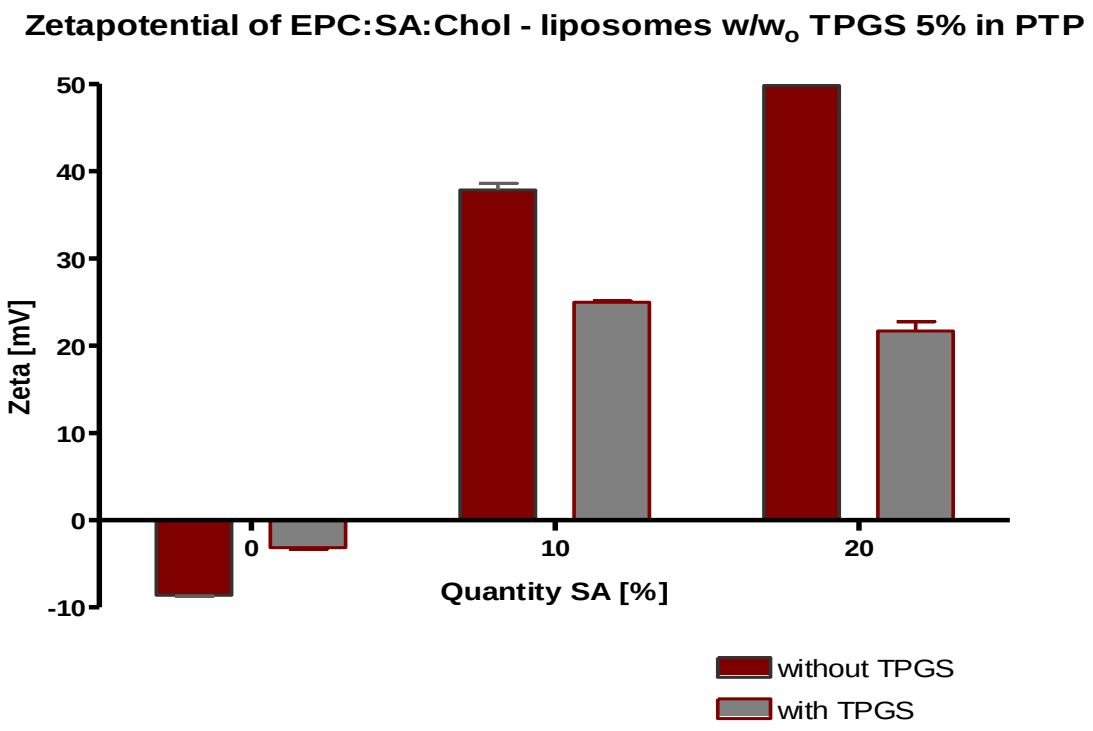


It was confirmed in these cases, too, that adding CS to liposomes causes their size to shrink. Just as with liposomes only containing EPC, CS and Chol, here too the shrinking was not gradual as could be expected according to theory. It was again not possible to clarify whether this was due to a part of CS not built directly into the liposomal membrane but instead only bound by its negative charge to the surface of the liposome. Using PCS, the average sizes of liposomes measured were 167.1 nm (10% CS) and 179.7 nm (20% CS). The negative charge of CS also manifested itself in the values of zeta potential, which shifted to negative territory in contrast to CS-free liposomes. Values as negative as -5.7 mV in PTP (20% CS) and –13.9 mV in water (20% CS) were measured. Paradoxical values were obtained from measurements in PBS. The zeta potential of liposomes containing only EPC, 5% TPGS and Chol measured in this buffer was –3.4 mV. Adding 10% CS changed the value to –4.4 mV, whereas liposomes with

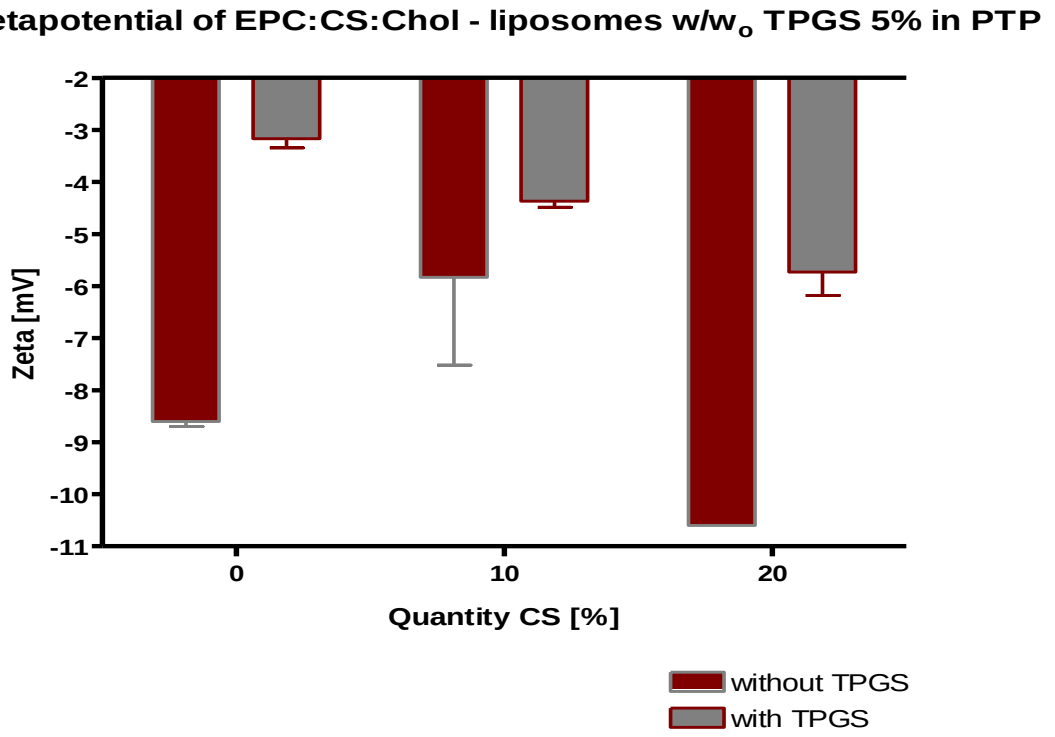
20% CS exhibited zeta potential of only –3.0 mV. We can again only speculate that a part of the CS was only bound to the surface of the liposomes by its negative charge, which was however suppressed by the buffer (similarly to liposomes only containing EPC, CS and Chol), thus making the role of PEG more prominent. The CS has under the chosen conditions no influence on the potential.

The impact of TPGS, or rather its hydrophilic part PEG, on the zeta potential of liposomes containing EPC, TPGS, Chol and SA vs. EPC, TPGS, Chol and CS can best be demonstrated by comparison of the zeta potential of these liposomes with liposomes containing EPC, SA, Chol and PC, CS, Chol with the same amount of SA and CS (i.e. 0, 10, and 20%).

Graph 6 EPC:SA:Chol – liposomes with and without TPGS, comparision of zeta potentials



Graph 7 EPC:CS:Chol - liposomes with and without TPGS, comparision of zeta potentials



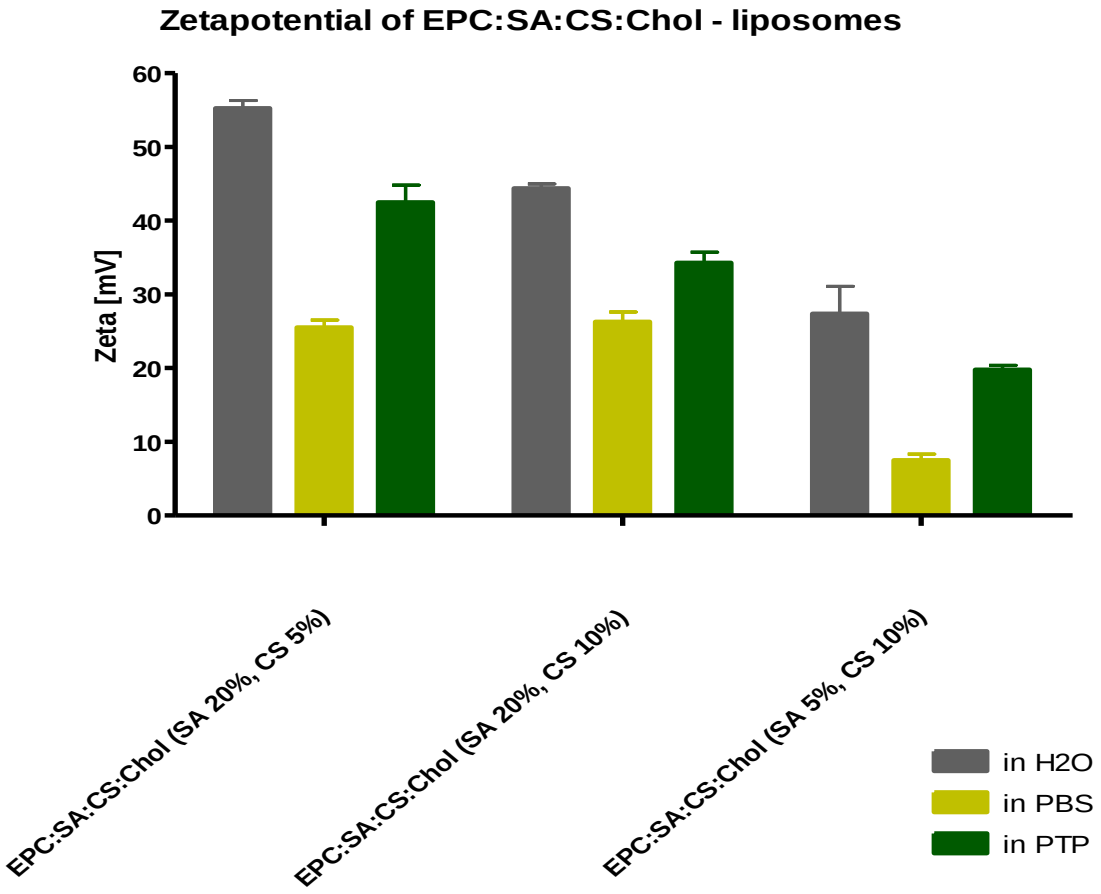
Similar to liposomes only containing EPC, TPGS and Chol, in this case as well PEG suppressed the zeta potential. PEG masks, so to speak, the zeta potential caused by the charge of SA or CS. This corroborates the conclusions reached in the studies of Yoshioka and Arnold and others (see 4.3 EPC:TPGS:Chol – liposomes).

4.5 EPC:SA:CS:Chol – liposomes

Table 7 EPC:SA:CS:Chol - liposomes, results

Liposomes	SA [%]	CS [%]	Z ave [nm]	Poly	Zeta [mV]		
					in PBS	in PTP	in H2O
EPC:SA:CS:Chol 10:4:1:5	20.0	5.0	155.8	0.198	25.5	42.5	55.2
EPC:SA:CS:Chol 5:2:1:2	20.0	10.0	151.1	0.122	26.3	34.3	44.4
EPA:SA:CS:Chol 10:1:2:7	5.0	10.0	190.6	0.088	7.5	19.8	27.4

Graph 8 EPC:SA:CS:Chol - liposomes, zeta potential



Extrusions through the 200 nm-pored membrane produced liposomes with sizes of 155.8 nm (20% SA, 5% CS), 151.1 nm (20% SA, 10% CS) and 190.6 nm (5% SA, 10% CS). Presence of SA and CS again affected the size of the liposome, liposomes with higher amounts of SA and CS being much smaller than liposomes with higher amount of Chol at the expense of SA and CS. This is because Chol increases fluidity of the membrane, which then produces larger liposomes.

Zeta potential measurements showed that SA can balance the negative charge of CS, even to the extent of completely covering it. Positive zeta potential was measured in all buffers, although the last group of liposomes contained higher amount of the negatively charged CS (10%) than of the positively charged SA (5%). This shows that it is possible to prepare liposomes with a positive zeta potential containing negatively charged substances in their membranes, such as bile acids, that increase permeability

of liposomes through biological membranes. Small amounts of SA are sufficient to achieve a positive zeta potential, which is welcome as it is relatively highly toxic.

Unfortunately, no literature was found dealing with joint applications of bile acids and cationic lipids in liposomes and their impact on zeta potential. These conclusions thus cannot be compared with results of other studies.

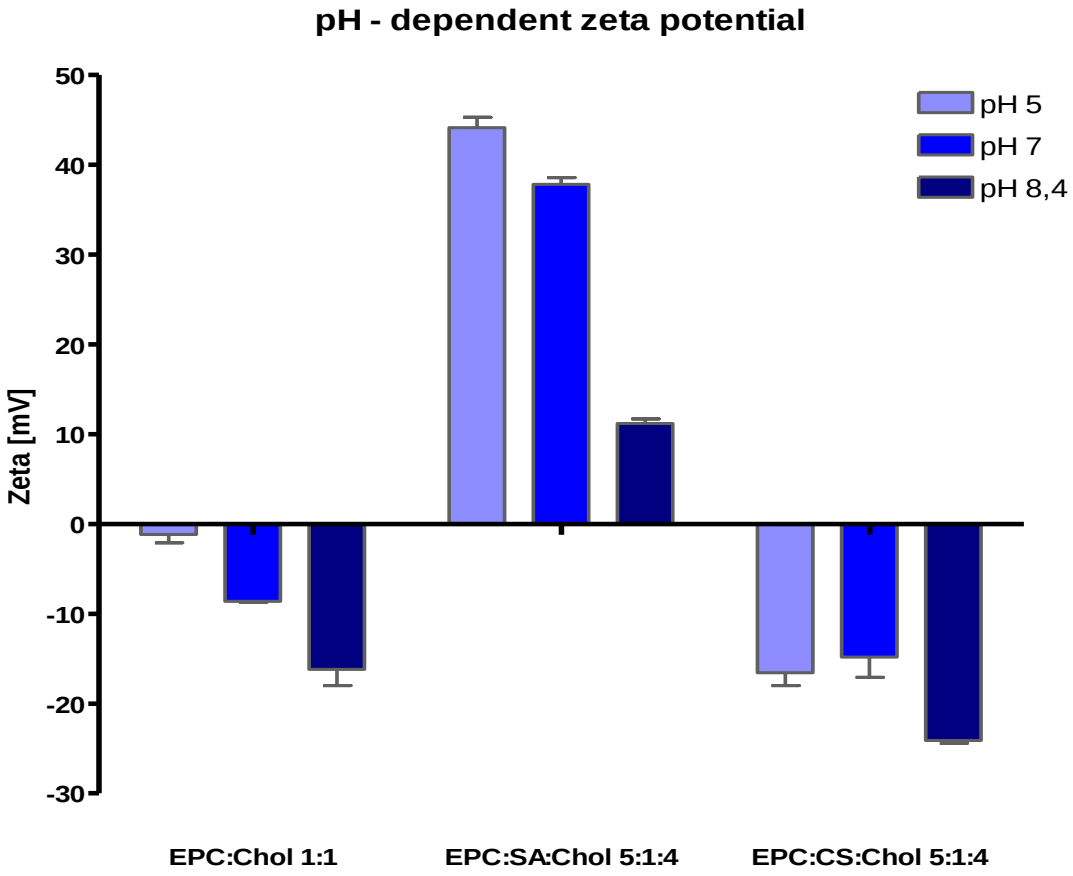
4.6 Influence of pH on zeta potential

Another problem worth notice is the impact of different pH on the zeta potential of identical liposomes. Measurements were carried out in PTP with pH set at 5.0, 7.0 and 8.4, respectively.

Table 8 pH - dependent zeta potential, results

Liposomes	Zeta [mV] in PTP		
	pH 5.0	pH 7.0	pH 8.4
EPC:Chol 1:1	-1.2	-8.6	-16.2
EPC:SA:Chol 5:1:4	44.1	37.8	11.2
EPC:CS:Chol 5:1:4	-16.6	-14.8	-24.1

Graph 9 pH - dependent zeta potential, in PTP



The influence of pH on zeta potential was observed with three different liposome types. It was possible to confirm that an acid pH shifts the zeta potential towards positive values and, conversely, a basic pH shifts the zeta potential towards negative values. Values measured for the liposomes EPC:Chol (1:1) were –1.2 mV (pH 5.0), -8.6 mV (pH 7.0), and –16.2 mV (pH 8.4). For the liposomes EPC:SA:Chol (5:1:4), zeta potential was 44.1 mV (pH 5.0), 37.8 mV (pH 7.0), and 11.2 mV (pH 8.4). Only liposomes of the composition EPC:CS:Chol (5:1:4) produced results at odds with the hypothesis. Zeta potential was more negative at pH 5.0 (-16.6 mV) than at pH 7.0 (-14.8 mV). At pH 8.4, the most negative value was measured as expected –24.1 mV. It can only be speculated whether this was again the influence of CS not being built into the liposomal membrane.

The hypothesis that acid pH shifts zeta potential towards positive values and basic towards negative values was also confirmed by Son and others [44], who studied changes of zeta potential of liposome/DNA complexes under changes of ionic strength

and pH. They reached this conclusion for complexes of both positive and negative zeta potential.

Svensson and others [45] studied the impact of pH on electrophoretic mobility and zeta potential for liposomes whose surface was modified by chitosane. For these particles, the zeta potential they measured at pH 3 was 16.0 mV against -7.0 mV at pH 8. The pH value was also important for interactions of these liposomes with mucine.

5 CONCLUSIONS

During the experimental stage of this work, it was achieved by manual extrusion to prepare liposomes of varying size, thus proving the presupposition that the liposome size significantly depends on its membrane composition. Higher cholesterol rate increases the fluidity of liposomal membrane, which causes formation of liposomes bigger than the membrane pores of the extruder; on the contrary stearylamine and cholylsarcosine cause smaller liposome size by their charges. TPGS also causes smaller size of liposomes, probably because of its PEG chains which increase liposome stability, inhibiting their aggregation, and so the liposomes may look smaller on the PCS.

It was achieved to prove the influence of SA, CS and TPGS on liposome zeta potential as well. SA with its positive charge induces positive zeta potential in liposomes, already in very small concentrations. Similarly, CS with its negative charge induces negative zeta potential. In liposomes containing both SA and CS, SA suppresses the negative charge of CS and so these liposomes show positive zeta potential. That is a particularly important discovery because it proves the possibility of preparing liposomes with a small amount of SA (5%) that show positive zeta potential together with the favourable features of CS (the ability to form complexes with cations, thus improving their absorption). Liposomes with positive zeta potential have far broader application in use; furthermore they are not taken up so fast by cells of reticuloendothelial system. TPGS causes reduction of zeta potential; it is possible to say that it disguises it. This is caused mainly by PEG chains which push the border of the Stern layer into the exterior, causing the measured zeta potential to be lower than in equal liposomes without PEG.

The next issue of this project was the influence of pH on zeta potential as zeta potential is an important indicator of liposome stability and pH changes rapidly in the gastrointestinal tract after administration by mouth. As expected, it was proved that an acid pH shifts liposome zeta potential towards positive values and basic pH towards negative values.

It could be shown that the researched substances, which have been used in this field only recently and rarely, are applicable in liposome preparation. With them it is

possible to achieve such surface properties as with commonly used phospholipids or bile acids, and some of their qualities are even better (e.g. lower toxicity of CS compared to bile acids [30]). A significant advantage is also their lower price which could become an important criterion for choosing them.

In studying the surface properties of liposomes prepared this way, we have made only the first step. The whole research should lead towards administrating peptide drugs (e.g. insulin or calcitonin) by mouth, liposomes serving as their protective sheath which also improve their absorption from the gastrointestinal tract. The next goal could therefore be studying interactions between those liposomes and biological membranes, first on artificial membranes with defined pore size and then on model cell membranes. Another step would be examining the liposomes' stability and the amount of drugs they are able to hold inside. It is also necessary to research possibilities of transforming the liposomes into a stable form, because freshly prepared liposomes stored only in buffers eventually succumb to degradation. This can be avoided by freeze drying; after that, the liposomes can be reconstituted by adding a suitable buffer or dissolvent in which they would be given to patients. Or it is also desirable to encapsulate the dried liposomes in capsules and to apply those capsules orally to the patient. Further research could look into conditions of freeze drying and consequent changes in the liposomes and drugs contained inside them.

6 SOUHRN

Perorální podání je nejpohodlnější aplikace léčiv. Již několik let probíhají mnohé výzkumy jak podávat perorálně například inzulín nebo kalcitonin. Bohužel mnohé látky, zejména bílkovinné nebo peptidické povahy, nejsou schopny vydržet agresivní podmínky, které se vyskytují v trávicí soustavě (GIT), jako je velmi rozdílné pH, působení kyselin nebo trávicích enzymů. Dochází tak k jejich degradaci ještě dříve, než se vůbec mohou vstřebat a začít terapeuticky působit. Tyto látky se kvůli velikosti své molekuly nebo náboji často ani nevstřebávají. Vyvstává tedy potřeba tyto látky chránit a zlepšit jejich absorpci. A jako vhodnými kandidáty na tuto funkci se jeví lipozómy.

Lipozómy jsou částice s fosfolipidovou dvouvrstevnou membránou, kterou lze připravit nejen ze široké škály fosfolipidů, ale i z mnohých povrchově aktivních látek a solí žlučových kyselin. Díky tomu lze připravit lipozómy s velmi různorodými vlastnostmi, které na složení membrány do jisté míry závisí, velikostí, povrchovým nábojem (zeta potenciálem), s různým charakterem povrchových skupin. Různé lipozómy mají i po podání do organismu různý osud, některé jsou rychleji vychytávány buňkami retikuloendoteliárního systému, některé jsou prioritně vychytávány mozkovými buňkami, jiné lépe pronikají střevní stěnou. Pro perorální podání je zvláště důležitá odolnost vůči agresivním podmínkám GIT a schopnost difundovat nebo se vstřebávat do střevní stěny. Tyto vlastnosti bohužel mnohé lipozómy nemají, spíše naopak, i lipozómy jsou v GIT často degradovány dříve, než dosáhnou svého cíle, proto neustále probíhá výzkum nových i známých kombinací již tradičních surovin, či se zkouší látky úplně nové.

Cílem této práce bylo připravit lipozómy s přídavkem v praxi zřídka používaných surovin, konkrétně cholylsarkosinu (CS), stearylaminu (SA) a d-α-tokoferolsukcinátu(TPGS), a zjistit některé jejich povrchové vlastnosti, které jsou rozhodující pro další použití lipozómů. Připravené lipozómy vždy obsahovaly fosfatidylcholin z vaječného bílku (EPC), cholesterol (Chol) a jednu nebo více výše uvedenou zkoumanou substanci. Naměřené údaje byly porovnávány s výsledky získanými za stejných podmínek u lipozómů o složení EPC:Chol (1:1).

Lipozómy byly připravovány filmovou metodou a následnou hydratací a ruční extruzí přes membránu s definovanou velikostí pórů 200 nm. Dále byla měřena jejich velikost a polydisperzita a nakonec zeta potenciál ve dvou pufrech a v destilované vodě.

Velikost lipozómů byla měřena pomocí Zetasizeru 3000 HS (Malvern). Při tomto měření byla navíc získávána i polydisperzita, která vypovídá o šíři distribuce velikosti lipozómů. Dále bylo pracováno pouze se vzorky, jejichž polydisperzita byla menší než 0,2. Velikost lipozómů závisí především na použitých substancích, na jejich náboji nebo rigiditě molekuly, dále na velikosti pórů membrány použité při extruzi, iontové síle použitého média a povrchové struktuře lipozómů.

U lipozómů složených pouze z EPC a Chol byla naměřena průměrná velikost 274,6 nm. Všechny ostatní lipozómy se vykazovaly velikostí menší, konkrétně u lipozómů se SA byla naměřena velikost až 187,3 nm (EPC:SA:Chol 2:1:1), s CS 185,3 nm (EPC:CS:Chol 2:1:1), s TPGS 190,7 nm (EPC:TPGS:Chol 10:1:9). Stejný trend se projevil i u lipozómů s kombinací čtyř substancí, opět konkrétně 140,3 nm (EPC:TPGS:SA:Chol 10:1:4:5), 167,1 nm (EPC:TPGS:CS:Chol 10:1:2:7) a 151,1 nm (EPC:SA:CS:Chol 5:2:1:2). Velkou velikost lipozómů, jejichž membrána byla pouze z EPC a Chol, lze považovat za důsledek poměrně velkého obsahu Chol, který zvyšuje fluiditu lipozomální membrány a při extruzi tak vznikají lipozómy větší. Zmenšující se velikost po přidání různých substancí má různá vysvětlení. Jedním, společným pro všechny případy, je fakt, že složení lipozomální membrány se měnilo vždy na úkor Chol. Ve všech lipozómech byl stále stejný podíl EPC, ale podíl Chol vždy klesal se vzrůstajícím množstvím přidávané zkoušené substance. Tím tedy i klesala fluidita lipozomální membrány a extruzí byly získávány lipozómy o menší velikosti. I zkoušené látky ale působily na velikost lipozómů svými vlastnostmi. SA a CS nesou náboj, SA kladný, CS záporný. Tento náboj se projevuje i na lipozomálním povrchu, lipozómy se pak navzájem odpuzují, je tak zabráněno jejich agregaci a na PCS se mohou jevit jako menší. Součástí molekuly TPGS je polyethylenglykol (PEG), který je v ní vázán esterovou vazbou. Je – li molekula TPGS zabudována do lipozomální membrány, řetězce PEG trčí ven směrem do rozpouštědla, čímž se molekula na PCS může jevit jako větší, neboť se tak snižuje její hybnost. PEG také zabraňuje agregaci lipozómů. Zmenšení velikosti lipozómů tak nejspíš bude

důsledkem sníženého obsahu Chol a antiagregačního působení PEG, které převládají nad zvětšujícím efektem PEG vlivem snížení hybnosti lipozómů.

Další povrchovou vlastností lipozómů, která byla zkoumána, byl zeta potenciál. Zeta potenciál je náboj lipozómu, který částice získává ve vodném médiu. Není to ale náboj přímo na povrchu lipozómu, ale náboj na povrchu jeho solvatačního obalu, tzv. Nernstův potenciál. Závisí na povaze povrchu částice a na vlastnostech média, ve kterém je částice dispergována. Měření vždy probíhalo ve dvou fosfátových pufrech a v destilované vodě. Fosfátovými pufry byly PBS (Phosphate Salined Buffer) o pH 7,4 a PTP (Phosphat-Trennpuffer) o pH 7,0. Zeta potenciál byl měřen pomocí Zetasizeru 3000 HS (Malvern). Opět byly získané hodnoty porovnávány s výsledky lipozómů o složení EPC:Chol 1:1, u kterých byl naměřen zeta potenciál -3,9 mV (v PBS), -8,6 mV (v PTP) a -10,4 mV (ve vodě).

Zkoumané substance ovlivňovaly svými vlastnostmi i zeta potenciál. SA nese kladný náboj, který posunul zeta potenciál do kladných hodnot – konkrétně byly dosaženy až hodnoty 38,2 mV (v PBS), 53,3 mv (v PTP) a 57,9 mV (ve vodě). Zeta potenciál postupně stoupal se stoupajícím obsahem SA v lipozómech. Při vyšších koncentracích SA (nad 20 mol%) ale dochází k nasycení zeta potenciálu, se zvyšujícím se podílem SA se hodnota zeta potenciálu již příliš nemění.

CS je sůl žlučové kyseliny, nese záporný náboj a zeta potenciál posouvá do hodnot záporných. Teoreticky by se zeta potenciál měl postupně snižovat se zvyšujícím se podílem CS (obdobně jako u SA), ale naměřené hodnoty tomuto předpokladu úplně neodpovídaly. V průběhu dalších měření se objevil problém, že se zřejmě jen část CS váže do lipozomální membrány a část je navázána na povrchu lipozómů jen díky svému náboji. To by mohlo vysvětlovat vlnovitý tvar křivky závislosti zeta potenciálu na množství CS v lipozómech. Tuto hypotézu se ale v průběhu experimentální části této práce již nepodařilo potvrdit ani vyvrátit. Jako nepříliš vhodný pro měření zeta potenciálu u lipozómů s CS se ukázal PBS, což je pufr poměrně silný a zřejmě dokázal náboj CS potlačit. V PBS byla naměřena největší hodnota zeta potenciálu pouze -4,5 mV. V ostatních médiích byly naměřeny největší hodnoty zeta potenciálu -12,8 mV (v PTP) a -18,5 mV (ve vodě).

TPGS také ovlivňuje zeta potenciál. Hlavně jeho řetězec z PEG. Ten není zabudován do lipozomální membrány, trčí z ní ven do rozpouštědla a posouvá hranici Sternovy vrstvy směrem od lipozómu. To vede ke zmenšení zeta potenciálu. U lipozómů s TPGS tedy byly naměřeny hodnoty zeta potenciálu -2,5 mV (v PBS), - 2,9 mV (v PTP) a -5,9 mV (ve vodě). Podobně se vliv TPGS na zeta potenciál projevil i u lipozómů, jejichž membrána byla složena ze EPC, Chol, TPGS a SA nebo CS. Bylo připraveno několik druhů lipozómů, které vždy obsahovaly 50 mol% EPC, 5 mol% TPGS a proměnlivý obsah Chol a SA nebo CS. U všech vzorků byl naměřený zeta potenciál menší než zeta potenciál lipozómů bez TPGS, které jinak měly stejné složení membrány a byly měřeny ve stejných pufrech za stejných podmínek. Dá se tedy říci, že TPGS zeta potenciál lipozómů maskuje.

Dalším zkoumaným problémem byl vliv SA a CS, které byly společně v lipozomální membráně. Měření zeta potenciálu ukázalo, že SA je schopen vyvažovat negativní náboj CS, dokonce jej i úplně zakrýt. Ve všech pufrech byl naměřen kladný zeta potenciál, i když poslední připravená skupina lipozómů obsahovala větší podíl negativně nabitého CS (10%) než podíl kladně nabitého SA (5%). Ukázalo se tedy, že lze připravovat lipozómy s kladným zeta potenciálem, které ve své membráně obsahují např. negativně nabité soli žlučových kyselin, které zvyšují prostupnost lipozómů skrz biologické membrány. Na dosažení kladného zeta potenciálu navíc stačí velmi málo SA, což je důležité zjištění vzhledem k jeho poměrně vysoké toxicitě.

Dalším problémem, který stál za povšimnutí, byl vliv různého pH na zeta potenciál stejných lipozómů. Měření bylo prováděno v PTP, jehož pH bylo upraveno na hodnoty 5,0; 7,0 a 8,4. Byl pozorován vliv pH na zeta potenciál u tří různých druhů lipozómů. Podařilo se potvrdit, že kyselé pH posouvá zeta potenciál do kladnějších hodnot a naopak zásadité pH posouvá zeta potenciál k hodnotám záporným. Konkrétně u lipozómů o složení EPC:Chol (1:1) byly naměřeny hodnoty -1,2 mV (pH 5,0), -8,6 mV (pH 7,0) a -16,2 mV (pH 8,4). U lipozómů EPC:SA:Chol (5:1:4) pak 44,1 mV (pH 5,0), 37,8 mV (pH 7,0) a 11,2 mV (pH 8,4). Pouze lipozómy o složení EPC:CS:Chol (5:1:4) poskytly výsledky, které daný předpoklad nepotvrdily. Zeta potenciál při pH 5,0 byl zapornější (-16,6 mV) než zeta potenciál při pH 7,0 (-14,8 mV). Při pH 8,4 ale byla naměřena očekávaná nejzápornější hodnota -24,1 mV. Zda se opět projevil vliv CS, který nebyl zabudován do lipozomální membrány, lze pouze spekulovat.

Podařilo se tedy prokázat, že zkoumané substance, které se v této oblasti používají poměrně řídce a krátkou dobu, jsou vhodné k přípravě lipozómů. Lze s nimi dosáhnout takových povrchových vlastností, které vykazují tradičně používané fosfolipidy nebo žlučové kyseliny, mnohdy je svými vlastnostmi i předčí (např. nižší toxicita CS oproti klasickým žlučovým kyselinám). Nezanedbatelnou výhodou je i jejich nižší cena, která může být v jejich budoucím použití v praxi jedním z důležitých kritérií pro jejich volbu. K perorálnímu podání bílkovinných léčiv ale ještě vede dlouhá cesta, na níž bylo touto prací uděláno jen pár prvních kroků.

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