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# "Design, development and evaluation of liposomal nanoparticles. In vitro toxicity and permeability study with Transwell methodology."

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**ABBREVIATIONS**

| | |
|---|---|
| HSPC | Hydrogenated Soy Phosphatidylcholine |
| EPC | Egg Phosphatidylcholine |
| DPPG | 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) |
| DOTAP | 1,2-dioleoyl-3-trimethylammonium-propane |
| CHOL | Cholesterol |
| PBS | Phosphate-Buffered Saline |
| RhB | Rhodamine B |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| DSC | Differential Scanning Calorimetry |
| NP | Nanoparticle |

**ABSTRACT**

The aim of this study was to prepare liposomal systems, examine their physicochemical characteristics and thermal behavior overtime and evaluate their toxicity and permeability properties *in vitro* using a co-culture of intestinal epithelial cells Caco-2/TC7 and HT29-MTX. Liposomal systems were prepared using different lipids and physicochemical characterization was performed over a period of 31 days, showing satisfactory results in terms of physicochemical stability. Differential scanning calorimetry was performed to evaluate the thermal behavior of liposomes, displaying the role of each lipid in the formation of the thermodynamic characteristics of each system. Their toxicity was evaluated using MTT assay and all liposomes presented high viability of cells. Particularly, none of the systems caused viability lower than 80% even at the highest concentrations used (15% v/v). Therefore, the systems were non-toxic to the epithelial cells and permeability studies were performed using rhodamine B as a tracer dye. Permeability studies showed 2-2.5 folds permeability enhancement by the use of liposomes compared to the solution of rhodamine B. Among these systems, the liposomes containing the positively charged lipid DOTAP presented the highest percentage of transport of rhodamine B across the epithelial monolayer. Liposomes can be promising drug delivery systems across the intestinal epithelium, however further research needs to be done to examine the exact mechanisms of liposomal absorption and overcome *in vivo* barriers that eliminate the efficiency of liposomes.

# 1 INTRODUCTION/ THEORY

## 1.1 Nanotechnology and Nanomedicine

Despite the fact that Nanotechnology first appeared in the mid-1970s and as a science was established since the 1990s, Nanotechnology is not a new science but has its roots in antiquity, where craftsmen unknowingly used methodologies which were based on nanoparticles.

Nanotechnology mainly deals with particles or structures with dimensions ranging from a few nanometers to about 100-150 nm, in the order of $10^{-9}$ meters. Dimensions become easier to understand by indicating that a nanometer is roughly equal to 1/80000 a human hair or the length of 10 hydrogen atoms in a row (Figure 1.1). In these dimensions, unusual physical, chemical and biological phenomena emerge that are not observed in the macrocosm and are linked to quantum behavior of matter. Nanotechnology is evolving at a rapid pace and is being implemented in a wide range of scientific fields, including Medicine, which led to the creation of Nanomedicine.

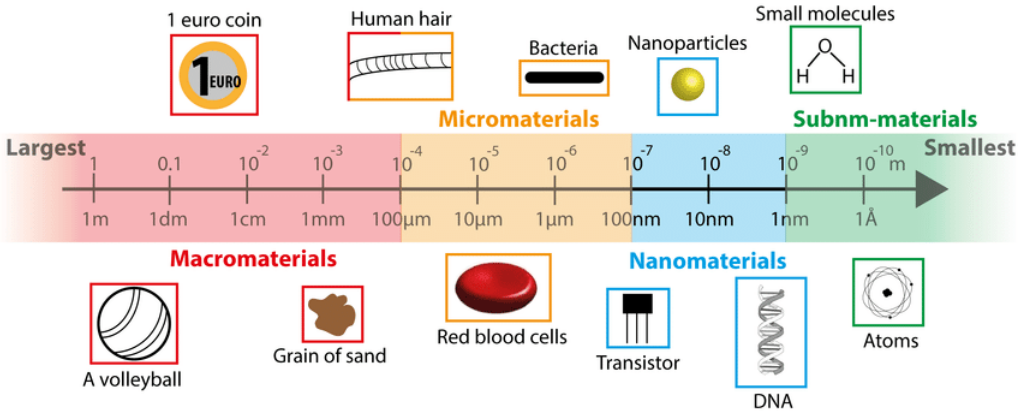


Figure 1.1: Comparison of nanomaterials to molecules of other sizes

Pharmaceutical Nanotechnology deals with the manufacturing and processing of drug delivery systems, their physicochemical characterization, as well as their applications and their biological evaluation. Nanoparticles are used for the delivery of drugs and other biological molecules. Drugs can either be encapsulated inside the nanoparticle or attached to their surface; hence the fate of the nanoparticles within the body greatly determines the fate of the drug. Among drug delivery nanosystems, the more

widely used are micelles, liposomes, dendrimers, as well as nano-suspensions, carbon nanotubes, polymeric and gold nanoparticles, fullerenes, nanogels etc. (D. Attwood 2012)(S.Soni 2014)(Figure 1.2).

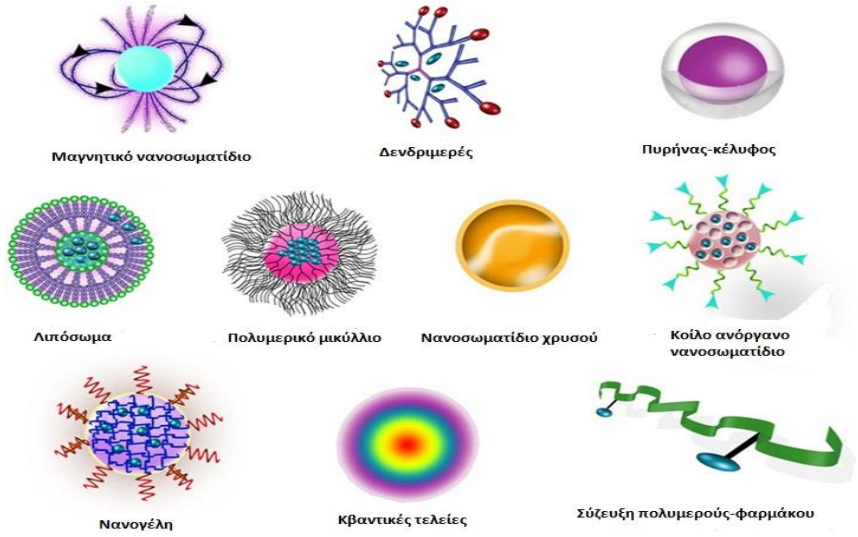


Figure 1.2: Categories of Nanosystems

The characterization of nanosystems includes the ability to encapsulate the drug, its release rate and the physical and chemical stability of both the drug and the carrier.The biological evaluation of nanosystems involves in depth knowledge of the absorption, distribution and excretion of both the released drug and the vector to which the drug is trapped or linked.New drug delivery technologies offer the possibility of developing new medicines and improving existing ones, thus promoting the market of medicines and the pharmaceutical industries.The need to invest in rapidly growing areas such as Nanotechnology seems imperative as it will have multiple benefits for both science and society through numerous and different applications. (Figure1.3)

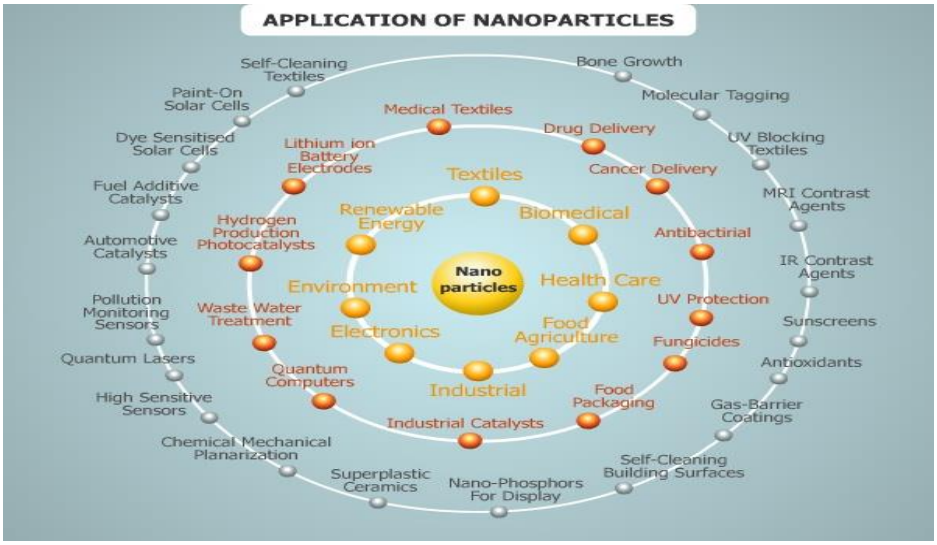


Figure1.3: Applications of Nanotechnology

## 1.2 Liposomes

### 1.2.1 Historical background of liposomes

The conception that the layers forming the lipids in an aqueous system could be useful as a simulation model of the cell membrane has a long history. However, only in the early 1960 emphasis was placed on studies concerning the properties of systems consisting of lipid and aqueous phase as models of cell membrane simulation. They were originally named "Bangosomes" after the special interest given by the scientific world to A.D.Bangham, but later renamed "liposomes".

Subsequently, innovators in the field of simulation of the biofilm model were Chapman, Dervchian and Luzzti who studied the system from different angles. In 1967, Papachadjopoulos and Miller described the structure of ultrasonic vesicles, which later became known as Small Unilamellar Vesicles (SUVs).

### 1.2.2 Introduction

During the recent decades, liposomes have been proven one of the most promising drug delivery and targeting systems, and liposomal technology is a dynamically growing field of research.

Liposomes are formed from naturally occurring phospholipids such as lecithin (phosphatidylcholine). The dispersion of phospholipids in aqueous medium results in the spontaneous formation of vesicular lipid structures (Figure 1.4). The type of lipid

structures depends on the concentration of phospholipids and on their geometry related to their chemical structure.

If the concentration and chemical structure of the phospholipids lead to the formation of bilayers, then the hydrophobic ends of the phospholipids come into energetically unfavorable contact with the aqueous environment, which creates a flexion so that the ends combine and minimize the exposure of the lipid chains to aqueous medium.Part of the aqueous environment in which the phospholipids have been dispersed is now trapped inside the structure that was formed. In addition, the energy required to bend the bilayer is covered by the increase in energy that brings to the system the exposure of the hydrophobic portions of phospholipids to the aquatic environment (N. Monteiro 2014). As a result, lipid vesicles, namely liposomes are formed.

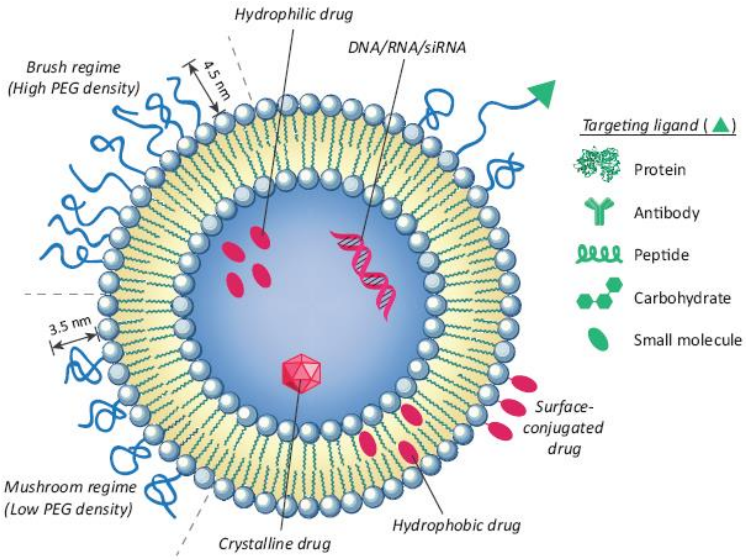


Figure 1.4: Structure of liposome, possible drug and molecule trapping sites according to their nature and bioactive molecules on the surface of the liposomes

### 1.2.3 Categories of Liposomes

Based on the composition of their lipid bilayer, liposomes are categorized in (Y.Perrie 2012)(Figure 1.5):

- **Conventional.** They are composed of neutral phospholipids.

- **pH-sensitive.** They contain in their mass phospholipids or free fatty acids which, depending on the pH of the aqueous environment to which they are exposed, exhibit a different charge and behavior.(N.Naziris 2016)
- **Cationic.** They carry net positive charge and are used in intracellular transport of macromolecules.
- **Immunoliposomes.** They are directed targeting liposomes that carry on their surface an antibody that functions as a surface antigen recognition center of the target cells.
- **Long-circulating liposomes** (or sterically stabilized or stealth or PEGylated). They contain in their bilayer phospholipids in which the polar ends are covalently linked to a hydrophilic polyethylene glycol (PEG) polymer. PEG creates steric stabilization, preventing the liposome from interacting with the biological systems. Thus, liposomes remain for longer in the bloodstream, as their macrophage destruction rate decreases (N. Pippa 2016)
- **Ligand-targeted liposomes, LTLs**. They may carry on their surface monoclonal antibodies, peptides, polysaccharides, hormones, vitamins etc.

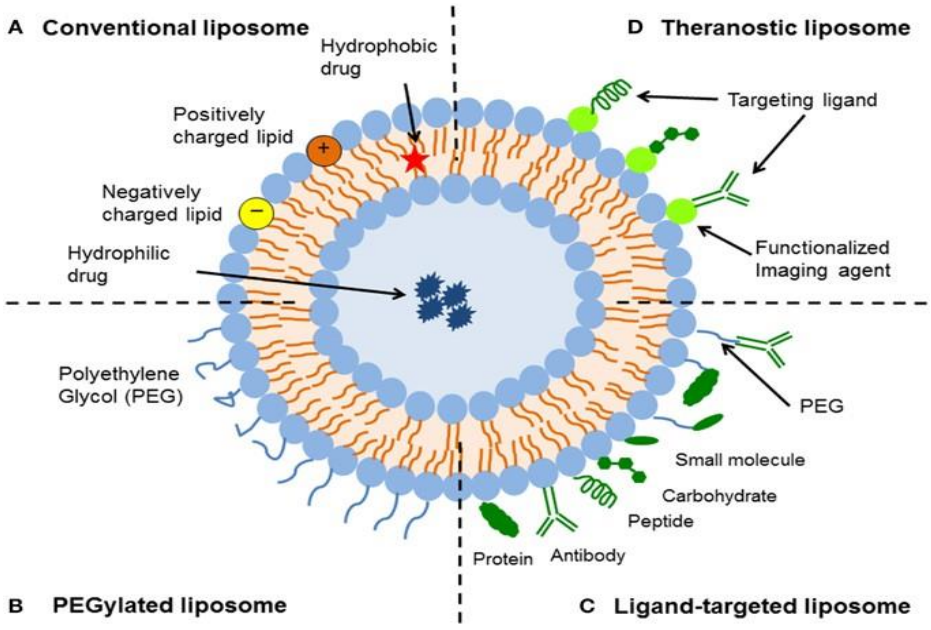


Figure 1.5: Modifications of the surface of liposomes in different categories

### 1.2.4 Advantages of liposomes

Liposomes have the ability to carry a wide range of molecules as they present the following advantages (N.Pippa 2016):

- They are biocompatible and biodegradable
- They protect the encapsulated drug by the effect of degradative enzymes
- They make water-soluble all hydrophobic compounds
- They provide the possibility of controlled release of the drug, keeping the therapeutic levels of the drug stable in the body without causing toxic effects
- They modify the absorption and biodistribution of drugs
- They achieve the reduction in the toxicity of very toxic drugs (e.g. doxorubicin)
- They avoid the action of immune cells as the entrapped substance becomes invisible by the immune system (stealth liposomes)
- They facilitate the entry of substances into the cells, which otherwise could not enter because of different physicochemical characteristics (M.T.Allen 2013)

## 1.3 The role of different lipids in the formation of liposomes

In order to prepare liposomal formulations with specific physicochemical characteristics, biophysical behavior and, by extension, in vivo behavior, it is important to select and combine the appropriate lipids. The properties of liposomes result from the properties of the lipids and the interactions developed between them in the bilayer. Also, lipids play an important role in understanding the role of inter- and intra-molecular interactions on liposome superstructure formation and behavior.

The understanding of the characteristics of lipids that form liposomes is of crucial importance regarding the understanding of membrane biophysics and stability. Additionally, stability is directly linked to $T_{m,}$ the temperature where the bilayer undergoes a gel to liquid phase transition. The addition of specific lipids (e.g. cholesterol) can eliminate this transition and provide a more stable system.

Numerous efforts have been done in order to develop novel lipids with properties that improve delivery while maintaining low cytotoxicity and immunogenicity.

However, nature also provides a variety of lipids that are being used successfully until today for the preparation of liposomes. The existing classification of lipids according to their physicochemical characteristics facilitates the selection of the most suitable ones for each liposomal preparation. (A. G. Kohli 2014)(R.K.Yandrapati 2012)(M. Anderson 2004)

## 1.4 Charged Liposomes

The presence of charge on the surface of liposomes favors the development of electrostatic forces between them and affects their ζ-potential. As a result, surface charge is able to control liposomal aggregation, flocculation, stability and also their biodistribution and in vivo effectiveness.

According to studies, cationic charge can augment the targetability of liposomes regarding angiogenic vessels in tumors. Besides the surface charge, the selected lipid dose and ratio may also affect biodistribution and targeting properties of positively charged liposomes *in vivo*. In addition, cationic liposomes are able to interact with circulating blood cells, leading to changes of membrane fluidity or affecting the formation of aggregates. Due to the preference of cationic liposomes to bind to angiogenic endothelial cells, it can be assumed that the surface of these cells has negatively charged receptors. It has been observed that cationic surface charge for most nanoparticles correlates with higher cellular uptake and increased cytotoxicity in non-phagocytic cells.(F.J.Carrion 1994)(E.Fröhlich 2012)

On the other hand, anionic and neutral liposomes could be used as drug nanocarriers to the extravascular compartment of tumors because of their extravasation. Additionally, the negatively charged lipids attract electrostatically the drug cation, which may be expected to push the head groups of phospholipids apart, hence increasing the diameter of the nanoparticle.(S. Krasnici 2003)(S. Villasmil-Sánchez 2010)

## 1.5 The role of cholesterol in lipid bilayers

Cholesterol is a membrane constituent found in biological membranes, widely used in model membranes as well. It is a lipid that presents numerous advantages that make it suitable for incorporation into phospholipid bilayers of liposomal formulations. Due

to its unique properties, cholesterol can modulate membrane fluidity, elasticity and permeability. Also, its incorporation into phospholipid bilayers is widely known to affect the gel to liquid crystalline phase. Therefore, the amount of cholesterol added in model membranes and liposomes should be carefully selected in proportion to other lipids added in each formulation.

The effect of cholesterol on lipid bilayers is attributed to the properties of its structure. More specifically, the 3-hydroxyl group is oriented towards the polar head region of the phospholipid bilayer, while the hydrophobic part of the molecule is oriented towards its non-polar part.Cholesterol can fit between alkyl chains of phospholipids with its non-polar part, causing disordering of the packing of the phospholipids. It interacts with the hydrophobic acyl chains of phospholipids through hydrogen bonds and hydrophobic interactions. The thermal effects caused by the presence of cholesterol depend on the concentration used.(I. Kyrikou 2005)(S.C. Lee 2005)

The structural characteristics described above as well as the numerous interactions developed explain the ability of cholesterol to provide a well-organized structure and a stable formulation. Cholesterol can ameliorate the packing of phospholipids while reducing bilayer permeability to undesired solutes and preventing the liposome from aggregation. Furthermore, cholesterol affects the fluidity of the bilayer by making it more rigid and resistant to stressful circumstances. The amount of cholesterol added can control the interactions developed with biomolecules at the water-membrane interface as well as the binding of proteins and small molecules on the surface of the liposome. However, little is known about the role of cholesterol on the interaction between the bilayer and ions found in the intercellular and intracellular media. According to studies, the addition of cholesterol reduces the binding of Na+ ions to the liposomal membrane due to increase of hydrophobicity of the membrane. Hence, its interaction with ions needs to be further examined because it is crucial for the activation of the immune system when liposomes enter the blood circulation.(A. Magarkar 2014)(N. Sadeghi 2017)(M.L. Briuglia 2015)

The stabilization effects of cholesterol on lipid membranes emerge from the increase in the cohesion of lipids and the promotion of the liquid-ordered phase. Studies confirm that cholesterol influences the phase transition between gel and liquid crystalline phases of the lipid bilayers by increasing the cohesion of lipids. The maintenance of the liquid phase of liposomes is extremely important in order to preserve the functionality and effectiveness of the liposomal carrier in vivo. Therefore, the presence of cholesterol influences the fate of the nanocarrier in vivo and processes such as influence blood clearance and uptake of liposomes by the reticuloendothelial system.(I. Kyrikou 2005)

### 1.6 Rhodamine B

Rhodamine B (RhB) is a hydrophilic fluorescent dye used to monitor nanosystems such as liposomes during *in vitro* experiments. The ability of RhB to act as a tracer facilitates the monitoring of *in vitro* behavior as well as biodistribution of liposomal systems. The detection of RhB in specific targets, such as organs, reflects the biodistribution and the amount of nanoparticles in these targets. In this way, RhB indicates the pathway followed by liposomes in the body as well as the target in which they accumulate. Therefore, it becomes feasible to know exactly how liposomes behave in living organisms and get qualitative and quantitative results. Usually, both NP-encapsulated RhB and free-RhB NPs are used in such studies, in order to observe the differences. (R.M.Samstein 2007)

### 1.7 Physicochemical Characterization of Liposomes

The characterization of the physical characteristics of liposomes includes techniques and measurements to determine their size and size distribution, surface charge and ζ-potential. The stability of the liposomal suspension is related to the physical, chemical, biological as well as to the stability of the liposome morphology controlled by electron microscopy. (N.Pippa 2016)

*Measurement of size and size distribution of liposomes*

The particles dispersed in a liquid are in constant motion, the Brownian motion. Brownian motion depends on their size, solution temperature and solution viscosity. The distribution of the size of liposomes reflects their stability. When the liposome

size distribution is maintained constant overtime, the liposomal suspension is characterized as stable. If the size distribution of the liposomes increases over time, the liposomal suspension is characterized as unstable and therefore unsuitable for use as a pharmaceutical formulation.

With the Dynamic Light Scattering (DLS) method, it is possible to measure this movement through the data collected for scattered light.From the Brownian motion measurement, the diffusion coefficient is determined and the particle size is then calculated.The diffusion coefficient and the particle size, as expressed by the mean hydrodynamic diameter,$D$ are mathematically linked to the Stokes-Einstein equation (N.Pippa 2013):

$$D = \frac{kT}{6\pi\eta R} \quad (1)$$

Where T is the absolute value of the temperature, k is the Boltzmann constant, $\eta$ is the solvent's viscosity, R is the hydrodynamic radius of the particle. Dynamic Light Scattering (DLS) method measures the particle size associated with the motion of the particle into a liquid, namely the Brownian motion.

The results obtained are the diameter of an equivalent sphere having the same diffusion coefficient as the particles present in the sample to be studied. The diameter of the equivalent sphere is called hydrodynamic diameter, and is slightly larger than the actual particle diameter of the particles of the sample due to the solubilization phenomenon and the presence of interactions between the particles.

## 1.8 Liposome Preparation Techniques

Liposomes can be prepared by various techniques, which are based on the same principle, namely the energy required to bend the phospholipid bilayers and form closed spherical structures, the liposomes. The technique followed for the preparation of liposomes depends on the desired characteristics and sizes. The main technique widely used is the hydration of a thin lipid film described in detail below.

**Thin-film hydration:** This is the simplest and most widespread method for the preparation of multilamellar liposomes (MLVs). It is based on phospholipid dispersion in the form of a film that occupies as large a surface as possible and then hydrating it

at a temperature above the main transition temperature of the phospholipids. The molecule to be incorporated is added either to the lipid film during its preparation if it is hydrophobic or to the aqueous medium upon hydration if it is hydrophilic. This results in the formation of multilamellar liposomes with heterogeneity in size, which can be sonicated and converted to SUVs or MLVs and therefore obtain greater homogeneity in size. (N.Pippa 2013)(Figure 1.6)

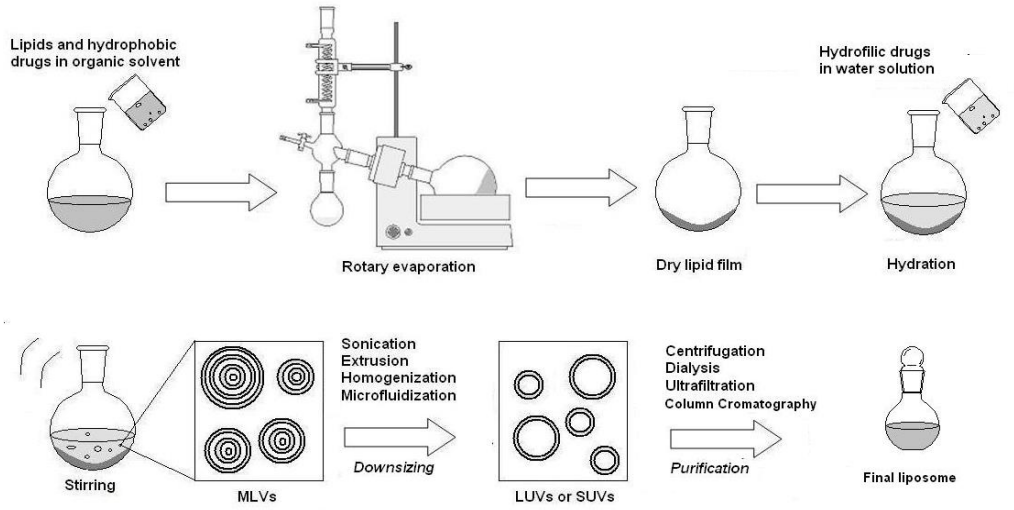


Figure 1.6: Thin film hydration method

Other techniques for the preparation of liposomes are Solvent Injection, Reverse-phase evaporation (REV), Heating Method, etc.

## 1.9 Differential Scanning Calorimetry, DSC

Differential scanning calorimetry (DSC) is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. It is one of the most effective thermal analysis techniques to determine the energy absorbed or released by a sample as it is heated or cooled. This technique is able to provide accurate information, easily and fast, about both physical and energetic properties of a substance. Differential Scanning Calorimetry (DSC) is widely used for determining the thermal behavior, the polymorphic forms and the melting point of molecules and lipid bilayers, as well as measuring thermodynamic parameters (i.e. $\Delta H$ and $T_m$), that affect directly the stability of liposomal systems.(W.M.Groenewoud 2001)

DSC is a valuable technique to acquire information about polymorphism, stability, interactions during research and drug development process. Regarding liposomes, DSC provides information about the thermal behavior and stability of lipid bilayer by determining the cooperativeness of the mixed lipids and indicating the appropriate liposomal composition to be used. Thermodynamic changes of lipid bilayers with encapsulated drug can also be detected with DSC, revealing information about possible interactions of the drug with the carrier. The thermodynamic parameters measured (i.e., *D*H and $T_m$) are affect the stability of the liposomal suspension under given storage conditions but also the pharmacokinetics and bioavailability of the liposomal system in vivo.(C. C. Matsingou 2006)(P. Gill 2010)

More specifically, the amount of energy provided, is associated with the phase transition of phospholipids and the conformation properties of the phospholipids. These factors affect the stability of the liposomal system and changes of enthalpy are associated with the thermal energy added. The phase transitions of phospholipids from the gel to liquid crystalline state are related to the decrease of the hydrophobic van der Waals interactions between lipid acyl chains. Calorimetric techniques are used to determine the enthalpy of this transition and are measured as enthalpy per mol of phospholipid. (N. Pippa 2013) (N. Pippa 2014)

Furthermore, according to studies, alterations of the lipid behavior of lipid bilayers can also be observed by the presence of cholesterol. The thermal effects of cholesterol depend on its concentration used. Results from the DSC experiments based on thermodynamic parameters could be useful to select appropriate concentration of cholesterol and phospholipids for the preparation of liposomal suspensions. (C. Matsingou 2007)

## 1.10 MTT Assay

Cytotoxicity studies are a major step towards the development of new drugs and new drug delivery systems. Cell viability assays measure the cytotoxic effect of a drug on the growth of cell lines towards the incubation time by indicating the percentage of viability of cells. MTT assay is a commonly used method for the assessment of cell proliferation while providing quantitative results easily obtained

photometrically. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is a water-soluble tetrazolium dye that gives yellow solutions in aqueous media. Dehydrogenase enzymes of living cells are able to reduce MTT to an insoluble formazan product, which has a purple color (Fig.1.7)(D. Gerlier 1986)(J.A.Plumb 1999). This conversion cannot be conducted by dead cells due to the absence of active enzymes, so this method is adequate for the measurement of the percentage of living cells. Therefore, MTT assay is a method for sensitive qualification of viable cells offering results regarding the cytotoxicity of drugs or drug delivery systems for *in vivo* experiments. A drawback of the described assay is that the possible cytotoxic or cytostatic effect of a drug or system cannot be distinguished. For that reason, Laser Scanning Cytometry (LSC) can be used in order to get information about both cytotoxic and cytostatic effects of a drug. (P. Pozarowski 2003)

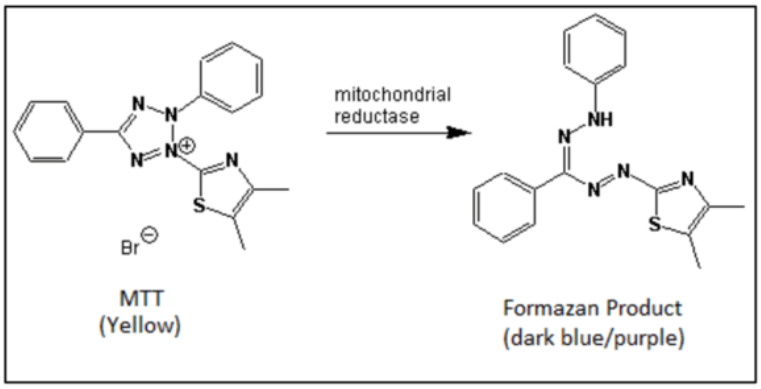


Figure 1.7: Chemical reaction of the reduction of MTT by the mitochondrial reductase of living cells to purple formazan crystals.

## 1.11 Permeability studies

Permeability studies are performed in order to monitor and quantify the transport of substances across cells monolayers. Drug permeability assay through constructed epithelia in semipermeable filter inserts is one of the most commonly used methods. Among others, Caco-2 cells have been used for permeability studies of drugs, food nutrients such as iron, glucose, but also antibodies, SLNs and other nanoparticles.

Intestinal epithelial cell models have also been used for the transport of liposomes. Studies have demonstrated that the use of nanoparticles enhances the permeability and bioavailability of encapsulated compounds in intestinal cell models (L. Du 2016).

Therefore, appropriate surface modifications and properties of liposomes allow their association with Caco-2 cells and the efficient transport of the substances that they deliver. The existence of a nano-vector is crucial regarding the transport of adequate amounts of the substance across the intestinal cell model. The permeability of encapsulated compounds in liposomal systems can also be measured by using a co-culture model of Caco-2/TC7 and HT29-MTX cancer cells that form tight junctions and mimic the intestinal epithelium. This co-culture acts as a monolayer through which compounds move in a paracellular or transcellular way. The cells of the co-culture are placed in semi-permeable filters as supporting materials for the final construction of intestinal epithelium after 21-days of cell culturing. (L. Yin 2009)(T.L.Watts 2013)

The formation of tight junctions is studied with Transepithelial Electrical Resistance (TEER) technique in order to reassure the membrane's integrity which is essential in order to proceed to transport of substances and drug testing. More specifically, TEER is the measurement of electrical resistance of a cellular monolayer and is a very sensitive and reliable method to examine the integrity and permeability of the monolayer. TEER technique is useful in order to measure the ionic conductance of the paracellular pathway in the epithelial monolayer. As it is a non-invasive technique, it can be applied to living cells, in every stage of their growth. So far, TEER measurement has been mainly applied to examine the permeability of tight junctions or to check the membrane disruption by components on intestinal and kidney epithelial cell lines (S. Chen 2015). Then, the studied compound is added and the permeability of the compound in cells is monitored overtime and quantitative results are obtained with the adequate method.

In the present study Rhodamine B was used as a tracer dye, encapsulated in the liposomal system which provides an easy and quick way for quantification of the transported compound through the intestinal epithelium. Numerous studies report the use of Rhodamine B for the monitoring of the absorption of nanoparticles into intestinal epithelial cells. Rhodamine B has been used to monitor the transportation of $Fe_3O_4$ NPs in Caco-2 cells under inflammatory conditions and the results indicated a transportation of NPs approximately 0,8-1,2 times higher compared to the control (G. Zhou 2017). Other studies suggest the use of Rhodamine B as a tracer dye to control

the transport of insulin encapsulated in Trimethyl chitosan-cysteine conjugate (TMC-Cys) NPs through intestinal cells. Compared to insulin solution, TMC-Cys NPs enhanced by numerous folds the insulin transport (L. Yin 2009). Furthermore, Caco-2 cell monolayer has been used for the transport of PLGA NPs into deoxycholic acid emulsion, using once more Rhodamine B and achieving enhanced permeability. (R.M.Samstein 2007)

It is important to examine the pathway that liposomes follow across the intestinal cell model. It should be mentioned that oral delivery of liposomes faces many challenges including mainly crossing barriers and membranes and instability of liposomes in the gastrointestinal tract. The extent and pathway of nanoparticle uptake is different in different parts of the intestine (T.L.Watts 2000). Among others, liposomes can be transferred across the intestinal epithelium with mucoadhesion, via M cells (endocytosis) or enhancer-facilitated absorption using for example surfactants. Also, the use of polymers has been reported to enhance liposomes permeability by slightly opening the tight junctions of the epithelium (H. He 2018).

However, the poor understanding of the absorption mechanisms of liposomes in the intestinal epithelium remains a challenge that needs to be addressed in order to develop and market liposomal products for per os use. Research into the mechanisms that regulate their transport is currently an active area of investigation. Therefore, Rhodamine B constitutes a valuable tool regarding the monitoring of the permeability of nanoparticles across intestinal cell models.Permeability and TEER studies are important in order to monitor the permeability of drugs and compounds *in vitro* and predict their behavior *in vivo*.

# 2 MATERIALS AND METHODS

## 2.1 Preparation and physicochemical characterization of liposomes

*Materials*

- Chloroform (Lab-Scan analytical sciences Ltd, Dublin, Ireland)
- HPLC-grade H2O (PRO TMPSLabconco System)
- Phosphate Buffer Saline (PBS) tablet (Sigma-Aldrich)
- Cuvettes 10x10 x48 mm (Sarstedt, Germany)
- 1µl Hamilton microliterTMsyringes (Bonaduz AG, Switzerland)
- L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC) (Avanti Polar Lipids Inc., Albaster, AL, USA) (Figure 2.1)
- L-α-phosphatidylcholine, hydrogenated (Egg, Chicken) (EggPC) (Avanti Polar Lipids Inc., Albaster, AL, USA) (Figure 2.2)
- 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG) (Avanti Polar Lipids Inc., Albaster, AL, USA) (Figure 2.3)
- 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Avanti Polar Lipids Inc., Albaster, AL, USA) (Figure 2.4)
- Cholesterol (Avanti Polar Lipids Inc., Albaster, AL, USA) (Figure 2.5)
- Rhodamine B (Sigma-Aldrich)(Figure 2.6)

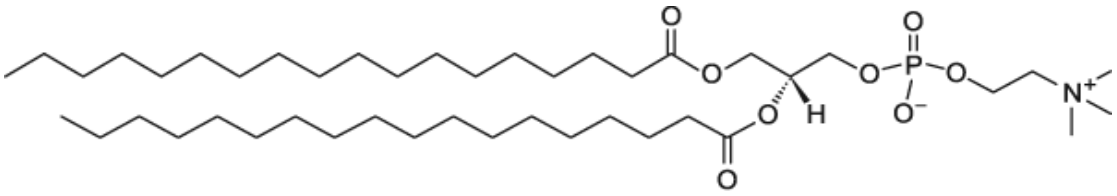

Figure 2.1: Chemical structure of HSPC. MW: 783.774. CAS number: 97281-48-6. Transition temperature: 52 °C

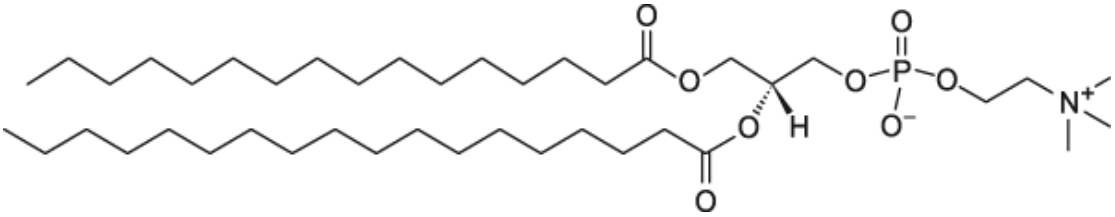

Figure 2.2: Chemical structure of EggPC. MW: 779.762. CASnumber:97281-45-3. Transitiontemperature: -15 - 10 °C. EPC, is a mixture of different in chemical nature phospholipids of L-α-phosphatidylcholine in terms of their lipid chain length and of the number and position of double bonds

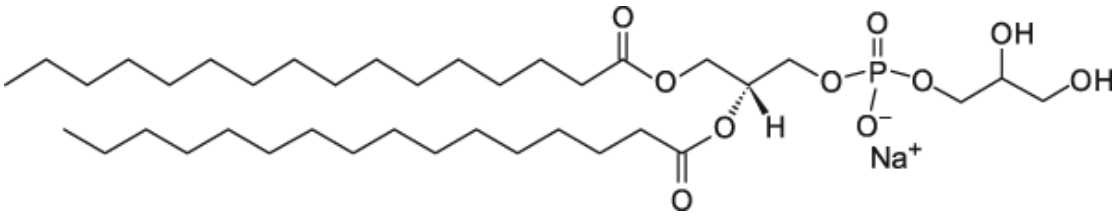


Figure 2.3: Chemical structure of DPPG. MW: 744.952. CAS number: 200880-41-7.

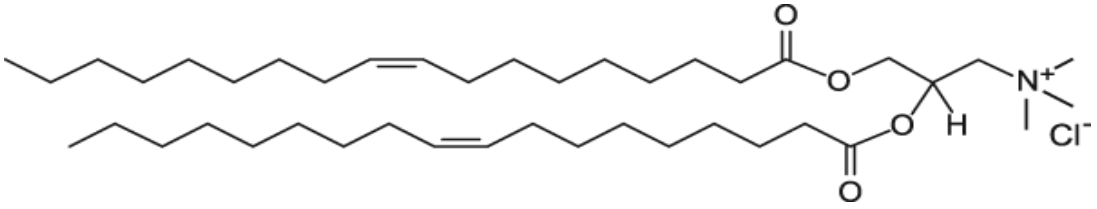


Figure 2.4: Chemical structure of DOTAP. MW: 698.542.CAS number: 132172-61-3.

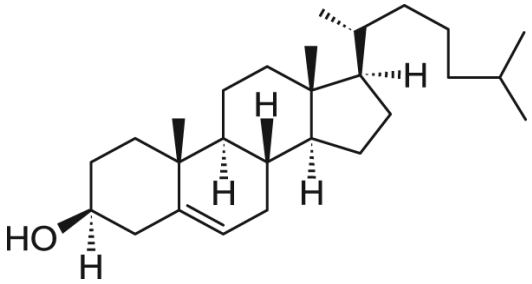


Figure 2.5: Chemical structure of cholesterol. MW: 386.654. CAS number: 57-88-5.

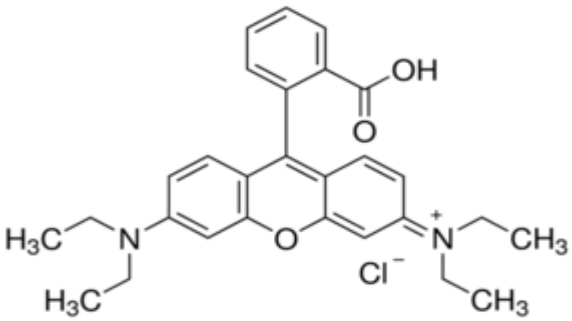


Figure 2.6: Chemical structure of Rhodamine B. MW: 479.01. CAS number: 81-88-9

*Instrumentation*

- **Precision scale** (College B154, Mettler-Toledo, Switzerland)

- **Rotary evaporator** – A device used for the efficient and gentle removal of solvents from samples by evaporation (Laborota 40 A00, Heidolph Instruments) (Figure 2.7)
- **Sonication Device** with Sensor (UP200s Ultraschallprozessor dr. Hielscher GmbH, Berlin, Germany)
- **Photon correlation spectrophotometer** (Zetasizer 3000 HAS, Malvern UK)

Figure 2.7: Rotary Evaporator

Working principles of instrumentation mentioned above are further analyzed below.

- **Probe sonicator**

Model: UP 200 S (Ultrasonic processor for stationary operation)

Company: Dr Hielscher GmbH

*Working principle*: Energy transfer takes place from the sensor of the organ (the Ti spike) in the form of ultrasounds, through the sample (e.g. liposomal suspension). The operating conditions of the organ, namely the frequency and intensity of the ultrasounds, are regulated according to the kind of the sample, through two potentiometers controlled by the operator of the organ.

*Operating conditions:*

Emitted energy: 200W (150W in liquid media)

Power of intensity of emitted energy (amplitude): 20% - 100%

Frequency of emitted pulse: 0.1 -1 cycles/sec (cycle)

Operating frequency: 24kHz

Changing range of frequency: ±1kHz

*Operating conditions during the experiments:*

Emitted energy: 200W (150W in liquid media)

Power of intensity of emitted energy (amplitude):100%

Frequency of emitted pulse: 0.7 cycles/sec (cycle)

Operating frequency: 24kHz

Changing range of frequency: ±1kHz

- **Photon Correlation Spectrophotometer** (Figure 2.8)

Photon correlation spectroscopy organ is a device that measures the size distribution (PDI) and ζ-potential of particles.

Figure 2.8: Photon correlation spectrophotometer.

Model: Zetasizer Particle Size and Zeta Potential Analyser 3000

Company: Malvern Instruments Ltd.

*Working principle*: Particles with size 1-5000 nm can be measured with Photon correlation spectroscopy method (PCS). One of the main characteristics of the particles is that they are in constant, random, thermal motion (Brown motion). This

kind of movement leads to the formation of a model of moving stigma, due to the intensity of light scattered by the particles of light. This movement is detected as change of the intensity in relation to time, using a detector and a photomultiplier. Bigger particles move slower from smaller ones, therefore the rate of fluctuation of the scattered light is slower. Photon correlation spectroscopy uses the rate of change of fluctuations of light to determinate the distribution of size of particles that cause scattering of light.

In this organ, the production of the beam of the emitted light is made from a lamp He-Ne and the wave length of light is 632 nm. The radiation that falls on the particles (e.g. liposomes) is then scattered and the scattered photons are detected by a photomultiplier.

In the software of the organ there is a program of algorithms that analyzes the changes of intensity of the scattered light in order to define the size distribution of the particles in the sample. Additionally, the use of a specific mathematical analysis that consists of the ISO 13321 international method, allows the calculation of the average number of sizes. In these measurements, size is defined as the hydrodynamic diameter of particles (liposomes) and distribution of the range is defined as dispersion index.

*Electrophoretic light scattering (ELS)*

Electrophoretic light scattering (ELS), is a method commonly used to characterize the electrostatic properties of nanosystems. ELS technique studies the motion of charged particles which are dispersed in a liquid, in an electric field. Usually, it is applied to examine and monitor changes in the ζ -potentials of biological systems and particles.

Z-potential of the particles that the organ measures, is the ζ-potential of the coverage surface of the particle, which is formed between the charge of the particle and the media around it. Z-potential does not represent the surface charge of the particle. (S. P. Strand 2001)

*Application of the sample*

The sample is diluted with HPLC-grade filtered $H_2O$ (from filter Millipore with diameter 200nm) and then:

a) It is transferred into a one-use cuvette made of polystyrene. The cuvette is placed into a specific position of the organ, which can be closed with a cap, so as the scattered light is not diffused. The size is measured.
b) It is injected at a slow pace in the specific position of the organ, in order to avoid the formation of bubbles that would block the correct measurement of ζ-potential. The injection places the liposomal suspension at the interior cuvette of the organ and ζ-potential is measured.

*Preparation of HSPC liposomes*

Thin Film Hydration (TFH) method was used for the preparation of all liposomal formulations. The thin lipid film, that is intended for the formation of L-α-phosphatidylcholine hydrogenated (Soy) (HSPC) liposomes, is prepared by mixing L-α-phosphatidylcholine hydrogenated (Soy) (HSPC) with chloroform. The solution is transferred into a round-bottom flask of 100 ml and the solvent is slowly evaporated in a rotary evaporator under vacuum, until a homogeneous film is formed (T = 40 $^oC$). The formed film is left in the refrigerator for at least 24 hours (T = 4 $^oC$). Afterwards, the lipid film is hydrated with PBS. The hydration is done in a water bath above the transition temperature of the lipids by slowly rotating the flask for one hour (T = 60 $^oC$). Then, the multilamellar liposomes formed (MLVs), are subjected to two cycles of sonication of 5 min- 5 min with a break of 5 min in between, into a test tube, using a sonication probe (oscillation width 100%, palm duration 0.7 sec). The sonication results in the formation of small unilamellar vesicles (liposomes, SUVs), which are left to rest for 20 minutes.

*Preparation of EggPC liposomes*

Thin Film Hydration (TFH) method was used for the preparation of all liposomal formulations. The thin lipid film, that is intended for the formation of L-α-phosphatidylcholine, hydrogenated (Egg, Chicken) (EggPC) liposomes, is prepared by

mixing L-α-phosphatidylcholine, hydrogenated (Egg, Chicken) (EggPC) with chloroform. The solution is transferred into a round-bottom flask of 100 ml and the solvent is slowly evaporated in a rotary evaporator under vacuum, until a homogeneous film is formed (T = 40 $^{o}$C). The formed film is left in the refrigerator for at least 24 hours (T = 4 $^{o}$C). Afterwards, the lipid film is hydrated with PBS. The hydration is done in a water bath above the transition temperature of the lipids by slowly rotating the flask for one hour (T = 30 $^{o}$C). Then, the multilamellar liposomes formed (MLVs), are subjected to two cycles of sonication of 5 min- 5 min with a break of 5 min in between, into a test tube, using a sonication probe (oscillation width 100%, palm duration 0.7 sec). The sonication results in the formation of small unilamellar vesicles (liposomes, SUVs), which are left to rest for 20 minutes.

The same method was applied for the preparation of all liposomal systems.More specifically the following liposomal systems were prepared in this study: HSPC, HSPC:EggPC 8:1,5, HSPC:EggPC:CHOL 8:1,5 with 25%[1] cholesterol, HSPC:EPC:DPPG 8:1,5 with 5% DPPG and HSPC:EPC:DOTAP 8:1,5 with 5% DOTAP. Additionally, three systems with Rhodamine B with concentration 1mg/mL were prepared, HSPC:EPC:CHOL:RHOD 8:1,5 with 25% cholesterol, HSPC:EPC:DPPG:RHOD 8:1,5 with 5% DPPG and HSPC:EPC:DOTAP:RHOD 8:1,5 with 5% DOTAP, following the method described above. In each of these three systems 55 μL of Rhodamine B was added before the evaporation of chloroform.

*Physicochemical Characterization of liposomes*

Size (average hydrodynamic parameter, $Z_{Ave}$), Polydispersity Index (PDI) and ζ-potential (ζ-pot) of liposomes are measured immediately after their preparation as follows:

The liposomal suspension is diluted 29 times in a cuvette of capacity 3 ml in HPLC-grade $H_2O$ and the average hydrodynamic parameter ($Z_{Ave}$), the Polydispersity Index (PDI) and ζ-potential (ζ-pot) of liposomes are measured with photon correlation spectrometry. The samples are scattered (632nm) at an angle of 90° and the

[1]All percentages refer to the overall system

measurements take place at temperature 25°C. The results are analyzed using the method CONTIN of the software package MALVERN.

The preparation of all liposomal systems and their physicochemical characterization was performed 7 times. The prepared liposomes are kept refrigerated at 4 ° C and their stability is measured periodically.

## 2.2 Differential Scanning Calorimetry, DSC

*Materials*

- PBS solution (Phosphate Buffer Saline) pH=7.4
- Citric Acid (Sigma Aldrich)
- Sodium Citrate (Sigma Aldrich)
- pH indicator strips (Machery Nagel)
- Prepared HSPC:EPC and HSPC:EPC:CHOL liposomes

*Instrumentation*

- Precision scale (College B154, Mettler-Toledo, Switzerland)
- Micro syringes 100μL και 1000μL Hamilton microliter™syringes (Bonaduz AG, Switzerland)
- Sealed aluminum 40μL crucibles
- Crucible Sealing Press (Mettler Toledo)
- Differential Scanning Calorimeter (DSC) 822e (Mettler Toledo, Schwerzenbach, Switzerland)

The basic components of a DSC heat flow organ include:

1. The oven, which is programmed with linear increase or decrease of temperature or isothermally.

2. The system of receptors for the sample and the reference substance.

3. The sensor for measuring the temperature difference between sample and reference substance by means of thermocouples.

4. The adjustment system for the atmosphere of the sample and the reference substance.

5. The computational control system of the organ and data processing.

Heat flow systems usually operate in the area of -150ºC to 1500ºC.

*Parameters related to the operation of DSC*

In order to achieve correct and precise results with any type of Differential Scanning Calorimetry method chosen for the analysis of a sample, the most basic prerequisite is to select the appropriate device regarding the mass of the sample and the parameters of the measurements. Among the factors that need to be taken into consideration during this process, the most important ones are the rate of heating or cooling, the sensitivity to be programmed, the equilibrium temperature in isothermal change, as well as the temperature range selected and the capacity of the receptor.

Furthermore, the appropriate preparation of the sample, the mass and grain size of the sample, the oven atmosphere, namely static or dynamic, with inert or active gas (nitrogen, argon, helium, carbon dioxide, air, oxygen), by vacuum or pressure should be taken into consideration. Also, the design method for the base line, the calibration standard for the device to be used depending on the nature of the sample being tested are also important factors that need to be adjusted carefully, regarding the formation and record of the final thermal analysis curve.

*Working Principle*

The experiments of Differential Scanning Calorimetry (DSC) were performed using the organ DSC 822e Mettler-Toledo (Schwerzenbach, Switzerland), which was calibrated with pure indium (Tm=156.6 $^{o}$C).

Two and a half cooling-heating cycles were conducted in order to ensure the correct repeatability of the data. The temperature range was from 20 °C to 60 °C and the scanning speed was 5 °C / min. Before each cycle, the samples were subjected to a constant temperature of 20 ° C to ensure equilibration, while an empty aluminum crucible was used as a reference.

During the second cooling-heating cycles, the data from the first measurements (thermotropic parameters) were also taken into consideration, including characteristic transition temperatures, $T_m$, change of enthalpy, ΔH, temperature range corresponding to the half-height of the transition curve, $\Delta T_{1/2}$ as well as the temperature where the thermal phenomenon $T_{onset}$ begins.

All the above were analyzed with Mettler-Toledo STARe software (Mettler–Toledo LLC, Columbus, OH).

*Preparation of solutions for DSC*

**Citric acid / sodium citrate buffer solution (Citrate Buffer) pH=4.0**

2.1g of citric acid and 2.9g of sodium citrate are dissolved in 100mL HPLC grade H2O. The pH is measured using pH indicator strips. If pH is not equal to 4.0, then more citric acid or sodium citrate is added, depending on the pH value.

*Preparation of samples for DSC*

Differential Scanning Calorimetry method was applied to HSPC:EPC and HSPC:EPC:CHOL liposomal systems.

The lipid bilayer placed on the crucible should not weigh more than 8,000mg. Then, the dispersion media is added, so that for each mg of bilayer, 10 μL of dispersion media is added. The hydration took place 30 minutes before the measurement. The bilayer of each molar ratio were hydrated in two different dispersion media, PBS (Phosphate Buffer Saline) with pH=7.4 and Citrate Buffer with pH=4.0. The crucible was sealed by applying pressure to the press.

## 2.3 MTT Assay

*Materials*

- Caco-2/TC7 epithelial intestinal cancer cell lines (American Type Culture Collection, ATCC) were kindly provided by Dr. Monique Rousset (INSERM UMR 505, Paris, France)
- HT29-MTX human colon adenocarcinoma cell lines (American Type Culture Collection, ATCC) cell line by Dr. ThéclaLesuffleur (INSERM UMR S 938, Paris, France)
- Dubelccoo's Modified Eagle Medium (DMEM) medium, composed of 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1%non-essential amino acids (Invitrogen)
- Dimethyl sulfoxide(DMSO)(Sigma-Aldrich, Germany)
- Phosphate Buffer Saline (PBS) (Gibco- Life Technologies, Grand Island,NY, USA)
- 0,25% Trypsin + 1mM EDTA in PBS (Gibco- Life Technologies, Grand Island,NY, USA)
- Blue tetrazolium bromide, MTT stock solution 5mg/mL (Sigma-Aldrich, Germany)
- Isopropanol (Sigma-Aldrich, Germany)
- All cell culture plastic were obtained by Corning Costar, Lowell, MA, USA

*Methods*

**Thawing of cell lines**

Thawing procedure is applied to frozen Caco-2/TC7 and HT29-MTX cell lines. At first, cells are removed from -80 ºC, they are placed in ice for 15 minutes and then in a 37°C water bath. Cells and DMEM growth medium are then placed in cell culture flasks. This procedure takes place in the laboratory hood, under sterilization conditions. The flasks are placed in the incubator at 37°C for 24 hours, maintained under a 5 % $CO_2$ atmosphere at 37 °C.

**Cell Culture Growth**

Cell cultures are observed daily using electron microscope. Cell medium was changed every 48h. This procedure is followed if non-adherent, namely dead, cells are more

than 20-30%. After changing medium and washing with PBS for 2-3 minutes, new medium is added and the cells are placed in the incubator, so they can grow further overtime.

In the case of 80% confluency trypsinization is performed. Trypsinization is a process where the proteolytic enzyme trypsin is used in order to detach adherent cells from the bottom of the flask. Trypsin destroys the proteins which allow the cells to adhere to the flask. Trypsinization is applied to transfer the cells in a new flask. For this reason, a small amount of Trypsin/EDTA in PBS solution is added to the flask for a short period of time and the flask is placed in the incubator, so as the enzyme will act more efficiently. However, trypsin is a proteolytic enzyme and should not remain in the cell culture for a long period of time, as it may affect other cell surface proteins as well and cause degradation of cell functionality. In order to stop the action of the enzyme, double amount of DMEM is added, compared to the amount of trypsin solution added previously.

Afterwards, the content of the flask is transferred into falcons and centrifugation is applied (5 min at 1800 rpm) to separate cells from the rest of the solution. The supernatant is then removed and cells are resolubilized in DMEM in new flasks and incubated.

**MTT assay**

Cytotoxicity of systems was assessed 4h after treatment by MTT assay. In more detail, 200 $\mu$L of medium containing cells were added to the 96-well plate at a density of approximately $10^6$ per well and incubated for 24 h at 37° C under 5% CO2. Cells were exposed to liposomes at different concentrations of 0.01%, 0.05%, 0.1%, 0.5% , 1%, 5%, 10% and 15% v/v in the culture medium. After the incubation period, the supernatant is removed and 100 $\mu$L of PBS:MTT 9:1 solution was added to each well and cells were incubated for 4h. MTT stock solution (5 mg/mL) was added to each culture and incubated for 4h. At the end of the incubation period, the medium was removed and 100 $\mu$L of DMSO:isopropanol 1:1 were added to each well in order to dilute the insoluble purple formazan. The absorbance of the converted dye was

measured at a wavelength of 570nm with a Safire II Microplate Luminometer (TECAN, Switzerland) device. Eq. 1 was used to determine the cell viability.

$$Cell\ viability\ (\%) = \left(\frac{OD_{treated\ cells}}{OD_{control}}\right) \cdot 100 \quad (2)$$

Cells were placed in 96-well plates. According to the literature, a cell density of approximately $10^3$ cells per well is suggested as a starting point for cell lines with a doubling time of around 24 h. However, this number might vary between $5 \times 10^2$ to $10^4$ cells per well depending on the cell line. In order to simulate *in vitro* the conditions of the intestinal mucosa, a co-culture of Caco-$_2$/TC7 and HT29-MTX is required, in proportion 9:1. The required amount of each cell line is placed in DMEM solution and 200μL of it is placed in each well of the plate. At the completion of this procedure, the plates are incubated for 24h.

**Addition of liposomes**

The supernatant after 24h is removed carefully from each well, to not remove any cells. Then, different concentrations of the liposomal systems are prepared by appropriate dilutions using DMEM. The concentrations used were in a range from 0,01% v/v to 15% v/v with 6 repeats. DMEM is used as a blank (100% cell viability). 200 μL of the liposomal solution is added in each well. Then, the plates are placed in the incubator for 48 and 72h.

MTT assay was performed at 48h and 72h of incubation for all liposomal systems. Routinely, MTT stock solution (5 mg/mL) was added to each well (100μl/well) being assayed to equal one tenth of the original culture volume and incubated for 4h avoiding direct light as MTT is a photosensitive dye. The 4h incubation period is the time required for maximal MTT reduction. Afterwards, the supernatant solution is carefully removed and a purple crystal-like precipitate was observed at the bottom of each well. This precipitate is the result of the action of MTT dye to the cells. In order to measure the % viability of cells photometrically, the precipitate of MTT and cells needs to be dissolved. For this reason, 100 μL of DMSO:isopropanol 1:1 solution is added to each well. Then, the absorbance of each well is measured photometrically at a wavelength of 570 nm and with background subtraction at 630-690 nm.

## 2.4 Permeability studies

*Materials*

- Caco-2/TC7 epithelial intestinal cancer cell lines (American Type Culture Collection, ATCC) were kindly provided by Dr. Monique Rousset (INSERM UMR 505, Paris, France)
- HT29-MTX human colon adenocarcinoma cell lines (American Type Culture Collection, ATCC) cell line by Dr. ThéclaLesuffleur (INSERM UMR S 938, Paris, France)
- Dubelccoo's Modified Eagle Medium (DMEM) medium, composed of 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% non-essential amino acids (Invitrogen)
- Hank's Balanced Salt Solution (HBSS) composed of NaCl 0.14M, KCl 0.005M, $CaCl_2$ 0.001M, $MgSO_4$-$7H_2O$ 0.0004M, $MgCl_2$-$6H_2O$ 0.0005M, $Na_2HPO_4$-$2H_2O$ 0.0003M, $KH_2PO_4$ 0.0004M, Glucose 0.006M and $NaHCO_3$ 0.004M
- Dimethyl sulfoxide (DMSO)(Sigma-Aldrich, Germany)
- Phosphate Buffer Saline (PBS) (Gibco- Life Technologies, Grand Island,NY, USA)
- 0,25% Trypsin + 1mM EDTA in PBS (Gibco- Life Technologies, Grand Island,NY, USA)
- Ethanol 70% (Sigma-Aldrich, Germany)
- Isopropanol (Sigma-Aldrich, Germany)
- 12-well ThinCert™ polycarbonate inserts, 0.4 µm pore size and growth surface 4.67 $cm^2$ were purchased from Greiner Bio-One GmbH (Frickenhausen, Germany). All cell culture plastic were obtained by Corning Costar, Lowell, MA, USA
- Incubator conditions: 37 °C and 5% $CO_2$

*Methods*

DMEM medium is placed in both compartments of 12-well cell culture plates with specific ThinCert™ polycarbonate inserts. The medium is left for one hour so as the membranes are well hydrated and able to receive cells. Each plate corresponds to a different liposomal system and 4 repetitions of each system are performed. According

to the literature, an amount of approximately $10^5$ cells per well are required in order to obtain a coherent cell monolayer in the filter, so the amount of the co-culture solution is prepared accordingly. After one hour, the DMEM is removed; new medium is added as well as the solution of the co-culture. The plates are placed in the incubator for 24 hours. In order to examine the permeability of liposomal systems, 21 days of cell growth in Transwell plates are required.

Regular changes (every 48h) of the medium are performed throughout the period of 21 days. At the end of this period, Transepithelial electrical resistance (TEER) technique was performed to evaluate the integrity of tight junctions of the cell culture by measuring ohmic resistance. In this method, two electrodes are used, one electrode is placed in the upper compartment, the other in the lower compartment and the electrodes are separated by the cellular monolayer. The TEER measurements are highly dependent on the electrode positions in the compartments, and a careful handling of the electrodes is required so as to avoid any disturbance to the cells. The electrodes should remain still, in a vertical position relative to the bottom of the well during the measurement and with no contact with the side walls of the well.

To eliminate the influence of temperature, measures were performed within 15 min after taking the culture plates out of the incubator. At first, TEER is measured with DMEM which already existed in the plates, then the medium is removed, HBSS is added and TEER is measured again. Liposomes are administrated at 15% concentration and two wells among all plates are used as blank containing only Rhodamine B. For the TEER measurements a well without cells but with medium was used for the calculations. TEER was measured before and after administration of liposomes in order to examine their effect in epithelium integrity. According to the literature, the calculation of TEER is done using Ohm's Law:

$$TEER_{reported} = R_{tissue} \cdot M_{area} (\Omega \cdot cm^2) \quad (3)$$

More specifically, $R_{tissue}$= $R_{cells}$- $R_{blanc}$and $M_{area}$represents the effective area of the semipermeable membrane, namely the filter. For the filters used in this study, $M_{area}$ equals to 1,1 $cm^2$. $R_{BLANC}$ results from the mean value of three individual measurements of the well that contains the blank. According to the literature, a

satisfactory value of TEER in order to proceed to permeability measurements is >200 $\Omega \cdot cm^2$.

After the administration of liposomes, samples are received at 10 min, 30 min, 1h, 2h, 3h and 4h time intervals in order to examine the permeability of liposomes containing Rhodamine B at the lower compartment. Namely, the sample was taken from the lower compartment and was then replaced by the same amount of HBSS so as to preserve the same volume in both compartments. At the end of this procedure all samples were measured photometrically in a 96-well plate.

# 3 RESULTS

## 3.1 Physicochemical Characterization

In this study, six liposomal systems were prepared. Their physicochemical characteristics on the day of their preparation (T=0) are presented in the table below (Table 3-1).

Table 3-1: Physicochemical characteristics of all liposomal systems, T=0

| System | Dh | SD | PDI | SD |
|---|---|---|---|---|
| **HSPC** | 98,2 | 2,2 | 0,301 | 0,02 |
| **EggPC** | 108,6 | 1,7 | 0,396 | 0,008 |
| **HSPC:EggPC** | 117,5 | 2,5 | 0,501 | 0,017 |
| **HSPC:EggPC:Chol** | 179,1 | 4,8 | 0,609 | 0,013 |
| **HSPC:EggPC:DPPG** | 81 | 1,3 | 0,364 | 0,013 |
| **HSPC:EggPC:DOTAP** | 92,3 | 2 | 0,339 | 0,013 |

Figures 3.1 and 3.2 below show graphically the values of the size and polydispersity index, respectively, for all liposomal systems described in Table 3.1 for T = 0. The ζ-potential of all systems was measured on T=4 days.

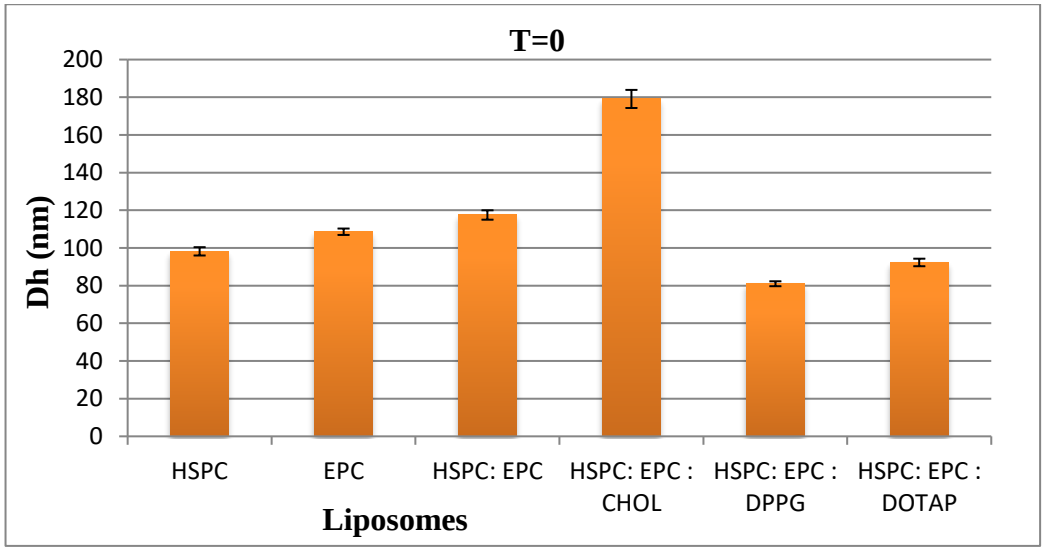


Figure 3.1: Size (Dh) of all liposomal systems on day T=0

On the day of their preparation, HSPC:EPC:CHOL had the largest size, while HSP:EPC:DOTAP the smallest one. (Figure 3.1)

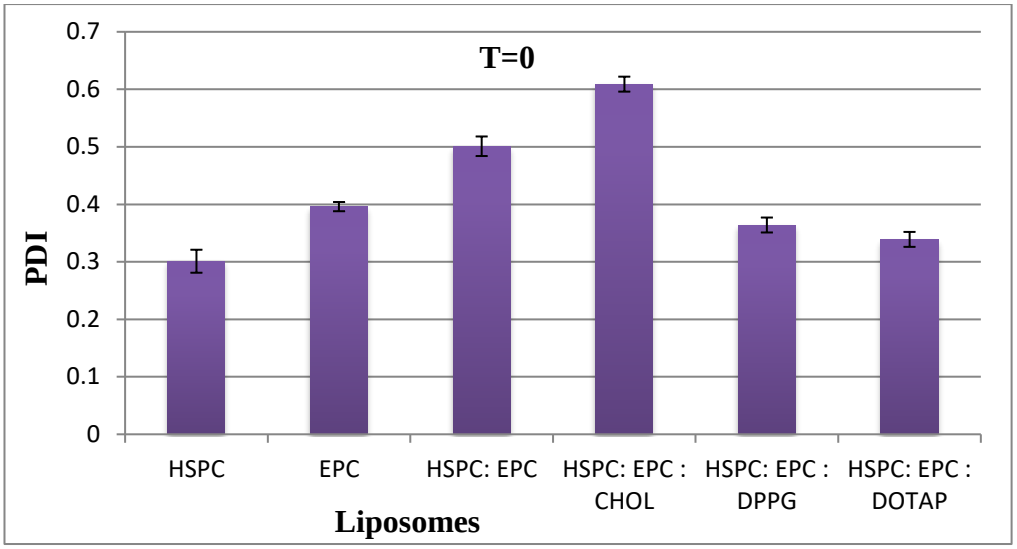


Figure 3.2: Polydispersity Index (PDI) of all liposomal systems on day T=0

On the day of their preparation, HSPC:EPC:CHOL had the largest PDI value, while HSPC liposomes the smallest one. (Figure 3.2)

Z-potential of all systems is presented in the table below, T=4 days. (Table 3-2)

Table 3-2: ζ-potential of all liposomal systems, T=4 days

| System | ζ-potential | SD |
|---|---|---|
| **HSPC** | 3 | 0,5 |
| **EggPC** | -0,8 | 1,9 |
| **HSPC: EggPC** | 1,9 | 0,1 |
| **HSPC: EggPC : CHOL** | 0,7 | 1,2 |
| **HSPC: EggPC : DPPG** | -20,7 | 6,5 |
| **HSPC: EggPC : DOTAP** | 43,1 | 4,6 |

Figure 3.3 shows graphically the values of ζ-potential for all liposomal systems described in Table 3.2, on T=4 days. It is observed that HSPC:EPC:CHOL liposomes presented the smallest absolute value of ζ-potential, while HSPC:EPC:DOTAP the largest one.

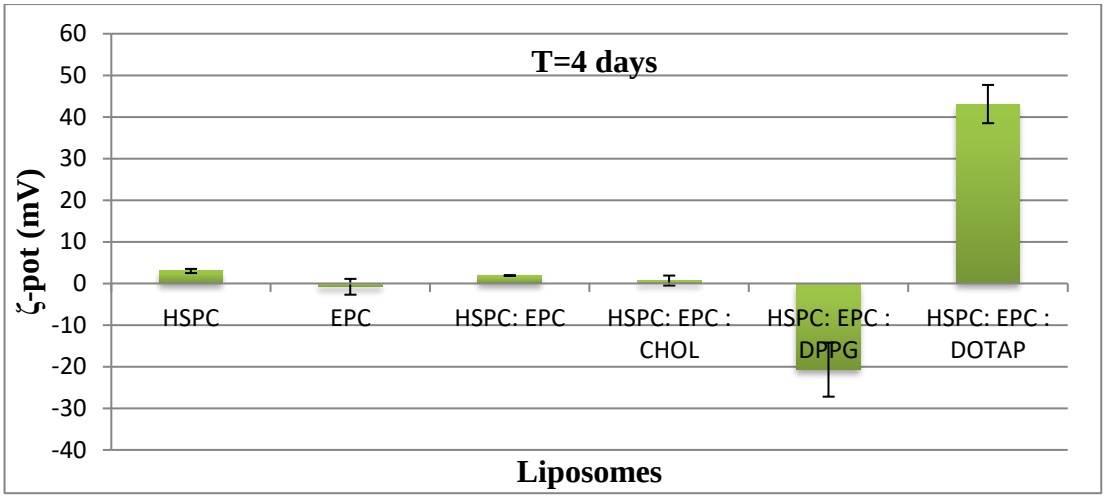


Figure 3.3: ζ-potential of all liposomal systems, T=4 days

## 3.2 Stability Study

All liposomal systems were fully characterized in terms of physicochemical characteristics and stability over time at a storage temperature of 4 ° C.

**HSPC liposomes**

The physicochemical characteristics of HSPC liposomes overtime are presented in the table below. (Table 3-3)

Table 3-3: Physicochemical characteristics of HSPC liposomes over 31 days.

| $T_{ave}$(days) | $Z_{Ave}$ | SD | PDI | SD |
|---|---|---|---|---|
| **0** | 98,2 | 2,2 | 0,301 | 0,02 |
| **4** | 97,4 | 2,3 | 0,294 | 0,006 |
| **7** | 118,5 | 2,8 | 0,29 | 0,008 |
| **12** | 96,2 | 1,9 | 0,286 | 0,008 |
| **19** | 117,1 | 4,4 | 0,309 | 0,006 |
| **24** | 89,2 | 3,5 | 0,291 | 0,004 |
| **31** | 92 | 2,2 | 0,285 | 0,009 |

All HSPC liposomes prepared were small unilamellar (SUVs) with an average Dh around 98 nm on the day of their preparation. PDI value, that represents the size distribution in relation to the Dh, had a value around 0,3 on the day of their

preparation (Table 3-3). The physicochemical characteristics of HSPC liposomes were measured over 31 days.

After 31 days, Dh slightly decreased, from 98 to 92 nm, showing several fluctuations and PDI also decreased, from 0,3 to 0,285.

In the following figures 3.4 and 3.5, the changes in the physicochemical characteristics of the liposomes described in Table 3.3 are graphically illustrated, in relation to time.

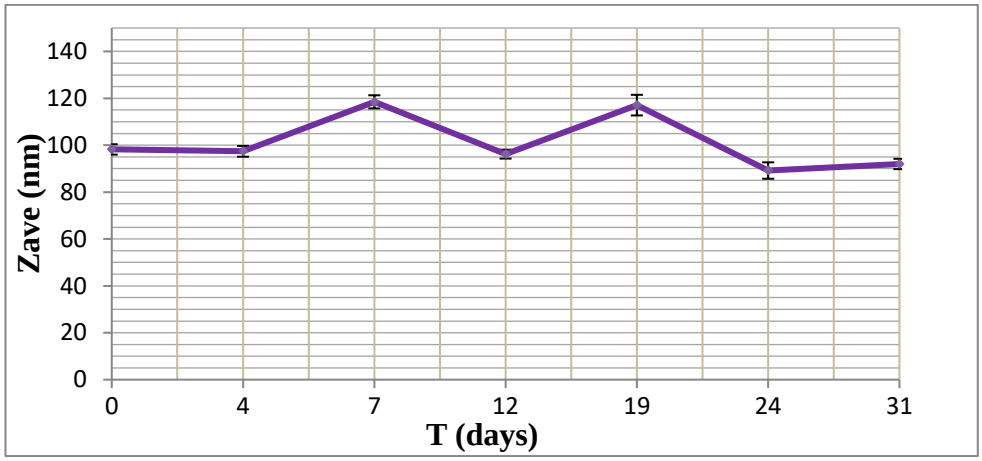


Figure 3.4: Average hydrodynamic diameter of HSPC liposomes over 31 days

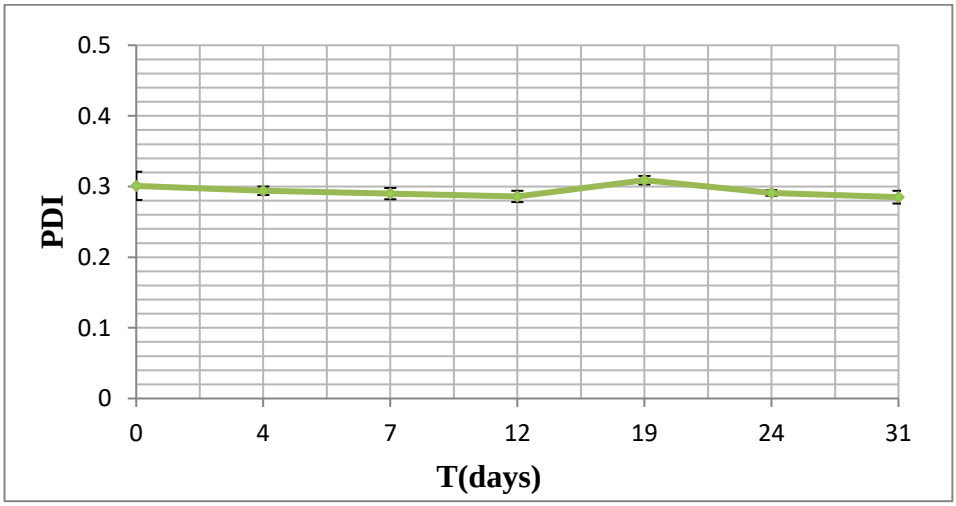


Figure 3.5: Polydispersity Index (PDI) of HSPC liposomes over 31 days

Overall, after 31 days, both Dh and PDI values showed a slight decrease.

**EggPC liposomes**

The physicochemical characteristics of EggPC liposomes overtime are presented in the table below. (Table 3-4)

Table 3-4: Physicochemical characteristics of HSPC liposomes over 31 days.

| $T_{ave}$(days) | $Z_{Ave}$ | SD | PDI | SD |
|---|---|---|---|---|
| **0** | 108,6 | 1,7 | 0,396 | 0,008 |
| **4** | 107,6 | 3,2 | 0,39 | 0,008 |
| **7** | 107,2 | 2,3 | 0,37 | 0,019 |
| **12** | 106,3 | 2,4 | 0,37 | 0,02 |
| **19** | 106,9 | 3,9 | 0,383 | 0,005 |
| **24** | 109,7 | 4,1 | 0,397 | 0,005 |
| **31** | 93,6 | 2,9 | 0,369 | 0,017 |

All EPC liposomes prepared were small unilamellar (SUVs) with an average Dh around 108 nm on the day of their preparation. PDI value, that represents the size distribution in relation to the Dh, had a value around 0,396 on the day of their preparation (Table 3-4). The physicochemical characteristics of HSPC liposomes were measured over 31 days.

After 31 days, Dh slightly decreased, from 108,6 to 93,6 nm, showing no fluctuations and PDI slightly decreased as well, from 0,396 to 0,369.

In Figure 3.6 and Figure 3.7, the changes in the physicochemical characteristics of the liposomes described in Table 3-4 are graphically illustrated, in relation to time.

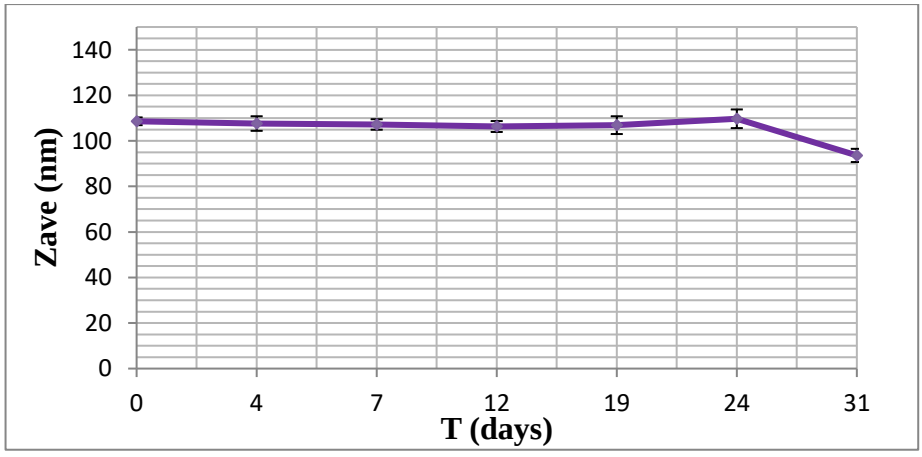


Figure 3.6: Average hydrodynamic diameter of EggPC liposomes over 31 days

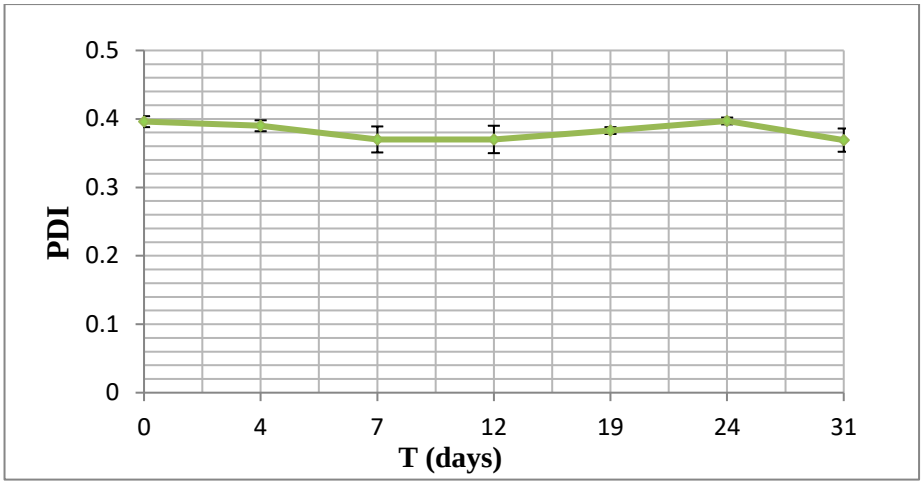


Figure 3.7: Polydispersity Index (PDI) of EggPC liposomes over 31 days

Overall, after 31 days, both Dh and PDI values showed a slight decrease overtime with almost no fluctuations.

**HSPC:EggPC liposomes**

The physicochemical characteristics of HSPC:EggPC liposomes overtime are presented in the table below. (Table 3-5)

Table 3-5: Physicochemical characteristics of HSPC:EggPC liposomes over 31 days.

| $T_{ave}$(days) | $Z_{Ave}$ | SD | PDI | SD |
|---|---|---|---|---|
| **0** | 117,5 | 2,5 | 0,501 | 0,017 |
| **4** | 130,3 | 2 | 0,533 | 0,015 |
| **7** | 186,3 | 4,9 | 0,71 | 0,024 |
| **12** | 205,9 | 8,3 | 0,871 | 0,139 |
| **19** | 236,4 | 16,8 | 0,944 | 0,098 |
| **24** | 232,9 | 12,8 | 0,875 | 0,109 |
| **31** | 230,1 | 7 | 0,996 | 0,007 |

All HSPC:EPC liposomes prepared were small unilamellar (SUVs) with an average Dh around 117 nm on the day of their preparation. PDI value, that represents the size distribution in relation to the Dh, had a value around 0,5 on the day of their preparation (Table 3-5). The physicochemical characteristics of EPC liposomes were measured over 31 days. After 31 days, Dh value almost doubled, from 117 to 230 nm, showing a constant, steady increase and PDI also increased, from 0,50 to 0,99.

In Figure 3.8 and Figure 3.9, the changes in the physicochemical characteristics of the liposomes described in Table 3-5 are graphically illustrated, in relation to time.

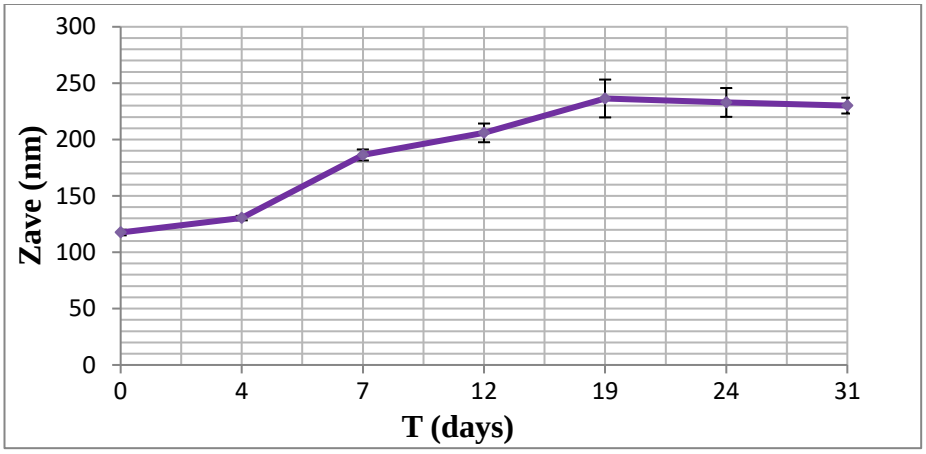


Figure 3.8: Average hydrodynamic diameter of HSPC:EggPC liposomes over 31 days

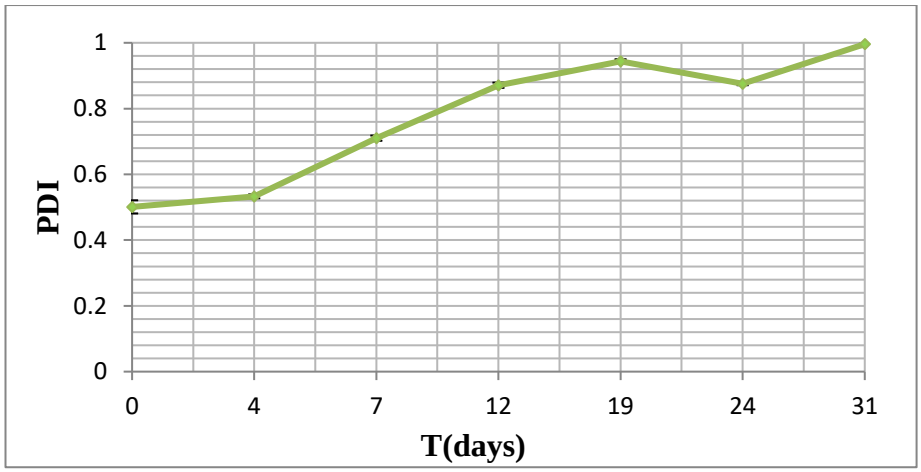


Figure 3.9: Polydispersity Index (PDI) of HSPC:EggPC liposomes over 31 days

Overall, after 31 days, both Dh and PDI values almost doubled.

**HSPC:EggPC:Chol liposomes**

The physicochemical characteristics of HSPC:EggPC:Chol liposomes overtime are presented in the table below. (Table 3-6)

Table 3-6: Physicochemical characteristics of HSPC:EggPC:Chol liposomes over 31 days.

| $T_{ave}$(days) | $Z_{Ave}$ | SD | PDI | SD |
|---|---|---|---|---|
| **0** | 179,1 | 4,8 | 0,609 | 0,013 |
| **4** | 186,2 | 7,5 | 0,609 | 0,05 |
| **7** | 155,1 | 4 | 0,485 | 0,019 |
| **12** | 124,1 | 1,7 | 0,313 | 0,029 |
| **19** | 189,5 | 5,9 | 0,588 | 0,038 |
| **24** | 193,2 | 4,4 | 0,66 | 0,07 |
| **31** | 191 | 5,8 | 0,537 | 0,004 |

All HSPC:EPC:CHOL liposomes prepared were small unilamellar (SUVs) with an average Dh around 179 nm on the day of their preparation. PDI value, that represents the size distribution in relation to the Dh, had a value around 0,6 on the day of their preparation (Table 3-6). The physicochemical characteristics of HSPC:EPC:CHOL liposomes were measured over 31 days.

After 31 days, Dh value slightly increased, from 179,1 to 191 nm. PDI slightly decreased, from 0,609 to 0,537. Both Dh and PDI values showed few fluctuations.

In the following Figures 3.10 and 3.11, the changes in the physicochemical characteristics of the liposomes described in Table 6 are graphically illustrated, in relation to time.

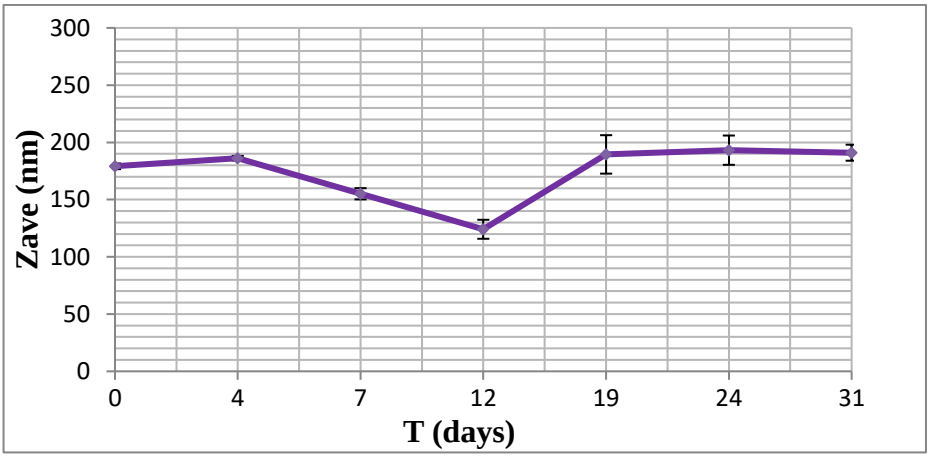


Figure 3.10: Average hydrodynamic diameter of HSPC:EggPC:Chol liposomes over 31 days

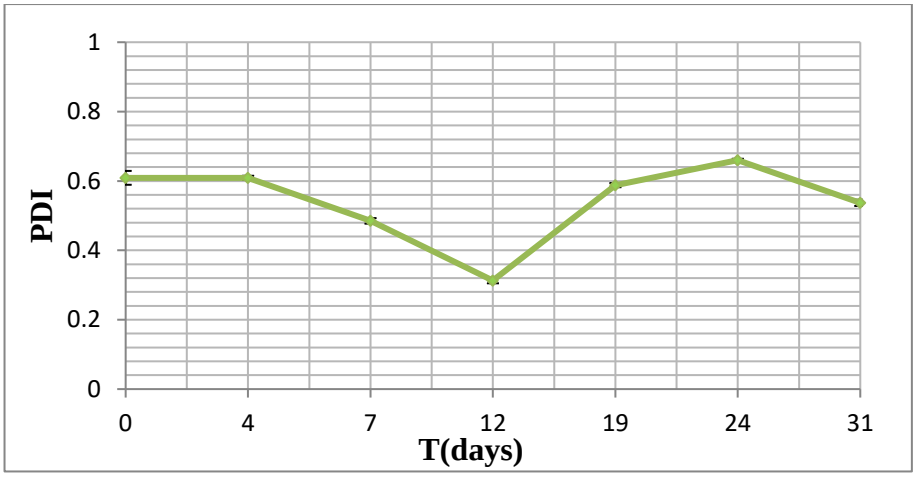


Figure 3.11: Polydispersity Index (PDI) of HSPC:EggPC:Chol liposomes over 31 days

Overall, after 31 days, Dh slightly increased and PDI value slightly decreased.

**HSPC:EggPC:DPPG liposomes**

The physicochemical characteristics of HSPC:EggPC:DPPG liposomes overtime are presented in the table below. (Table 3-7)

Table 3-7: Physicochemical characteristics of HSPC:EggPC:DPPG liposomes over 31 days.

| $T_{ave}$(days) | $Z_{Ave}$ | SD | PDI | SD |
|---|---|---|---|---|
| **0** | 81 | 1,3 | 0,364 | 0,013 |
| **4** | 107,2 | 1,9 | 0,43 | 0,004 |
| **7** | 119,7 | 2,6 | 0,464 | 0,003 |
| **12** | 135,6 | 2,8 | 0,579 | 0,007 |
| **19** | 150,6 | 3,6 | 0,666 | 0,008 |
| **24** | 145,8 | 5,8 | 0,619 | 0,004 |
| **31** | 145,9 | 5 | 0,613 | 0,014 |

All HSPC:EPC:DPPG liposomes prepared were small unilamellar (SUVs) with an average Dh around 81 nm on the day of their preparation. PDI value, that represents the size distribution in relation to the Dh, had a value around 0,364 on the day of their preparation (Table 3-7). The physicochemical characteristics of HSPC:EPC:DPPG liposomes were measured over 31 days. After 31 days, Dh increased from 81 to 145,9 nm. PDI also increased, from 0,364 to 0,613 nm.

In the following Figure 3.12 and Figure 3.13, the changes in the physicochemical characteristics of the liposomes described in Table 3-7 are graphically illustrated, in relation to time.

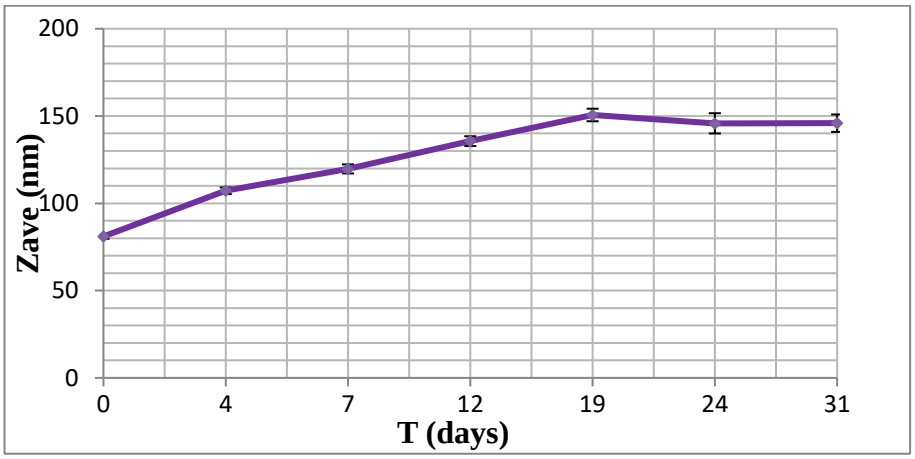


Figure 3.12: Average hydrodynamic diameter of HSPC:EggPC:DPPG liposomes over 31 days

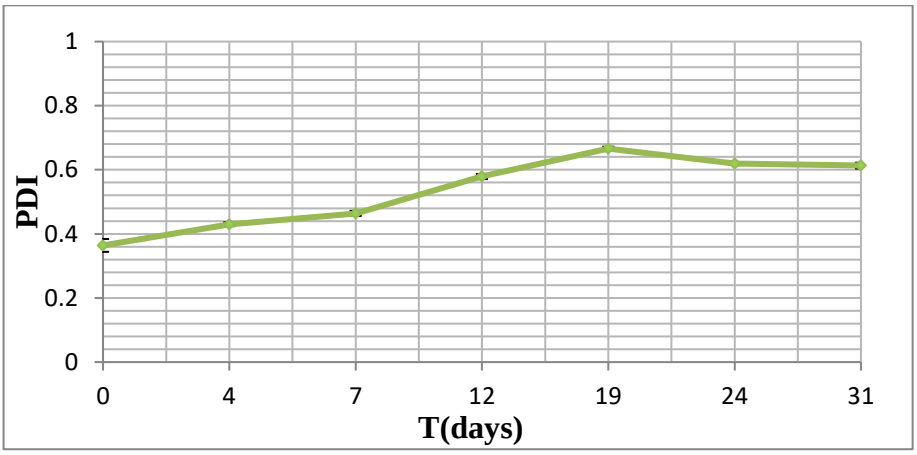


Figure 3.13: Polydispersity Index (PDI) of HSPC:EggPC:DPPG liposomes over 31 days

Overall, after 31 days, both Dh and PDI values increased.

**HSPC:EggPC:DOTAP liposomes**

The physicochemical characteristics of HSPC:EggPC:DOTAP liposomes overtime are presented in the table below. (Table 3-8)

Table 3-8: Physicochemical characteristics of HSPC:EggPC:DOTAP liposomes over 31 days.

| $T_{ave}$(days) | $Z_{Ave}$ | SD | PDI | SD |
|---|---|---|---|---|
| **0** | 92,3 | 2 | 0,339 | 0,013 |
| **4** | 123,1 | 4,1 | 0,538 | 0,014 |
| **7** | 140,3 | 4,1 | 0,617 | 0,03 |
| **12** | 133,3 | 3,7 | 0,592 | 0,016 |
| **19** | 153,8 | 1,4 | 0,695 | 0,016 |
| **24** | 141 | 6,6 | 0,647 | 0,009 |
| **31** | 162,2 | 5,8 | 0,719 | 0,023 |

All HSPC:EPC:DOTAP liposomes prepared were small unilamellar (SUVs) with an average Dh around 92 nm on the day of their preparation. PDI value, that represents the size distribution in relation to the Dh, had a value around 0,339 on the day of their preparation (Table 3-8). The physicochemical characteristics of HSPC:EPC:DOTAP liposomes were measured over 31 days. After 31 days, Dh increased from 92,3 to 162,2 nm. PDI also increased, from 0,339 to 0,719 nm. Both showed few fluctuations.

In the followingFigure 3.14 and Figure 3.15, the changes in the physicochemical characteristics of the liposomes described in Table 3-8 are graphically illustrated, in relation to time.

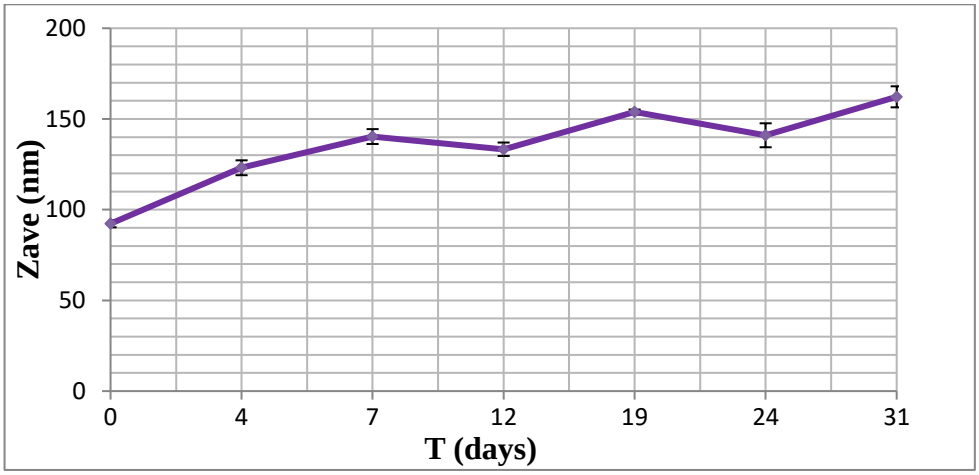


Figure 3.14: Average hydrodynamic diameter of HSPC:EggPC:DOTAP liposomes over 31 days

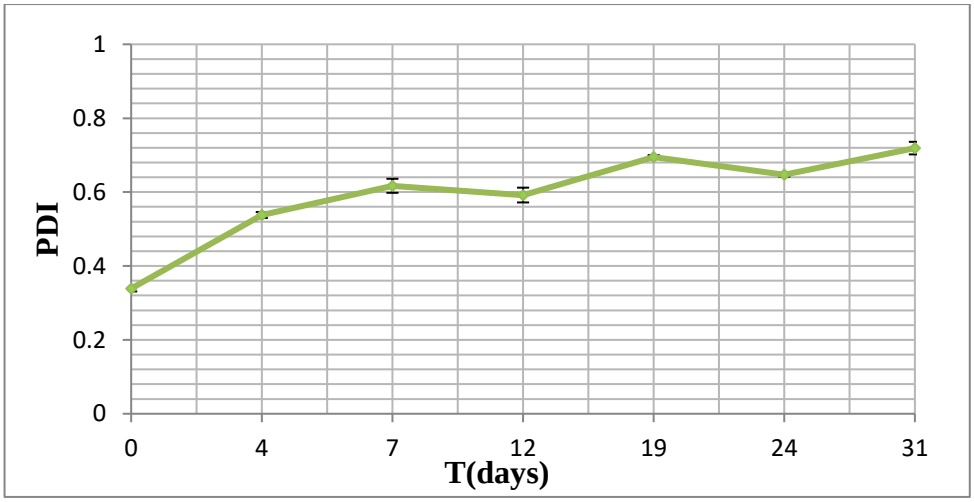


Figure 3.15: Polydispersity Index (PDI) of HSPC:EggPC:DOTAP liposomes over 31 days

Overall, after 31 days, both Dh and PDI values increased.

REMARK: All fluctuations observed were due to the fact that a period of about five days is required to bring thermodynamic stability to the system.

## 3.3 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was performed to study the thermodynamic parameters and thermotropic behavior of three liposomal systems, HSPC, HSPC:EPC and HSPC:EPC:CHOL. The results of the thermal analysis are presented in the thermogram and the tables below. (Figure 3.16 & Table 3-9)

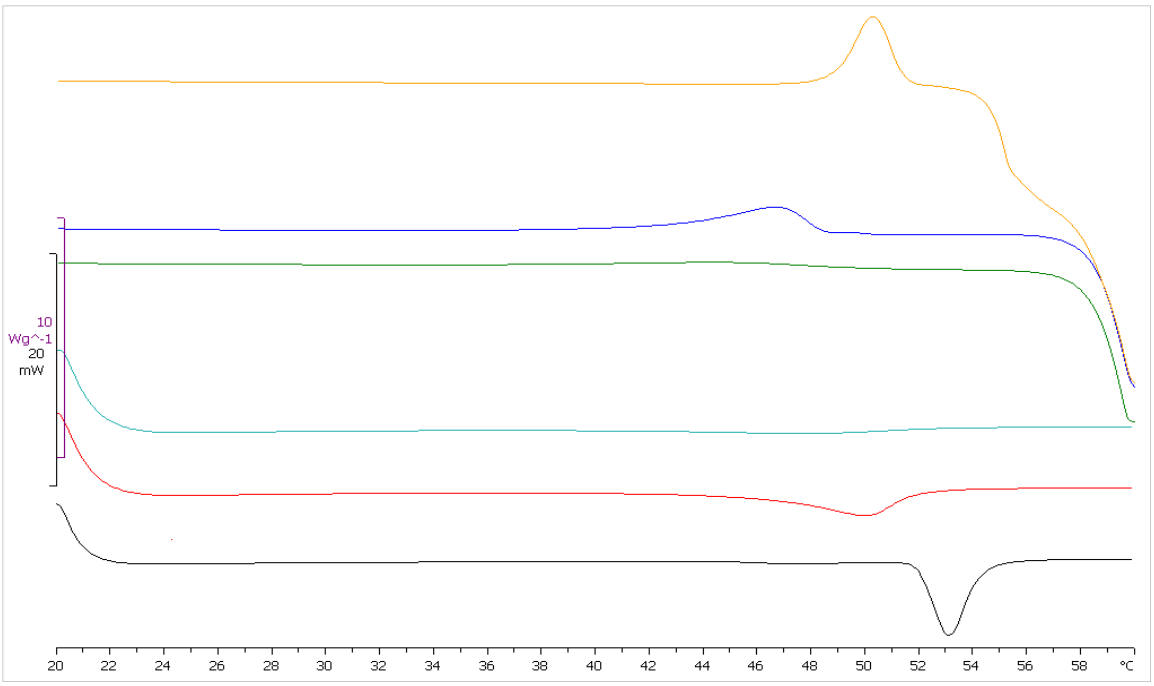


Figure 3.16: DSC thermogram of HSPC, HSPC:EPC and HSPC:EPC:CHOL liposomes

**Thermodynamic changes during heating**

The thermodynamic changes of liposomal characteristics during heating are presented below. (Table 3-9)

Table 3-9: Calorimetric characteristics of HSPC, HSPC:EPC and HSPC:EPC:CHOL liposomes in PBS (pH = 7.4). (Heating)

| Sample | Molar ratio | $T_{onset,m}$ (°C ) | $T_m$ (°C) | $\Delta T_{1/2,m}$ (°C ) | $\Delta H_m$ (J/g) | $T_{onset,s}$ (°C ) | $T_s$ (°C) | $\Delta T_{1/2,s}$ (°C ) | $\Delta H_s$ (J/g) |
|---|---|---|---|---|---|---|---|---|---|
| HSPC | - | 51.8 | 52.85 | 1.35 | -56.81 | 45.27 | 47.36 | 2.43 | -2.33 |
| HSPC:EPC | 8:1.5 | 45.7 | 49.79 | 3.55 | -45.41 | - | - | - | - |
| HSPC:EPC:Chol | 8:1.5,25% | 42.93 | 47.84 | 5.28 | -6.52 | - | - | - | - |

$T_{onset}$ : temperature at which the thermal effect starts

$T_m$ : temperature at which the thermal capacity under constant pressure (ΔCp) is maximum

$\Delta T_{1/2}$ : half-width at half the height of the transition peak

ΔH: change of enthalpy normalized per mole of the liposomal system

m: main transition

s: secondary transition

Thermal analysis is performed to evaluate the thermotropic behavior of liposomes and examine their stability. At first, HSPC liposomes present two phase transitions. The first on is a broad, low-enthalpy pre-transition from gel phase ($L_{\beta'}$) to rippled phase ($P_{\beta'}$) at 47.4 $^{o}$C and the second one corresponds to the main transition, from rippled phase ($P_{\beta'}$) to liquid crystalline phase ($L_a$). These results are in agreement with literature. (R.Koynova 1998)(D.D. 1990)(A. Kapou 2006). This broad transition observed in the thermogram is provoked by the creation of domains in the liposomal membrane. The level of stability of HSPC liposomes according to DSC analysis is in agreement with the results obtained from the stability studies, where HSPC liposomes presented satisfactory stability overtime (size between approximately 90 and 120 nm and PDI=0.3).

Regarding HSPC:EPC liposomal system, the peak is wider compared to the peak of HSPC liposomes due to the cooperativity of lipid membranes. The system is composed of two different materials that have a different melting point. This binary system results in a lower melting temperature ($T_m$) compared to HSPC liposomes. The presence of domains in membranes is even more intense, due to the presence of EPC. The width of the peak reflects the level of heterogeneity of the system and indicates lack of thermodynamic stability.

Therefore, a decrease in the enthalpy is observed as well as in the energy content of the membrane. The result of this phenomenon is the decrease of transition as well as the fluidizing of the membrane. These results regarding HSPC:EPC system are also reflected in the stability studies which preceded DSC analysis and demonstrate the lack of stability. Namely, HSPC:EPC liposomes present a constantly increasing size overtime and PDI values that also increase and are equal to 1 at the end of the study. All the above confirm the thermodynamic instability of the system.

Moreover, the thermal phenomena observed in HSPC:EPC system become even more intense in the case of HSPC:EPC:CHOL liposomes. As shown in the thermogram, the transition almost disappears and no phase transitions can occur. The addition of cholesterol augments the heterogeneity of the system and is directly linked to the lack of thermodynamic stability which results in a solution-like model. According to the stability study, this system presents sizes approximately 130 to 190 nm and PDI around 0.5 that both indicate a rather instable system.

**Thermodynamic changes during cooling**

The thermodynamic changes of liposomal characteristics during cooling are presented below. (Table 3-10)

Table 3-10: Calorimetric characteristics of HSPC, HSPC:EPC and HSPC:EPC:CHOL liposomes in PBS (pH = 7.4). (Cooling)

| Sample | Molar ratio | $T_{onset,m}$ (°C ) | $T_m$ (°C) | $\Delta T_{1/2,m}$ (°C ) | $\Delta H_m$ (J/g) |
|---|---|---|---|---|---|
| HSPC | - | 51.64 | 50.62 | 1.51 | 56.7 |
| HSPC:EPC | 8:1.5 | 48.67 | 46.96 | 3.59 | 46.37 |
| HSPC:EPC:Chol | 8:1.5,25% | 49.06 | 45.33 | 5.12 | 6.05 |

$T_{onset}$: temperature at which the thermal effect starts

$T_m$: temperature at which the thermal capacity under constant pressure (ΔCp) is maximum

$\Delta T_{1/2}$ : half-width at half the height of the transition peak

ΔH: change of enthalpy normalized per mole of the liposomal system

m: main transition

s: secondary transition

According to the thermogram, the cooling curves presented a slight hysteresis for all liposomal systems, which is in agreement with the literature. (N.Pippa, 2014; 2015).

## 3.4 MTT Assay

In this study, the toxicity of nine different liposomal systems (S1-S9) was evaluated using the MTT assay. Cells were treated with various concentrations (0.01, 0.05, 0.01, 0.05, 0.1, 0.5, 1 and 5% v/v and in three systems additionally 10 and 15% v/v) of liposomes for 48 and 72 hours.

The results are presented in figures of % cell viability towards liposomal concentration in culture medium. Each column represents the mean ± SD of results from 4-6 individual measurements. The systems are presented per three below, and every triad is selected so as to compare the systems of each triad to each other.

**Systems 1, 2 and 3**

The % viability of cells for different concentrations in culture medium of systems 1-3 is presented in the Figures below at 48 and 72 hours.

System 1: HSPC

System 2: HSPC:EPC:CHOL

System 3: HSPC:EPC:CHOL:RHOD

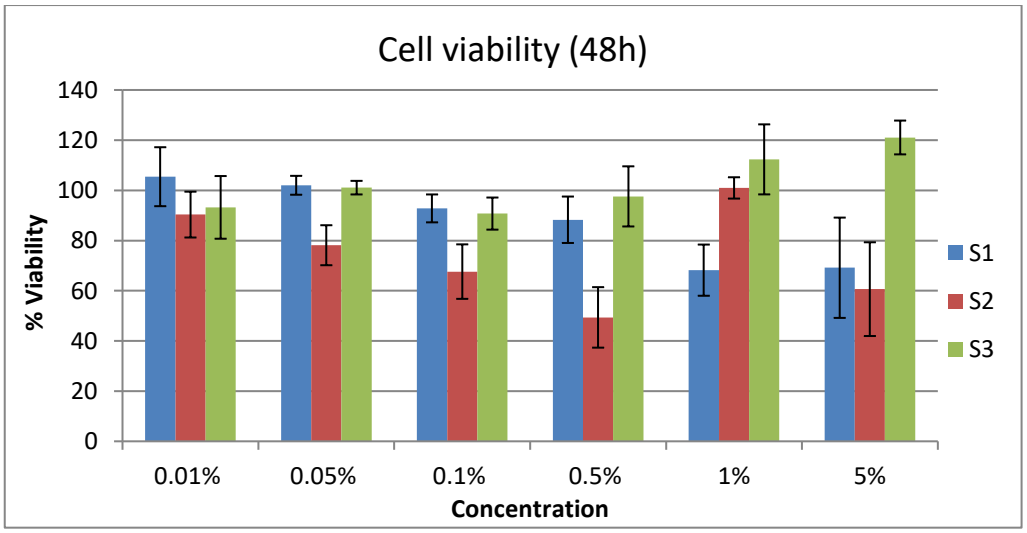


Figure 3.17: % viability versus concentration for S1, S2, S3 at 48h

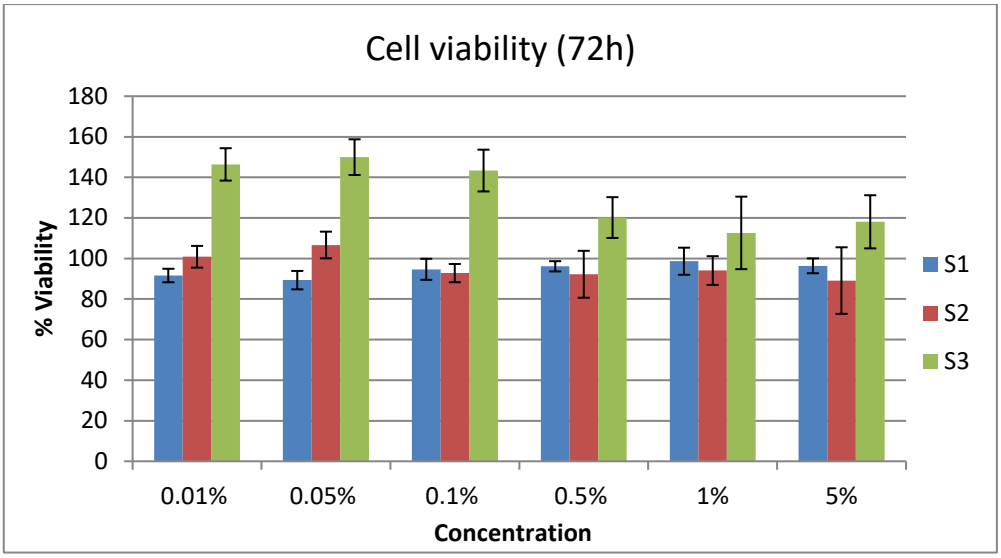


Figure 3.18: % viability versus concentration for S1, S2, S3 at 72h

Regarding systems 1, 2 and 3, viability of cells after 72 hours is over 80%. More specifically, both systems 1 and 2 lead to 80-100% viability of cells in all concentrations used. Therefore, systems 1 and 2 are almost non toxic for these cells. System 3 is associated with slightly higher percentage of cell viability compared to systems 1 and 2. At higher concentrations, system 3 presented approximately 100-120% viability of cells.

The above results are in agreement with the nature and toxicity profile of the substances that construct these liposomes. According to the literature, there are no data available for humans regarding carcinogenicity caused by Rhodamine B, and no systemic toxicity of Rhodamine B has been reported after oral administration. The lack of toxicity of the systems containing cholesterol was expected, as cholesterol is a structural element of cell membranes of organisms. Additionally, Hydro Soy Phosphatidylcholine (HSPC) and Egg Phosphatidylcholine (EPC) are lipids derived from animal sources that are widely consumed by humans, so they are not toxic.

**Systems 1, 4 and 5**

The % viability of cells for different concentrations of systems 1, 4 and 5 is presented in the Figures below at 48 and 72 hours. System 1 is presented again to be used in comparison with systems 4 and 5.

System 1: HSPC

System 4: HSPC:EPC:DOTAP

System 5: HSPC:EPC:DOTAP:RHOD

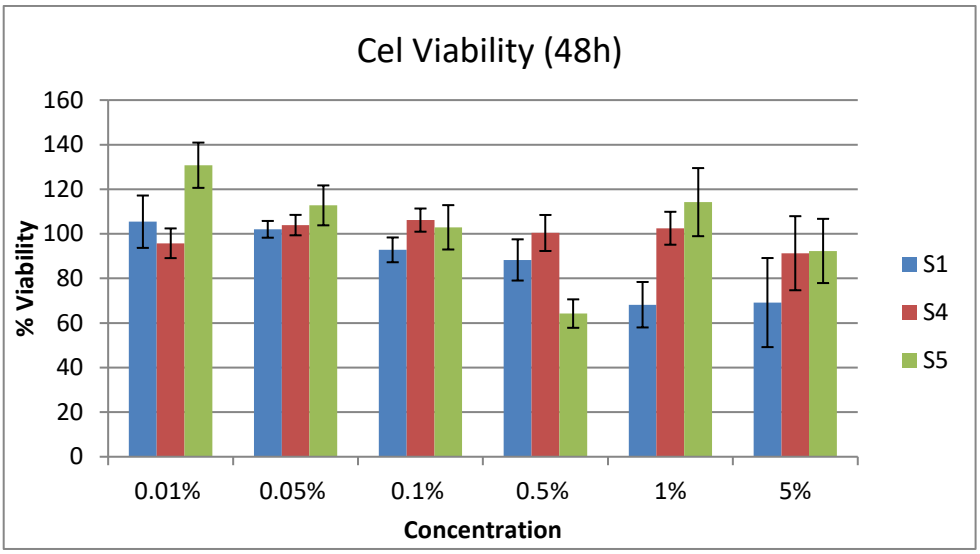


Figure 3.19: % viability versus concentration for S1, S4, S5 at 48h

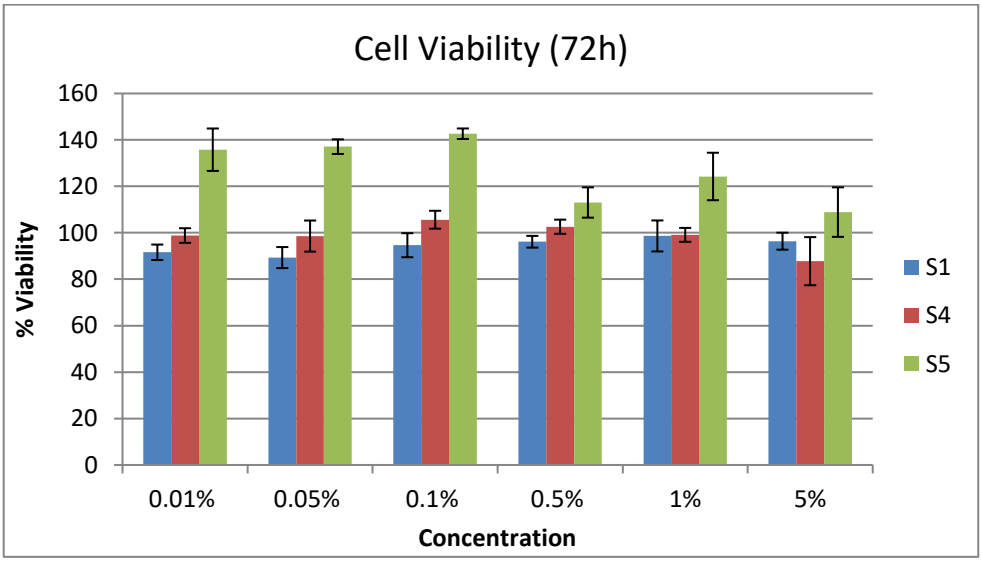


Figure 3.20: % viability versus concentration for S1, S4, S5 at 72h

Systems 4 and 5 presented no toxicity to cells after 72 hours. More specifically, system 4 caused similar cells viability to system 1, ranging between approximately 85 and 105%. System 5 presented slightly higher viability of cells which probably results from the existence of a higher amount of cells at the beginning of the experiment. It should be mentioned that the presence of DOTAP adds a positive charge to the liposomes which,

according to figure 3.20, did not affect negatively the proliferation of cells. DOTAP is a widely used cationic lipid in both in vitro and in vivo applications. According to the literature, cationic nanocarriers composed of DOTAP provoke limited toxicological effects to cells(K.Lappalainen 1994). Only at higher concentrations DOTAP liposomes are able to cause some toxic effects, probably due to the cationic surface charge which interacts with cell membranes through electrostatic interactions. Additionally, high concentration of positively charged liposomes may induce the creation of reactive oxygen species due to the presence of cationic head groups, which can lead to inflammatory and toxicological effects (K. B. Knudsen 2015). This observation, along with the higher liposome uptake by cells, explains the decline of viability of cells at the highest concentration used of system 4 (5%).

**Systems 6, 7 and 8**

The % viability of cells for different concentrations of systems 6, 7 and 8 is presented in the Figures below at 48 and 72 hours.

System 6: EPC

System 7: HSPC:EPC

System 8: HSPC:EPC:DPPG

System 9: HSPC:EPC:DPPG:RHOD

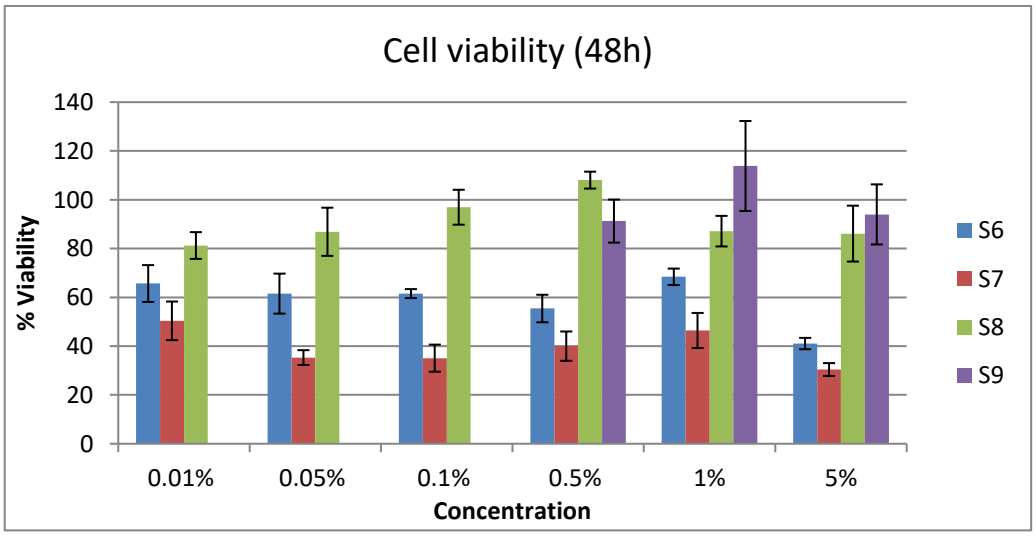


Figure 3.21: % viability versus concentration for S6, S7, S8, S9 at 48h

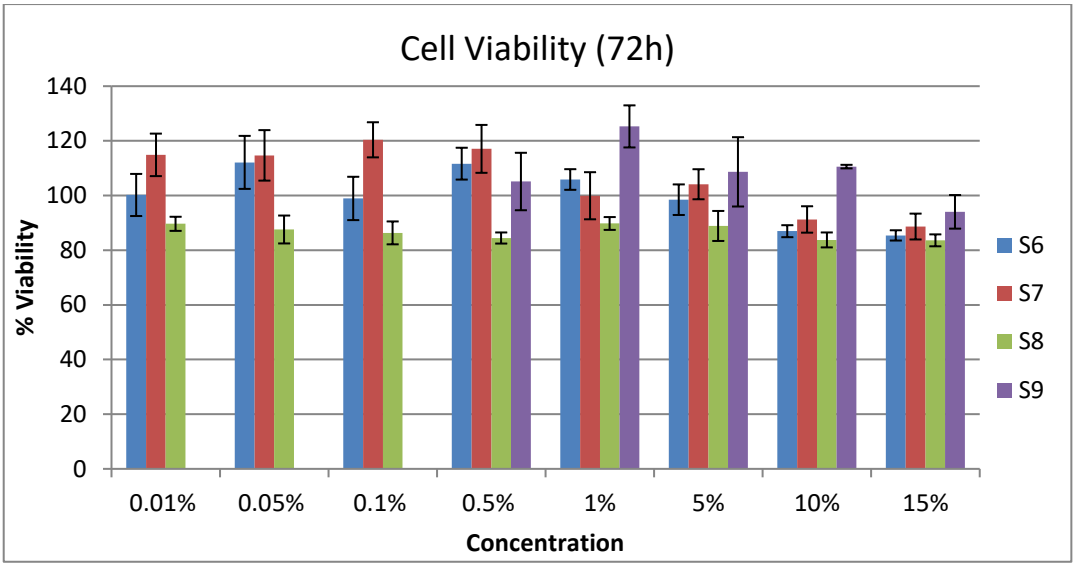


Figure 3.22: % viability versus concentration for S6, S7, S8, S9 at 72h

Regarding systems 6, 7 and 8, they all present viability of cells higher than 80% after 72 hours, even at 10% and 15% concentrations. More specifically, system 6 presented 100-120% viability for concentrations up to 5%, which slightly decreased at around 90% at concentrations 10 and 15%. System 7 presented similar fluctuations of viability to system 6 and had the highest percentages of cells viability at concentrations 5-15%, compared to both systems 6 and 8. System 8 caused 80-90% viability of cells at all concentrations, showing almost no fluctuations and system 9 showed slightly higher percentages of viability.

It is important to note that the presence of DPPG lipid (system 8) adds a negative charge to the liposomes which, compared to system 7 that is composed of the same lipids as system 8 except DPPG, slightly affects the viability of cells. It is observed that between systems 7 and 8, the presence of DPPG slightly decreases the percentage of viability of cells, especially at lowest concentrations (0,01-0,5%, 72h).

Regarding toxicological information, there is no data available for DPPG, but according to the literature no component of this product at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen. Phosphatidylglycerol, the precursor of DPPG, can be found in organisms, particularly in cell membranes, in lung surfactant and in the amniotic fluid.

Furthermore, it should be mentioned that lipids with different charge presented different effects on the viability of cells. Specifically, HSPC:EPC:DOTAP (System 4) liposomes with positive charge presented 100% viability of cells except the highest concentration which decreased the viability at 85%, while anionic HSPC:EPC:DOTAP liposomes (System 8) provoked 80-85% viability of cells at all concentrations. It has to be mentioned, that all the systems present are appropriate for drug delivery due to the reported cell viability. Namely, positively charged liposomes have almost no effect on the viability of cells; in contrast with negative ones that present approximately 20% lower viability. This can be attributed to the fact that the interactions between cells and liposomes highly depend on the kind of surface charge of liposomes, as different electrostatic interactions are developed.

## 3.5 Permeability studies

### 3.5.1 TEER Measurements

Cell culture on Transwell plates requires approximately 21 days so as tight junctions between cells are developed adequately. For this reason, measurements of TEER are performed to check the connection between cells on day 21, in both DMEM and HBSS media. Each measurement includes three plates. For each plate one table is presented according to Ohm's Law described above.

The results from TEER measurements are presented below. Three individual measurements were performed for each well.

Plate 1: HSPC:EPC:CHOL:RHOD

Plate 2: HSPC:EPC:DPPG:RHOD

Plate 3: HSPC:EPC:DOTAP:RHOD

*Day 21*

Measurements on day 21 were performed in both DMEM and HBSS media. Both of the measurements were performed before the administration of liposomes. As

presented below in tables 3-11 and 3-12, TEER values were satisfactory on day 21 (TEER>200 Ω*cm$^2$), so the administration of liposomal systems was possible.

A. TEER measurement in DMEM medium

| | | TEER (Ω*cm$^2$) | STDEV |
|---|---|---|---|
| Plate 1 | 1st well | 194 | 19 |
| | 2nd well | 259 | 10 |
| | 3rd well | 293 | 13 |
| | 4th well | 279 | 10 |
| Plate 2 | 1st well | 281 | 4 |
| | 2nd well | 226 | 20 |
| | 3rd well | 252 | 5 |
| | 4th well | 250 | 15 |
| Plate 3 | 1st well | 305 | 13 |
| | 2nd well | 235 | 23 |
| | 3rd well | 287 | 9 |

Table 3-11: TEER of cells in DMEM according to Ohm's Law method, day 21.

B. TEER measurement in HBSS medium

| | | TEER(Ω*cm$^2$) | STDEV |
|---|---|---|---|
| Plate 1 | 1st well | 170 | 36 |
| | 2nd well | 229 | 7 |
| | 3rd well | 248 | 8 |
| | 4th well | 194 | 19 |
| Plate 2 | 1st well | 254 | 3 |
| | 2nd well | 206 | 15 |
| | 3rd well | 231 | 12 |
| | 4th well | 231 | 9 |
| Plate 3 | 1st well | 273 | 12 |
| | 2nd well | 216 | 21 |
| | 3rd well | 244 | 7 |

Table 3-12: TEER of cells in HBSS according to Ohm's Law method, day 21.

According to the above results, almost all TEER values were above 200 Ω*cm$^2$ before the administration of liposomes. It should be mentioned that TEER values were slightly lower in HBSS medium compared to DMEM medium.

*Day 22*

Measurement of TEER on day 22 was performed in HBSS medium, after washing with HBSS. This measurement was performed in order to examine the condition of tight junctions following the administration of liposomes. The results are presented below.

| | | TEER($\Omega$*cm$^2$) | STDEV |
|---|---|---|---|
| Plate 1 | 1st well | 93 | 19 |
| | 2nd well | 105 | 11 |
| | 3rd well | 185 | 11 |
| | 4th well | 103 | 20 |
| Plate 2 | 1st well | 99 | 5 |
| | 2nd well | 120 | 15 |
| | 3rd well | 132 | 4 |
| | 4th well | 174 | 16 |
| Plate 3 | 1st well | 229 | 27 |
| | 2nd well | 132 | 26 |
| | 3rd well | 114 | 12 |

Table 3-13: TEER of cells in HBSS according to Ohm's Law method, day 22.

*Day 23*

Measurement of TEER on day 23 was performed in HBSS medium. The results are presented in Table 3-14 below.

| | | TEER ($\Omega$*cm$^2$) | STDEV |
|---|---|---|---|
| Plate 1 | 1st well | 71,49 | 16,03 |
| | 2nd well | 181,13 | 25,09 |
| | 3rd well | 201,29 | 29,15 |
| | 4th well | 148,13 | 16,33 |
| Plate 2 | 1st well | 128,33 | 33,6 |
| | 2nd well | 176,73 | 26,52 |
| | 3rd well | 225,49 | 19,31 |
| | 4th well | 180,76 | 36,37 |
| Plate 3 | 1st well | 250,06 | 16,61 |
| | 2nd well | 147,76 | 7,21 |
| | 3rd well | 111,83 | 25,99 |

Table 3-14: TEER of cells in HBSS according to Ohm's Law method, day 23.

According to the above results, TEER values on day 23 were higher than the ones on day 22. The decrease of the values on day 22 can be attributed to the opening of the tight junctions after the administrations of liposomes. However, the augmentation of TEER values on day 23 (Table 3-14) indicates that the opening of tight junctions was temporary and the epithelium was not destroyed by the permeation of liposomes.

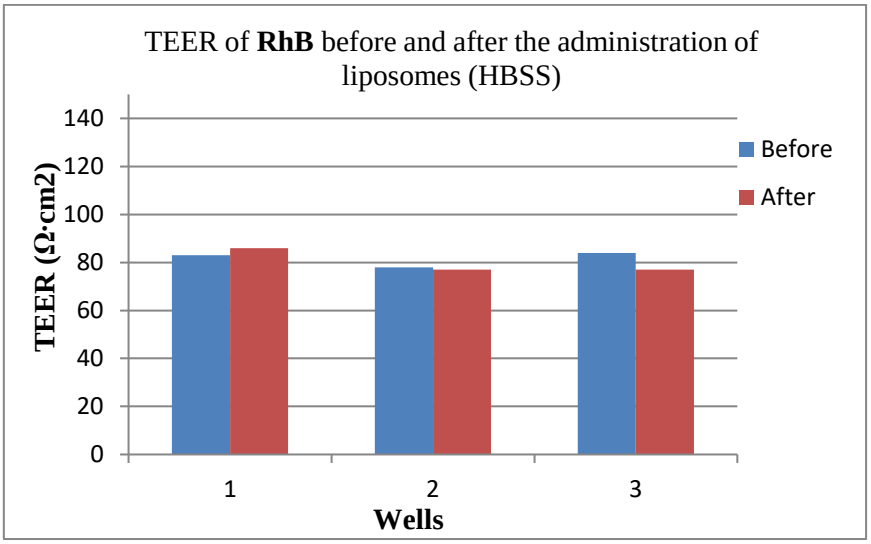


Figure 3.23: TEER of RhB before and after the administration of liposomes

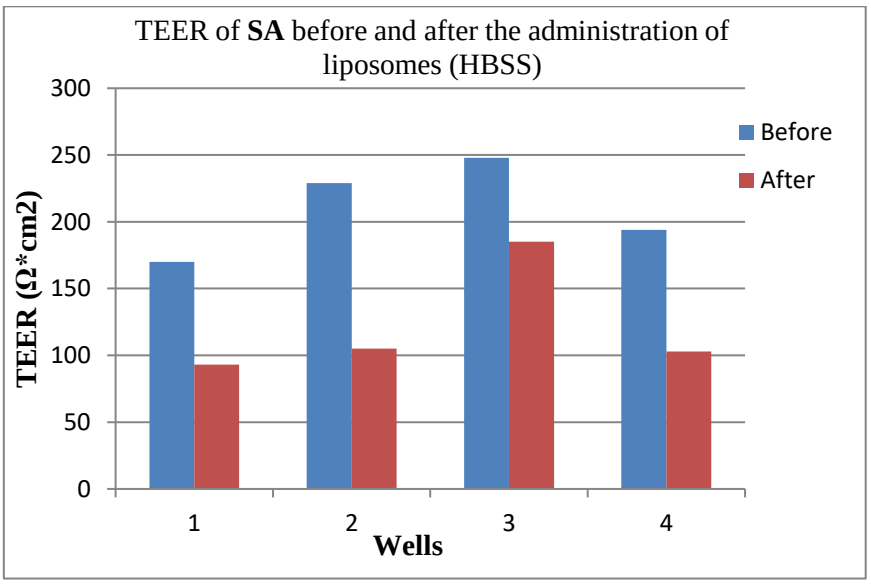


Figure 3.24: TEER of SA before and after the administration of liposomes.

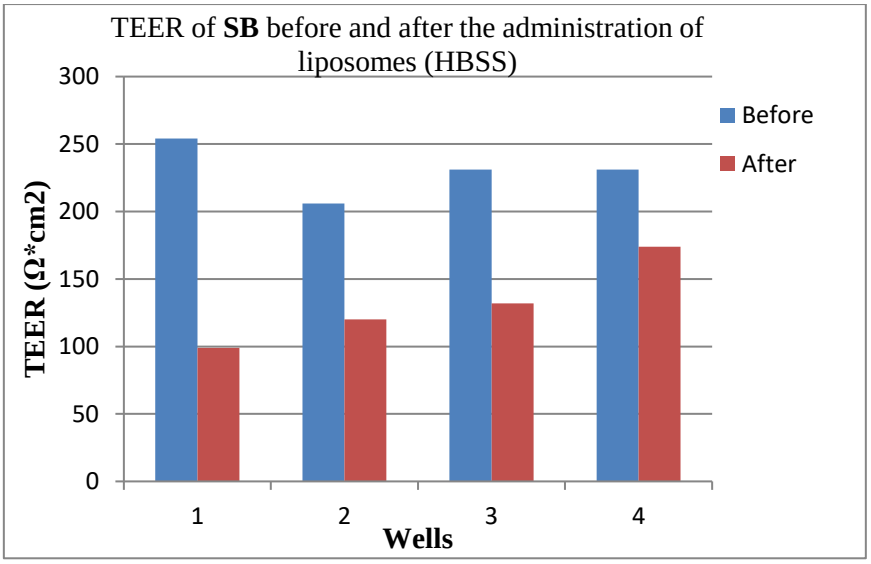


Figure 3.25: TEER of SB before and after the administration of liposomes

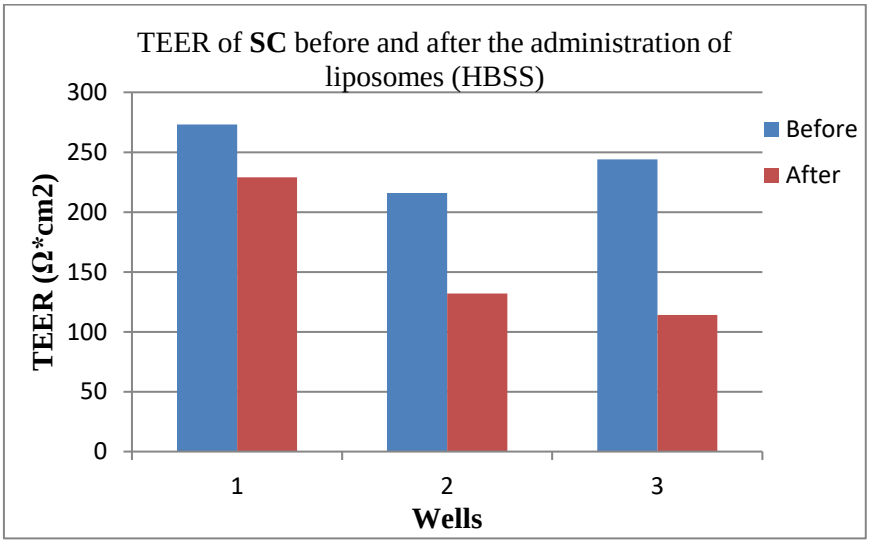


Figure 3.26: TEER of SC before and after the administration of liposomes

According to the results presented above, TEER values decreased compared to the values of day 21 (Table 3-12). This decrease is probably due to the washing of cells and changes of media, as these procedures may cause a rupture in the membrane of the cell culture insert or detaching of cells. Therefore, changes of media may lead to unequal density through the cell membrane and disturbance of tight junctions. Variations in TEER value can also arise from factors such as temperature, medium formulation and passage number of cells (B. Srinivasan 2015). Additionally, according to the literature, an important factor regarding drop of TEER values is the formation of pores on the cell membrane and not the binding of compounds to the cell membrane itself (M. M. Cajnko 2015). A small decline of TEER value during liposome permeability experiments using

small intestinal epithelial cell models has been reported in precious studies and is attributed mostly to alterations of media that cause increased cell activity (X. Zhang 2014). However, the decrease of TEER value observed on day 22 does not indicate an opening of tight junction through which liposomes could permeate the monolayer.

### 3.5.2 Intestinal Permeability Assay

By virtue of the satisfactory results from TEER measurements on day 21, permeability measurements were performed on this day. The transport medium in the wells was HBSS. HBSS provides the appropriate salts and pH for the intestinal epithelium in order to maintain its integrity along the permeability experiments. Due to the fact that the liposomal systems were almost non-toxic for the cells even at the highest concentration (15% v/v) used during the MTT assay, the same concentration was used for the permeability study. In order to have this concentration in the upper compartment, 15% of the HBSS volume of the apical compartment was replaced with the same volume of the liposomal system. After the administration of liposomes, samples were taken from the basolateral compartment at 10min, 30min, 1h, 2h, 3h and 4h time intervals. The samples were finally measured photometrically at 544nm in order to quantify the amount of Rhodamine B that permeated the constructed epithelia. Two wells were used as blank and contained only Rhodamine B. Three repetitions (1-3) for each system were performed.

System A (1-3): HSPC:EPC:CHOL:RHOD

System B (1-3): HSPC:EPC:DPPG:RHOD

System C (1-3): HSPC:EPC:DOTAP:RHOD

The results from permeability studies are presented below.

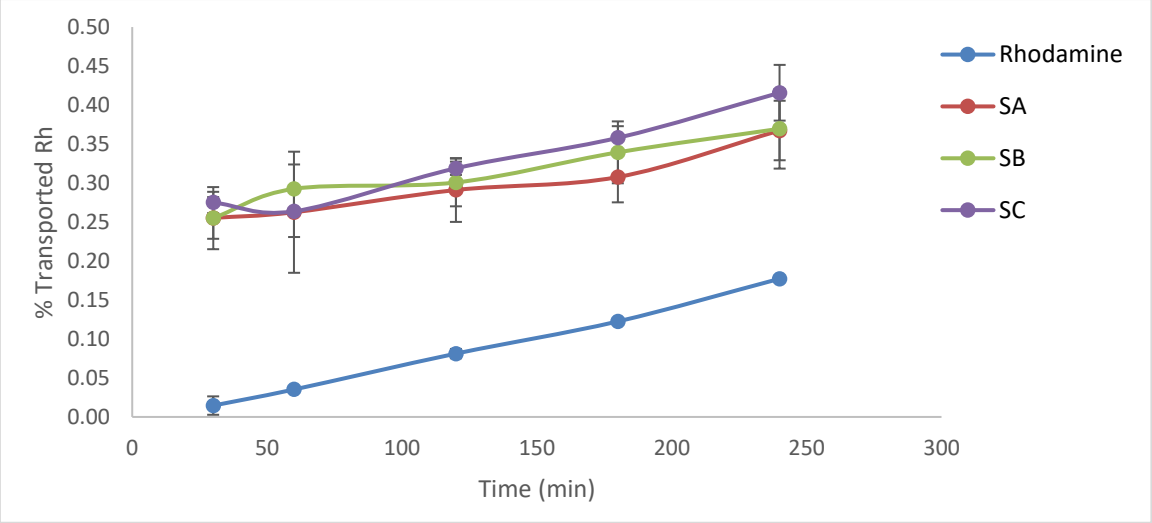


Figure 3.27: % transported Rhodamine B solution and Rhodamine B in systems SA, SB and SC versus time

| | | % Transported RhB | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Time(min)** | RhB | | SA | | SB | | SC | |
| 30 | 0,02 | 0,012 | 0,26 | 0,08 | 0,25 | 0,04 | 0,28 | 0,01 |
| 60 | 0,04 | 0,002 | 0,26 | 0,08 | 0,29 | 0,03 | 0,26 | 0,03 |
| 120 | 0,08 | 0,006 | 0,29 | 0,04 | 0,3 | 0,03 | 0,32 | 0,01 |
| 180 | 0,12 | 0,002 | 0,31 | 0,03 | 0,34 | 0,04 | 0,36 | 0,01 |
| 240 | **0,18** | 0 | **0,37** | 0,04 | **0,37** | 0,05 | **0,42** | 0,03 |

Table 3-15: % transported Rhodamine B solution and Rhodamine B in systems SA, SB and SC versus time

According to Figure 3.27, all systems A, B and C were transported across the epithelial cells at a much higher percentage compared to the solution of Rhodamine B. More specifically, at the end of the experiment (240 min) only 17% of RhB solution had been transported in contrast with liposomal systems A,B and C that managed to transport approximately 37 to 42% Rhodamine B. Among the three systems, system C, namely the system containing the positively charged lipid DOTAP presented the highest percentage of transport of Rhodamine B (approximately 41%) but not much higher than systems A and B. These two latter systems managed to transfer the same amount of Rhodamine B across the cell monolayer at the end of the study (approximately 37%). The slightly higher percentage associated with system C can be due to electrostatic forces developed between the

liposomal system and Caco-2 cells, however the exact mechanism of the permeation needs to be further investigated.

Therefore, the extent of permeability of Rhodamine B encapsulated in liposomes was approximately 2-2,5 times higher compared with that of RhB solution.

# 4 DISCUSSION

On the day of their preparation, all liposomal systems presented sizes between 80 and 180 nm and PDI between 0,3 and 0,6. The values of ζ-potential on the day of their preparation indicate that the most stable systems where the ones containing charged lipids, namely DPPG and DOTAP, probably due to the development of electrostatic forces on the liposomal surface.

All liposomal systems prepared in this study where fully characterized in terms of physicochemical characteristics. The liposomal systems composed of only one lipid, namely HSPC and EPC liposomes showed a satisfactory physicochemical profile with sizes up to 120 nm and PDI until 0,6. The similarity of the profile of these systems can be explained by the similarity of the structure of the phospholipids that compose them. The combination of these lipids in one liposomal system, HSPC:EPC resulted in larger sizes, high PDI values and poor stability. However, the addition of cholesterol in the latter system improves the physicochemical characteristics. HSPC:EPC:CHOL liposomes are smaller, more stable overtime, thanks to the presence of cholesterol that acts as fluidity regulator of the membrane. Additionally, the two charged liposomal systems, HSPC:EPC:DPPG and HSPC:EPC:DOTAP presented similar physicochemical profile, with sizes and PDI no larger than 160 and 0,7 respectively.

These results are in accordance with the literature and DLVO theory. The repulsive forces that are developed between the liposomes due to the charged phospholipids overcome their tendency to aggregate, namely the formation of larger particles. The fusion of liposomes into larger particles is a result of the dominance of the attractive van der Waals interactions over the repulsive hydration forces. The strong van der Waals attraction forces compete with the repulsive hydration forces, which develop at distances of less than 3 nm between adjacent bilayers. The predominance of attractive van der Waals interactions is due to the irreversible adhesion of the adjacent liposomes bilayers, while the repulsive hydration forces are thought to result from the attachment of water molecules to hydrophilic surfaces such as the polar surfaces of the liposomes.

Therefore, the predominance of repulsive forces on HSPC and EPC liposomes is probably higher than that of the rest of the liposomal systems prepared in this study, resulting in

the former having slightly improved physicochemical characteristics. It can be concluded that among the systems prepared in this study, HSPC and EPC liposomes presented the best stability.

Regarding HSPC:EPC system, the lack of stability can be attributed to the high rigidity and the lack of fluidity due to the presence of the two phospholipids and the absence of a fluidity regulator or a surfactant. The decrease of fluidity, along with an augmentation of attractive van der Waals interactions due to the absence of electrostatic interactions, leads to fusion of liposomes towards the creation of aggregations.

The liposomal system containing cholesterol, HSPC:EPC:CHOL showed better physicochemical characteristics than the system without cholesterol, HSPC:EPC, with sizes and PDI values no bigger than 200 nm and 0,7 respectively. This improvement is due to the presence of cholesterol and its unique role to act as a regulator of the fluidity of the liposomal membrane. Therefore, the suitable amount of cholesterol is able to ameliorate the physicochemical profile, enhance the stability and improve the functionality of a liposomal system.

Additionally, charged liposomes, HSPC:EPC:DPPG and HSPC:EPC:DOTAP presented a satisfactory physicochemical profile. Both systems did not exceed sizes of 160 nm and PDI value 0,7. They both presented high values of ζ-potential which indicate an adequate stability. Despite the coexistence of various different lipids, both systems showed good stability overtime, which can be attributed to the presence of charged lipids DPPG and DOTAP. These two lipids contribute to the development and maintenance of electrostatic forces able to overcome the tendency of liposomes towards aggregation. Therefore, these properties made them useful for further toxicity and permeability studies.

The application of DSC in the liposomal systems of this study demonstrated the thermal behavior and stability characteristics of the systems. DSC in this study confirmed the results acquired by the stability study and provided extra information on the thermodynamic characteristics of the systems. The analysis of liposomal systems with DSC offers in depth knowledge on the behavior and phase transitions of these systems. This information is important towards the comprehension of the cooperation of biomaterials and the preparation of stable and functional nanosystems. DSC is a useful

tool in order to examine the possible combinations and cooperation of different materials and therefore make steps towards the design and development of thermodynamically stable and effective drug delivery systems.

Furthermore, according to the results obtained by the MTT assay, all liposomal systems prepared in this study are not at all or are slightly toxic for the cell co-culture of Caco-2/TC7 and HT29/MTX, even at the highest concentrations used (15% v/v). The maximum toxicity observed provoked approximately 20% reduction in the cell viability, which is still a satisfactory percentage of viability. The majority of liposomal systems at 72 hours did not induce any cytotoxic effect, due to the biocompatibility of lipids that compose the liposomes. Overall, the toxicity profile of the liposomal systems and the results obtained are very encouraging for their use as delivery systems of drugs and bioactive compounds through the oral administration route. In order to evaluate their efficacy as bioavailability enhancers the systems HSPC:EPC:CHOL:RHOD, HSPC:EPC:DPPG:RHOD and HSPC:EPC:DOTAP:RHOD were used for permeability studies. More specifically, the co-culture model was cultured in special filters and the permeability of encapsulated Rhodamine was examined.

The results of the permeability study indicate that liposomal systems permeate intestinal cell models and transport Rhodamine B across Caco-2 cells at a higher rate compared to the solution of Rhodamine B. Despite the difficulties that liposomes have to face not only to permeate the intestinal epithelium but also to reach it passing through the gastrointestinal tract without degradation, these results suggest that once the liposomes reach the intestinal epithelium, the amount of the substance they carry can permeate the cells at a higher level compared to the solution of the substance itself. Appropriate modifications of the liposomal surface lipidic composition of the liposomal bilayer could probably be a way to further enhance the permeability through the intestinal epithelium and needs to be further investigated. However, in depth research of the mechanisms that govern liposomal absorption through intestinal cell models needs to be accomplished so as to design accordingly liposomes that target those mechanisms. Overall, the results of this study are encouraging regarding liposomal permeability across Caco-2 intestinal cell models. Future *in vivo* experiments are required to assess the significance of liposomal systems in intestinal permeability studies.

# 5 CONCLUSION

This study aimed to investigate the toxicity and permeability of liposomal systems in intestinal epithelial cell models. The results indicate that liposomes are a promising drug delivery system regarding the absorption via the intestinal epithelium as they enhanced the permeation of rhodamine B compared to the solution of rhodamine B. To conclude, liposomes were no toxic to the epithelial cells and permeability studies showed encouraging results regarding their future use per os. Nevertheless, further research and *in vivo* experiments are required in order to overcome barriers that limit the use of liposomes per os until today.

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