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Influence of environmental tonicity changes on lipophilic drug release from liposomes

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Thesis for the degree Master of Pharmacy 2018

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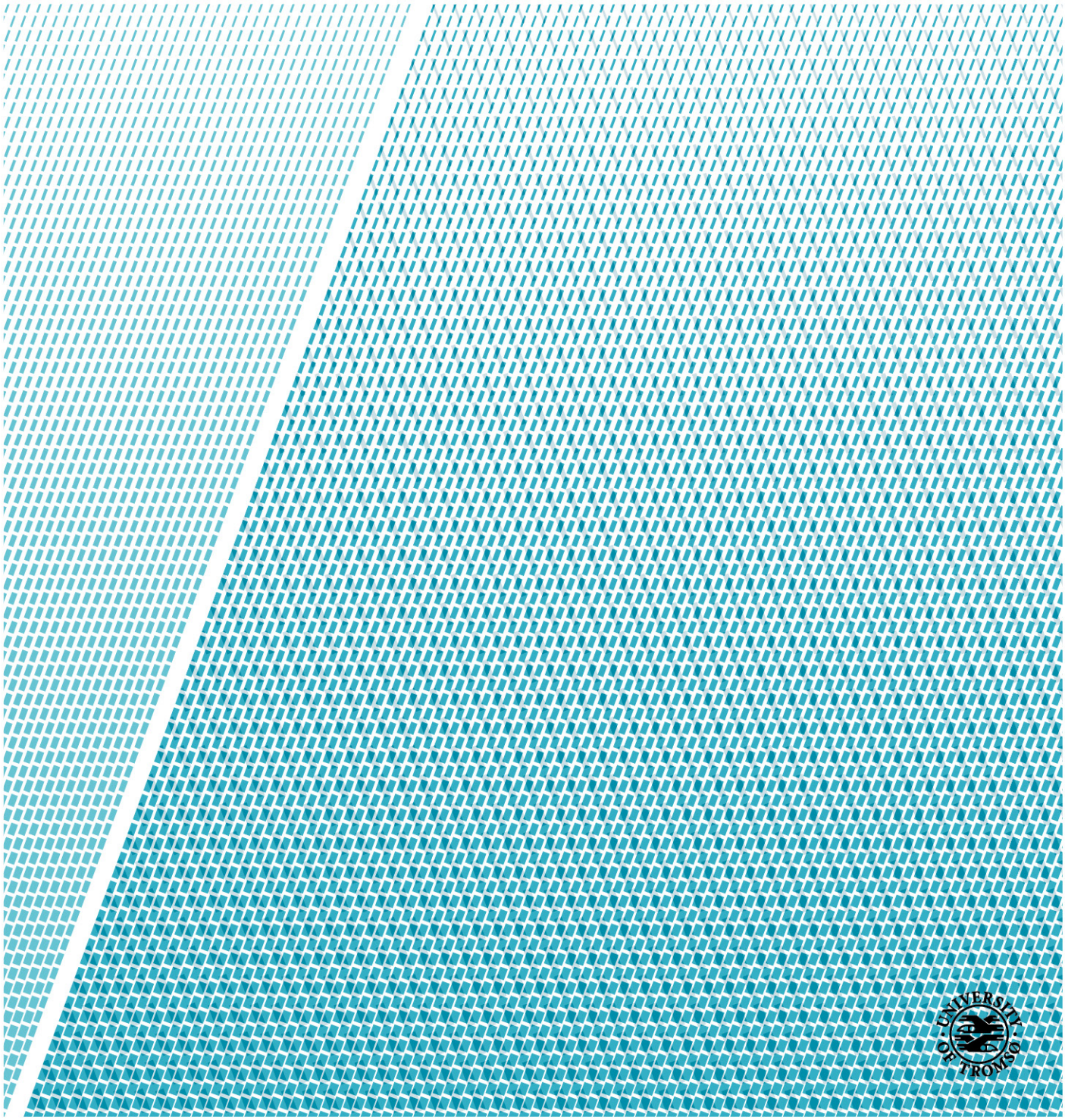

Acknowledgements

The research presented in this thesis was carried out at the Drug Transport and Delivery Research Group, Department of Pharmacy, University of Tromsø – The Arctic University of Norway from September 2017 to May 2018.

I would like to start by expressing my sincere gratitude to my supervisors associate professor Massimiliano Pio di Cagno and PhD student Iren Yeeling Wu for all their valuable guidance, advice, support and help during this project. I would also thank to the whole Drug Transport and Delivery Research Group for making me feel welcome and for all the help and support that has been given during this project.

I would like to thank InnoME GmbH for donating Permeapad® barriers and Lipoid GmbH for the donation of lipid samples.

I would like give thanks the graduating classes Bachelor of Pharmacy 2016 and Master of Pharmacy 2018 for many great years of studying together.

I would like to offer my sincerest thanks to the "The Office" we have had late nights together, lots of laughs and a few tears. The loving support and red wine have helped me to get through this period.

Last but not least, I am grateful to my family and friends for always supporting me with kind words of encouragement and always having my back when things seem most dire. I could not have made this without you!

Tromsø, 13.05.2018

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List of abbreviations

PDI	Polydispersity index
ZP	Zeta potential
SPC	phosphatidylcholine from soy.
PBS	phosphate buffered saline solution.
HPLC	High performance liquid chromatography
PBS300	PBS solutions with tonicity of 300 mOsm.
PBS190	PBS solutions with tonicity of 190 mOsm.
PBS65	PBS solutions with tonicity of 65 mOsm.
HC	Hydrocortisone
MP	Methylprednisolone
LUVs	large unilamellar vesicles
LUV300	large unilamellar vesicles made in PBS300.
LUV65	large unilamellar vesicles made in PBS65.
HC-LUVs	hydrocortisone incorporated LUVs.
HC-LUVs	hydrocortisone incorporated LUVs.
MP-LUVs	methylprednisolone incorporated LUVs.
MP-LUVs	methylprednisolone incorporated LUVs.
HC-LUVs	hydrocortisone incorporated LUV300.
HC-LUV65	hydrocortisone incorporated LUV65.
MP-LUV300	methylprednisolone incorporated LUV300.
MP-LUV65	methylprednisolone incorporated LUV65.
HC-SOLU300	Saturated solution of hydrocortisone in PBS300.
HC-SOLU65	Saturated solution of hydrocortisone in PBS65.
MP-SOLU300	Saturated solution of methylprednisolone in PBS300.
MP-SOLU65	Saturated solution of methylprednisolone in PBS65.

Sammendrag

Introduksjon: Liposomer er ofte kjent for å frakte legemiddel rundt i kroppen. De blir anvendt på grunn av deres egenskaper til å løse opp tungt løselige legemidler, forminsker bivirkninger ved blant annet kreft behandlinger og øker legemidlers levetid/terapeutisk effekt i kroppen før de blir eliminert. Første gangen liposomer ble nevnt var for mer enn 60 år siden. Det har tidlig vært diskutert at disse liposomene kan skrumpe eller svelle på grunn av miljøforandringer. Det er ikke før i de siste årene at dette fenomenet har blitt studert i større omfang, og nå er det blitt påvist at størrelsen på liposomer blir påvirket av osmotisk trykk.

Mål: Målet med oppgaven var å se hvordan fosfatbuffere av ulik tonisitet kunne forandre frigjøringsprofilen av to lipofile legemidler fra liposomer besående av ett dobbelt lag av lipid (LUVs).

Metoder: LUVs ble tillaget ved å fordampe av løsemiddelet til en fosfatbuffer-lipid-legemiddelløsemiddel-løsning. Liposomene ble deretter ekstrudert til ønsket størrelse og karakterisert ut ifra størrelse, størrelses distribusjon, overflatespenning og mengde inkorporert legemiddel i LUVs. For å forstå hvordan tonisitet påvirket størrelsen til liposomer, ble det ytre miljøet rundt LUVs forandret, og størrelse og størrelses distribusjon ble målt i isotont, hypertont og hypotont miljø. Permeabiliteten til hydrokortison og metylprednisolon fra løsninger eller LUVs ble studert ved hjelp av Franz celler igjennom en cellulose membran. I tillegg ble permeabiliteten av hydrokortison også studert igjennom en kunstig biologisk barriere, kalt Permeapad®.

Resultater og konklusjon: Vi observerte i denne oppgaven at LUVs skrumpet og svellet da tonisiteten i det ytre miljøet rundt liposomene forandret seg. Videre ble det observert at liposomene hadde en lettere tendens til å skrumpe enn å svelle. Vi kunne også observere at permeabiliteten til legemiddel (som var inkorporert i LUVs) igjennom cellulose membraner forandret seg dersom tonisiteten forandres. Det ble funnet signifikant lavere permeabilitet for både hydrokortison og metylprednisolon med et hypertont ytre miljø, men ingen signifikant forskjell i hypotont ytre miljø. Permeabilitetsstudiene gjennomført med Permeapad® fant en signifikant høyere permeabilitet av hydrokortison da det ytre miljøet ble forandret til et mer hypotont miljø.

Stikkord: Liposom, LUVs, hydrokortison, metylprednisolon, osmose, osmotisk trykk, tonisitet, isoton, hypertont, hypotont, permeabilitet, Permeapad®

Abstract

Introduction: Liposomes as drug delivery systems has been widely studied as a way to solubilize poorly soluble drugs, reduce side effects of chemotherapeutics and increase circulation time *in vivo*. Since the first descriptions of liposomes over 60 years ago, they have shown tendencies to shrink and swell when the external environment of the liposomes is altered. This phenomenon has been studied in recent years and it is now known that liposomes' shape is affected by osmotic pressure.

Aim: The aim of this thesis was to clarify if changing the tonicity (i.e. concentration of solutes) outside of large unilamellar vesicles (LUVs) would affect the release of lipophilic drugs from them.

Methods: LUVs were made by evaporating the solvent from a solution consisting of lipid, drug and solvents in phosphate buffered saline (PBS), and subsequently extruded to the desired sizes. The LUVs were characterized in terms of average diameter, size distribution, surface charge and percentage of drug entrapped inside the LUVs. To understand how tonicity affects the size of vesicles, the external environment of the LUVs was changed, and the size distributions of the LUVs were measured over time. The permeation properties of hydrocortisone and methylprednisolone were studied using Franz cells setup at different osmotic pressures using cellulose hydrate membranes or Permeapad® biomimetic barrier for both drug suspensions and drug-liposomal dispersions.

Results and conclusion: We demonstrated that the LUVs shrank and swelled when the tonicity of the external environment was altered. We also demonstrated that the permeability of drugs (incorporated into LUVs) through cellulose hydrate membranes were highly affected by changes in tonicity. A significantly lower permeability for both hydrocortisone and methylprednisolone from the liposomal formulation in hypertonic external environment was measured, whereas no significant changes were found for the hypotonic external environment. The permeability studies on hydrocortisone incorporated LUVs employing the, Permeapad® showed a significantly higher permeability when changing the tonicity to hypotonic environment.

Keywords: Liposomes, large unilamellar vesicles, LUVs, hydrocortisone, methylprednisolone, osmosis, osmotic pressure, tonicity, isotonic, hypertonic, hypotonic, permeability, Permeapad®

1 Introduction

1.1 Liposomes

1.1.1 What are liposomes?

Liposomes are a type of nano drug carrier which consists of spherical vesicles composed of phospholipid bilayers, typically dispersed in an aqueous medium. These vesicles can vary greatly in size and have one or more bilayer conformations in the barrier of the vesicles. Liposomal bilayers are primarily comprised of biocompatible constituents such as natural phospholipids, synthetic phospholipids, cholesterol and/or polymers (Alavi *et al.*, 2017; Xu *et al.*, 2016). Phospholipids used in liposomal formulations can be natural derivatives such as phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol. These are usually derived from a number of different sources such as chicken eggs, soy and bovine milk. Some vesicles can also be made by synthetic phospholipids such as dipalmitoyl phosphatidylglycerol, distearoyl phosphatidylcholine among others. There are also other constituents which can be incorporated within the phospholipid bilayers such as cholesterol which can be used to stabilize and increase the rigidity of phospholipid bilayers (Alavi *et al.*, 2017; De Gier *et al.*, 1968; Li *et al.*, 2015). Mucoadhesiveness of lipid bilayers can be increased with the addition of chitosan, whereas, increases *in vivo* circulation time of liposomal formulations can be achieved by PEGylation or addition of ligands for ligand based targeting (Allen and Cullis, 2013; Anwekar *et al.*, 2011; Li *et al.*, 2015).Liposomal formulations are appealing for drug delivery for different reasons. Liposomes are very efficient as solubilizing agents for poorly soluble drugs, inducing a drastic increase of apparent aqueous solubility for many poorly soluble drugs (di Cagno *et al.*, 2011; di Cagno and Luppi, 2013; Yang *et al.*, 2007). It has also been shown that liposomal formulations can reduce side effects of chemotherapeutic agents (Boulikas, 2004). Liposomal formulations can have longer circulation time *in vivo* and increase selectivity for particular areas of the body (Allen and Cullis, 2013; Brandl, 2001). When it comes to disadvantages of liposomal drug delivery systems there are higher production costs, problems with upscaling of preparation methods, time consuming preparation methods, oxidation and hydrolysis of the phospholipids and shorter half-life of the formulations *in vivo* (Alavi *et al.*, 2017; Anwekar *et al.*, 2011; Brandl, 2001; Samad *et al.*, 2007).

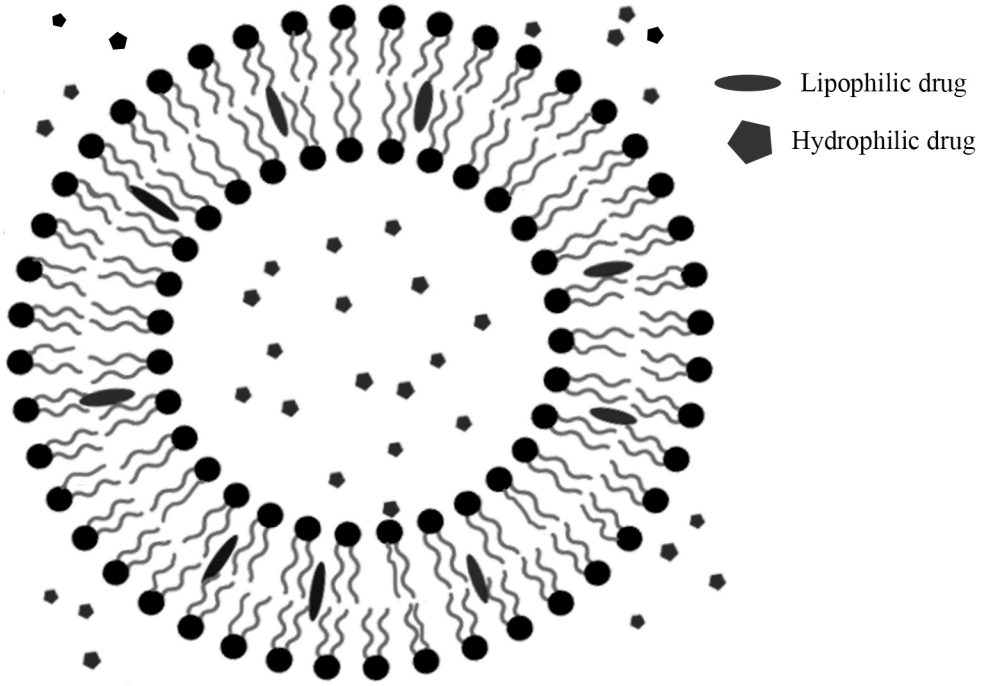

Figure 1: Graphic representation of a phospholipid-based liposome with hydrophilic and lipophilic drug incorporated into the aqueous core and lipid bilayer.

A variety of lipophilic, hydrophilic and amphiphilic drugs can be incorporated into liposomes. Hydrophilic drug would reside inside the aqueous core, hydrophobic drugs will be incorporated inside the hydrophobic region of the lipid bilayer, amphiphilic compounds will reside both inside the aqueous core and inside the lipid barrier (Brandl, 2001; Hupfeld *et al.*, 2006). The partitioning coefficients of drugs (logP) which indicates the ratio of drug in octanol/water mixture can be employed as a way to predict where drugs would be located into liposomes (inner core of bilayer). A high logP would mean that the drug is more lipophilic, and that drug is primarily incorporated into the lipid bilayer as opposed to the aqueous medium of the inner core. A logP value closer to zero could mean that the drug is of neutral partitioning properties and could be partly inside the aqueous core and partly inside the lipophilic area of the liposomal bilayer (Benet *et al.*, 2011; Leo *et al.*, 1971).

1.1.2 Size and shape of liposomes

Liposomal sizes generally range from 20 nm to several μm in diameter. Size plays a significant role in the biodistribution and circulation time of liposomal formulations *in vivo* as well as *in vitro* characteristics like aggregation of the individual liposomes, sedimentation and the amount of drug that can be entrapped inside the formulation (Hupfeld *et al.*, 2006; Samad *et al.*, 2007).

Liposomes are generally classified according their size and level of lamellarity as:

- Small unilamellar vesicles (SUVs)have a size range from about of 20-100 nm
- Medium sized unilamellar vesicles (MUVs) are >100 nm in diameter
- Large unilamellar vesicles (LUVs) have a size of >100 nm in diameter
- Giant unilamellar vesicles (GUVs) are > 1μm in diameter
- Multilamellar large vesicles (MLVs) size >500 nm, have between 5 and 25 lipid bilayers.
- Multilamellar vesicles (MV) size > 1μm in diameter and have multiple vesicles inside a lipid bilayer.

Photon correlation spectroscopy (PCS) or dynamic light scattering is a common approach to simultaneously measure vesicle sizes and polydispersity (size distribution) of liposomal dispersions. PCS works by scattering monochromatic light through a colloidal dispersion of particles. The light will then change the wavelength in correlation to the movement of the particles (Brownian movement) in the dispersion and size of the nanoparticles can be extrapolated from their movement (Kumar et al., 2016). The size and size distribution affects the amount of drug that is entrapped inside a formulation, aggregation and sedimentation of the liposomes. (Anwekar *et al.*, 2011; Brandl *et al.*, 1998; Hupfeld *et al.*, 2006)

1.1.3 Liposomal preparation methods

Many different methods have been designed for preparing liposomal formulations. The first method for making liposomes introduced was the "thin film method" by Bangham *et al.* (1965). It is the most common method utilized for liposomal preparations and it is based on the reconstitution of a thin film of lipids made by vacuum evaporation of solvents with aqueous media (Bangham *et al.*, 1965). Another method that can be used is the "ether vaporization method", where a lipid solution in highly volatile organic solvent (ethanol or ether) is injected into an aqueous medium through a fine needle to make liposomes (Samad *et al.*, 2007). An alternative method widely employed is the "reverse phase evaporation method". In this procedure, rotary evaporation is employed in order to evaporate solvents from a lipid/solvent mixture. The concentrated mixture is then rehydrated using a buffer solution to create liposomes (Samad *et al.*, 2007; Szoka and Papahadjopoulos, 1978). The preparation method used in this thesis was first described by Moscho *et al.* (1996), it is a variation of the "reverse-phase evaporation method". In short, chloroform/lipid solution and methanol are evaporated through an aqueous buffer solution by the means of rotary evaporation to form giant unilamellar vesicles (Moscho *et al.*, 1996).

1.1.4 Methods for size reduction of liposomes

Liposomal sizes could be reduced by a number of different methods, one of the most common ones is the extrusion of liposomes through polycarbonate membranes. In in short; an aqueous dispersion containing liposomes are extruded through polycarbonate membranes with fixed sized pores, some of the pore sizes available include 1.0, 0.8, 0.6, 0.4 and 0.2 μm. This method has been shown to give liposomal dispersions with a low polydispersity and an homogenous size distribution (Brandl, 2001; Olson *et al.*, 1979). The use of ultrasounds has been proven to be an efficient method in order to reduce vesicle sizes quite efficiently (>200 nm in diameter). The liposomal dispersion is sonicated using a probe for a set amount of time. This method works by generating shockwaves that form imploding gas/steam-bubbles. Upon implosion cavities are formed in the dispersions which breaks up the liposomes and makes them reconstitute into a smaller size (Brandl, 2001; Hupfeld *et al.*, 2006). An alternative technique for reducing the size of liposomal vesicles is the use of high pressure homogenizers, using the principle of cavitation same as the ultrasonication method. Here the liposomal dispersions are accelerated through a narrow gap which breaks up the liposomal vesicles and makes them reform into smaller vesicles. Microfluidizers use high pressure in combination with the principle of forming and imploding gas/steam-bubbles. The liposomal formulation is pressured through two micro channels which forces the liposomes to break up and reform in smaller sizes (Brandl, 2001).

1.1.5 Zeta potential

Surface charge of liposomes is generally quantified by the parameter zeta potential (ZP), This measurement of the electric potential (mV) is made on what is called the “slipping plane” in a colloidal dispersion. The “slipping plane” is the boundary between the stationary layer of charged particles around the dispersed particles and particles moving freely in the dispersion (Attard *et al.*, 2000; Coday *et al.*, 2015). ZP is important because charge in liposomal membranes either positive or negative can increase the electrostatic repulsion between the liposomes if they carry the same net charge, which could give a liposomal formulation better stability when it comes to aggregation and sedimentation (Narenji *et al.*, 2016). Electrostatic repulsion between liposomes which carry a net charge have been shown to reduce size and lamellarity of liposomes (Talsma *et al.*, 1992).

1.2 Osmosis and osmotic pressure

Osmosis is a natural phenomenon associated to the movement of water from a solution of low concentration through a semipermeable membrane to a solution of high concentration. This movement of water is induced by differences in chemical activity (i.e. concentration gradient) of solutes between two sides of a semipermeable membrane. Water molecules move from the side of the membrane with the lowest solute concentration (hypotonic environment) to the side with higher solute concentration (hypertonic environment) in order to equalize the chemical activity and therefore the concentration gradient. (Brandl *et al.*, 2008; Rasouli, 2016; Sinko, 2006) The phospholipid bilayer of liposomes is semipermeable, this means that osmosis can occur either from the inside of the liposome to the outside (efflux) or from the outside to the inside (influx) (Ohno *et al.*, 2009; Paula *et al.*, 1996; Rasouli, 2016). Osmotic pressure is a colligative property of solutions and is defined as the difference in pressure between a solution and pure liquid solvent across a membrane. It is the driving force of osmosis and is the force of water diffusing through a semipermeable membrane when trying to equalize the chemical activity of a system over a surface. J. Van 't Hoff were the first person to propose a law to calculate osmotic pressure. This is represented as Equation 1:

Equation 1

$$\pi V = nRT$$

Where π is the osmotic pressure, V the volume of the solution, n is moles of solutes, R is the gas constant and T is the absolute temperature.

The first van 't Hoff equation did however not take into account that molecules can be divided into sub particles in the case of e.g. ions which would give a higher osmotic pressure than non-ionizing solutes. The new equation given by van 't Hoff took in to account the van 't Hoff factor *i,* this is described as the number of electrolytes (ions) and nonelectrolytes yielded by dissociation of a molecule in a medium. Described as Equation 2:

Equation 2

$$\pi = iRTc,$$

where π is the osmotic pressure, i the number of ions a molecule dissociates into, R is the gas constant, t is the absolute temperature and c is the concentration of the solution (Sinko, 2006).

Osmolality is defined as mole of a solute dissolved in 1 kg of solvent and is measured in Osm/kg H_2O. The equation used for calculating the relative osmotic pressure (π_{rel}) between buffers of different osmolality is a variation of the van 't Hoff equation for calculation osmotic pressure (Koeppen and Stanton, 2013; Sinko, 2006) this is represented as Equation 3:

Equation 3
$$\pi_{rel} = R * T * (Osm_{(out)} - Osm_{(in)})$$

Where π_{rel} is the relative osmotic pressure, R is the gas constant, T is the absolute temperature and $Osm_{(out)} - Osm_{(in)}$ are the difference between external and internal osmolality (mOsm) (Wu *et al.*, 2017).

1.3 Liposomes and tonicity

Liposomes can shrink and swell due to altered tonicity. This phenomenon has been studied over the years and it has been showed that it is possible to induce changes in the size of liposomes by changing the tonicity on the outside of the liposomes in a dispersion. This happens as a result of water movement induced by changing the tonicity of the dispersing medium from an isotonic environment to either a hypertonic- or a hypotonic environment. The osmotic pressure caused by changing the tonicity can draw water out of the aqueous core of the liposome if the external environment is hypertonic, causing the liposomal bilayer to contract and shrink. Osmotic pressure can also draw water into the core when the external environment of the liposome is hypotonic, causing the liposomal bilayer to expand (Ahumada *et al.*, 2015; Alam Shibly *et al.*, 2016; Bangham *et al.*, 1967; Biondi *et al.*, 1991; Hallett *et al.*, 1993; Mui *et al.*, 1993; Polozov *et al.*, 2001; Rutkowski *et al.*, 1991; Sun *et al.*, 1986). These findings are corroborated by a recent study done employing the liposomal formulation and phosphate buffered solutions which is going to be used in this study (Wu *et al.*, 2017). Sabín *et al.* (2006) found that increasing the cationic (*e.g.* Na^+) concentration on the outside of a liposomal membrane, an osmotic force would be created driving water out through the liposomal membrane and create shrinkage of the liposomes. The authors suspected this was due to the low permeability of cations through the liposomal bilayer (Sabín *et al.*, 2006). However, membrane thickness and size of the lipophilic region of the lipid bilayer of liposomes seem to have an effect on the permeability of cations as well (Paula *et al.*, 1996).

1.4 Drug permeation across barriers

Passive diffusion is a process in which drug moves from areas of high concentration to areas of lower concentration spontaneously to equalize the concentration of a solution.

This allows molecules to pass through a membrane by following the concentration gradient of the drug when it moves from a place of high concentration to a place of lower concentration (Brandl *et al.*, 2008; Sinko, 2006). This movement of molecules over a membrane can be measured for instance by a Franz diffusion cell, where a donor chamber (place of high drug concentration) and an acceptor chamber (place of low drug concentration) are separated by a membrane in which the drug molecule can diffuse through. This movement of drug molecules from the donor chamber to the acceptor chamber per time unit, times the inverse of the membrane area (in contact with the solutions) is called the flux. The flux (j) of a drug can be calculated from the linearly part of a curve where the cumulative amount of diffused drug is plotted against the time (Brandl *et al*., 2008). The flux is represented by Equation 4:

Equation 4

$$j = \frac{\Delta m}{\Delta t} * \frac{1}{A}$$

Where j represents the flux, $\Delta m/\Delta t$ is the mass transfer (number of drug particles diffusing through a membrane per time unit) and A is the surface area of the diffusion membrane.

The apparent permeability (P_{app}) of a drug through a membrane normalizes the flux over the concentration in the donor compartment. P_{app} can be calculated by Equation 5 as:

Equation 5

$$P_{app} = \frac{j}{c}$$

j represents the flux and c the concentration of solubilized drug in the donor compartment.

1.5 Permeapad® barrier

The Permeapad® biomimetic barrier was first described by di Cagno *et al.* (2015) as a method for screening the permeability of drugs through artificial membranes. There are several different types of *in vitro* drug release assays to experimentally determine how permeable a drug can be *in vivo.* Some of the methods include the Parallel Artificial Membrane Permeability Assay (PAMPA), the Phospholipid Vesicle-based Permeation Assay (PVPA). These assays employ phospholipids either dissolved in solvent and dried (PAMPA) or as a phospholipid vesicle-based layer (PVPA) on a filter support (Flaten *et al.*, 2006; Kansy *et al.*, 1998). Another *in vitro* method for determining *in vivo* drug release is the caco-2 model which employs grown cell cultures in a monolayer formation on top of polycarbonate membranes (Hidalgo *et al.*, 1989).

The Permeapad® barrier is comprised of a thin layer of lipid in between two supporting layers. This model works on the principle of hydrating the lipids between the supporting layers to make a spheroid like structure with lipid bilayers running through it, mimicking a cell membrane (Berben *et al.*, 2018; di Cagno *et al.*, 2015; di Cagno and Bauer-Brandl, 2016). The background for developing the Permeapad® was to find a fast, cost effective and reliable method for determining permeability of drugs through barriers resembling *in vivo* biological barriers. The Permeapad® have been shown to give similar permeability estimates when compared with previously established methods of *in vitro* permeability assessment such as the PVPA and PAMPA Assays. The Permeapad® has been found compatible with a variety of different surfactants, solvents and biomimetic mediums as well as being resistant to fluctuations in pH and give reliable results at different pH levels (Bibi *et al.*, 2015; di Cagno *et al.*, 2015). For these reasons have the Permeapad® been chosen as the assay used in this work.

2 Aim of the study

The aim of this thesis was to clarify if changing the tonicity (i.e. concentration of solutes) on the outside of large unilamellar vesicles (LUVs) would affect the release of lipophilic drugs from them.

3 Drugs, materials, equipment and software

3.1 Drugs

Table 1: Molecular formula, molecular weight (MW), partition coefficient (LogP), ionization constant (pKa) and measured solubility for the investigated drugs.

	Formula	MW (g/mole)	Log P	pKa	Solubility (mg/mL)
Hydrocortisone	$C_{21}H_{30}O_5$[a]	362.46[a]	1.6[c]	12.48[e]	0.420[c]
Methylprednisolone	$C_{22}H_{30}O_5$[b]	374.47[b]	1.80[d]	12.46[f]	0.323[c]

[a]Sigma-Aldrich, (n.d.)
[b]Sigma-Aldrich, (n.d.)
[c]Benet *et al.* (2011)
[d]Grabowski *et al.* (2010)
[e]Dursch *et al.* (2014)
[f]Calculated value using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2017 ACD/Labs)

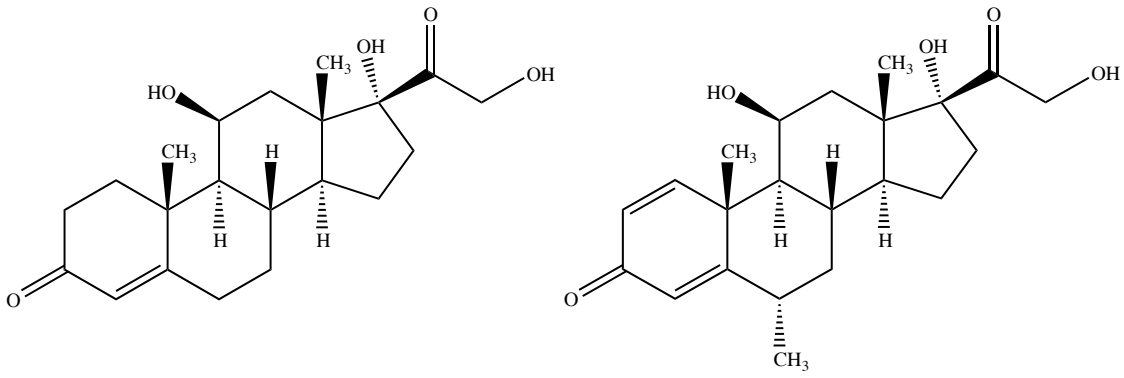

Figure 2: Chemical structures of hydrocortisone (left) and methylprednisolone (right).

3.2 Materials and chemicals

Table 2: Materials and chemicals used in the studies and their producers.

Materials	Producer
Acetic acid (≥99.8%)	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Cellulose hydrate membrane (Visking dialysis tubing, MWCO 12-14 kDa)	Medicell Membranes Ltd (London, UK)
Chloroform (≥99%)	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Hydrocortisone	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Lipoid S100 (soy phosphatidylcholine S100, >94%)	Lipoid GmbH (Ludwigshafen, Germany)
Methanol (≥99.9%)	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Methylprednisolone	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Permeapad® barrier	InnoME GmbH (Espelkamp, Germany)
Sodium chloride (≥99.5%)	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Sodium hydroxide (≥98%)	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Sodium phosphate dibasic dihydrate (98.5-101.0%)	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Sodium phosphate monobasic monohydrate (≥99.0%)	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)

3.3 Equipment

Table 3: Equipment used in the studies and their producers.

Equipment	producer
Acrodisc® Syringe filter with Supor® membrane (0.45 µm)	PALL (New York, USA)
Filter holder (Swinnex-25 Millipore) for Whatman® nuclepore™ membranes	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Folded capillary zeta cells	Malvern Instruments Ltd. (Worcestershire, UK)
Franz cell 5 mL acceptor volume (jacketed flat ground; pinch clamp and stir bar with a Standard diffusional area of 0.64 cm^2),	PermeGear Inc. (Pennsylvania, USA)
Franz cell 6 station stirrer (V6-CA)	PermeGear Inc. (Pennsylvania, USA)
Milli -Q® gradient, with Millipak® M 0.22µm filter	Millipore Corporation (Damstadt, Germany)
Osmometer (Semi-Micro k-7400)	Knauer (Berlin, Germany)
pH-meter (SensION™ + PH 31)	Hach (Barcelona, Spain)
Refrigerated/heating circulator (Julabo F12)	JULABO GmbH (Seelbach, Germany)
Refrigerated/heating circulator (Julabo 200F)	JULABO GmbH (Seelbach, Germany)
Rotavapor (Büchi R-124)	Büchi Labortechnik AG (Flawil, Switzerland).
Shaking water bath (GFL 1086)	Gemini BV (Apeldoorn, Netherlands)
Square polystyrene cuvettes (12 mm)	Malvern Instruments Ltd. (Worcestershire, UK)
Sterile syringe filter with polyetersulfone (PES) membrane (0.2 µm)	VWR International (Pennsylvania, USA)

Ultracentrifuge (Beckman model L8-70 M with SW 60 Ti rotor)	Beckman Instruments (California, USA)
UV reader (SpectraMAX® 190 Microplate reader)	Molecular devices (California, USA)
UV plate (Costar® 96-well)	Corning (New York, USA)
Vacuum pump (Büchi V-500)	Büchi Labortechnik AG (Flawil, Switzerland).
Water bath (Büchi B-480)	Büchi Labortechnik AG (Flawil, Switzerland).
Waters 2690 separation module	Waters Corporaton (Milford,USA)
Waters 996 Photodiode array UV-vis detector	Waters Corporaton (Milford,USA)
Whatman® Nuclepore™ Track-Etched Membranes (0.4 µm pore size)	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Whatman® Nuclepore™ Track-Etched Membranes (0.8 µm pore size)	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
XTerra™ RP 18[5] µm 3.9x150mm colum	Waters Corporaton (Milford,USA)
Zetasizer Nano Zen 2600	Malvern Instruments Ltd. (Worcestershire, UK)

3.4 Computer software

Table 4: Software used in the studies together with versions of the software and producers.

Name of software	Version of software	Producer
Millennium[32]	v3.20	Waters Corporaton (Milford,USA)
SoftMax® Pro	v5	Molecular devices (California, USA)
Zetasizer Software	v7.11	Malvern Instruments Ltd. (Worcestershire, UK)

4 Methods

4.1 Phosphate buffered saline (PBS) preparation

Phosphate buffered saline with an osmolality of 300 mOsm (PBS300) was prepared following a method previously described Wu *et al.*, (2017). In short, 22.5 g sodium phosphate monobasic monohydrate, 36.8 g sodium phosphate dibasic dihydrate, 22.9 g sodium chloride and 3.9 g sodium hydroxide were added to 5 L distilled water. The mixture was left stirred until the salts were completely dissolved. The pH of the buffer was adjusted to 7.4 (SensION™ + PH 31 pH meter, Hach, Barcelona, Spain) with sodium hydroxide. The tonicity was adjusted to 300 mOsm (Semi-Micro osmometer k-7400, Knauer, Berlin, Germany) with sodium chloride. PBS300 was diluted with distilled water in order to obtain two other buffer solutions (PBS190 (1.5:1 v/v), and PBS65 (1:4 v/v) respectively.

4.2 Preparation of large unilamellar vesicles

Large unilamellar vesicles were prepared following a method previously described by Wu *et al.* (2017). PBS solution (10 mL, PBS300 or PBS65) was added on top of an organic solution composed of drug/soy phosphatidylcholine (molar ratio 1: approx. 1.3) in $CHCl_3$ (1 mL) on top of methanol (0.2 mL) in a 50 mL round bottom flask. The organic solvents were gently removed by rotary evaporation (40°C, 40 rpm, Büchi R-124 rotavapor, Büchi vacuum pump V-500, Büchi B-480 water bath, Büchi Labortechnik AG (Flawil, Switzerland). The pressure was carefully reduced in increments of 100 mbar to 500 mBar, and then 10 mBar increments to 100 mBar (took approximately 20 min in total). After 90 min of evaporation at 100 mbar, giant unilamellar vesicles (GUVs) were formed. The GUVs were left over night at fridge temperature (2-8°C).

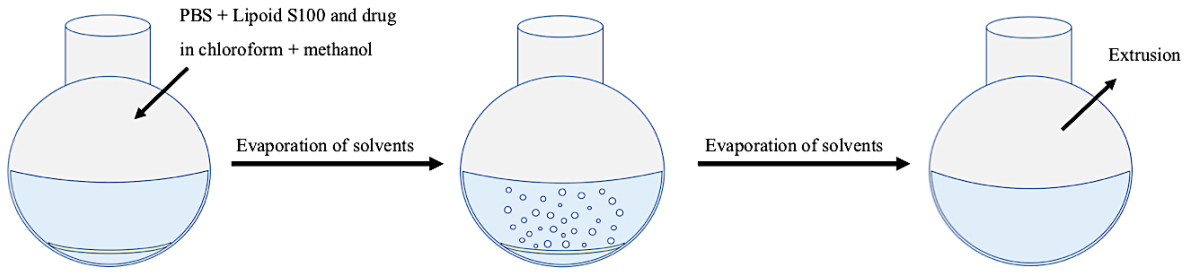

Figure 3: Graphic representation of liposomal preparation of GUVs by bubbling solvents containing drug + lipid through a phosphate buffered saline solution.

Room tempered (23-25°C) liposomal dispersions were then extruded through polycarbonate membranes (Nuclepore™ Track-Etched membrane, Sigma-Aldrich Chemie GmbH (Steinheim, Germany) of pore size 0.8 µm (5 times) and 0.4 µm (10 times) using a Swinnex-25 Millipore filter holder (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to reduce the liposomal sizes to large unilamellar vesicles (LUVs).

4.3 Osmotic activity determination

LUVs were exposed to different osmotic pressures in order to quantify at which extent LUVs sizes were affected by the exposure to different tonicities. Size analysis were performed employing the Zetasizer Nano Zen 2600 (Malvern industries, Worcestershire, UK). In brief, liposomal dispersions (500 µL) at room temperature (23-25°C) were filtered with 0.45 µm sterilized filter (Acrodisc® Syringe filter with Supor® membrane, PALL, New York, USA) and diluted 1:100 in PBS300 (alternatively, PBS65) in order to induce osmotic shock. Diluted LUVs were filtered through a 0.2 µm sterilized filter (Sterile syringe filter with PES membrane, VWR International, Pennsylvania, USA) prior to the size measurements. The experiments were conducted at time points: 0, 15, 30, 45 and 60 min in triplicates, each sample were measured thrice at each time point.

4.4 Characterization of liposomes

4.4.1 Liposomal size characterization

The sizes of the extruded LUVs were measured using photon correlation spectroscopy with an angle of 173°, using a square polystyrene cuvette (12 mm, VWR International) together with the Zetasizer Nano Zen 2600 (Malvern). The LUV dispersions were diluted 1:100 (v/v) in buffer solution of the same tonicity as the LUVs. Thereafter, the diluted dispersions were filtered using a 0.2 µm sterilized filter (VWR International) prior to measurement. Experiments were conducted in duplicates, and each sample were measured three times.

4.4.2 Zeta potential determination

The surface charge of the extruded LUVs was determined by measuring the zeta potential (ZP) using a folded capillary zeta cell from Malvern industries with the Zetasizer Nano Zen 2600 (Malvern) previously described by (Wu *et al.*, 2017)The LUVs were diluted 1:20 (v/v) with filtered deionized water (23-25°C, 0.2 µm pore size sterile syringe filter, VWR International) prior to measurement. Experiments were conducted in duplicates, and each sample were measured three times.

4.4.3 Entrapment efficiency determination

Drug entrapped inside the LUVs was quantified using a ultracentrifugation method previously described by Wu *et al.* (2017), in order to separate the entrapped drug inside the LUVs from unentrapped drug in the dispersion. Room tempered liposomal dispersion (23-25°C, 1 mL) were centrifuged for 30 mins with a speed of 38500 rpm at 10°C (*g*-force of 200 000) in a Beckman model L8-70 M ultracentrifuge with SW 60 Ti rotor, (Beckman Instruments, California, USA). The supernatant (with free drug) was carefully removed from the pellet containing the liposome-associated drug.

In order to determine the amount of drug inside the LUVs were the vesicles destroyed with a 50% methanol/PBS (v/v) solution. The drug concentrations were quantified using UV-visible spectroscopy on a clear polystyrene microplate with 96 wells (Corning, New York, USA) using a SpectraMAX® 190 microplate reader, with SoftMax® Pro v5 software (Molecular devices, California, USA). The analysis was performed at the maximum absorbance wavelength (λ_{max}) of 247 nm for hydrocortisone and 248 nm for methylprednisolone. The entrapment efficiency of the LUVs was calculated according to an equation described by Wu *et al.* (2017):

Equation 6

$$EE(\%) = \frac{M_{tot} - M_{free}}{M_{tot}} * 100$$

Where M_{tot} represent the total amount of drug in the liposomal dispersion, M_{free} the amount of unentrapped drug after ultracentrifugation. Experiments were performed in three parallels.

4.5 Standard curves

The standard curves for hydrocortisone (HC) were made form a 552 µM stock solution in PBS300 or PBS65. The stock solution was then diluted with pure PBS of same osmolality or 50% methanol/PBS (v/v) to make standard solutions with concentrations of 0, 5, 10, 20, 40, 60, 80 and 100 µM. For methylprednisolone (MP), 185 µM stock solution was prepared in 50% methanol/PBS300 (v/v), alternatively, PBS65 and diluted with 50% methanol/PBS to make a standard curve with the concentrations of 0, 1, 2.5, 5, 10, 20, 40, 60, 80 and 100 µM.

The standard curve concentrations were quantified by multi well plates for UV-visible spectroscopy employing Costar® UV 96 well plate (Corning) using the SpectraMAX® 190 microplate reader, with SoftMax® Pro v5 software (Molecular devices). The analysis was performed at the maximum absorbance wavelength (λ_{max}) of 247 nm for hydrocortisone and 248 nm for methylprednisolone.

4.6 Thermodynamic solubility determination

Saturated drug solutions (suspensions) of HC and MP (1 mg/mL) were prepared in PBS300 and PBS65. The suspensions were frequently vortexed and left in a water bath (GLF 1086 Shaking water bath, Gemini BV, Apeldoorn, Netherlands) at 35°C until thermodynamic equilibrium was reached. The drug suspensions (1 mL) were filtered using a 0.2 µm sterile syringe filter (VWR International). The concentration of the filtered solutions was quantified using UV-visible spectroscopy on a clear polystyrene microplate with 96 wells (Corning) using a SpectraMAX® 190 microplate reader, with SoftMax® Pro v5 software (Molecular devices). The experiments were done in triplicate for each of the drug suspensions.

4.7 Drug permeability and release studies

4.7.1 Drug permeability study of drug solutions using cellulose hydrate barriers

An *in vitro* drug permeability study was preformed using saturated drug solutions (to maintain sink conditions) from chapter 4.6 together with cellulose hydrate membranes. A Franz diffusion cell setup where employed according to a method previously described (Wu *et al.*, 2017). Jacketed flat ground Franz cells with 5 mL acceptor volume and standard 0.64 cm^2 diffusional area (PermeGear Inc., Pennsylvania, USA, USA) was used on a 6-station Franz Cell stirrer (PermeGear Inc., Pennsylvania, USA). This setup was connected to a Julabo refrigerated/heating circulator (JULABO GmbH, Seelbach, Germany). The temperature of the heating circulator was set to 40°C on the Julabo F12 (36°C on the Julabo 200F), to achieve a membrane temperature of approx. 35°C.

The saturated drug solutions from chapter 4.6 were used as the donor and 5 mL of PBS buffer of the same tonicity as the drug solutions were used in the acceptor chambers. The cellulose hydrate membranes (dialysis tubing, MWCO 12-14 kDa, Medicell membranes Ltd., London, UK) were hydrated in buffer (PBS300/PBS65) according to which buffer the solutions were made in. The study began by adding 0.8 mL of the saturated drug solutions to the donor chamber of the Franz cells. The study was preformed over 4 hours with sampling (0.5 mL) from the acceptor chamber every 0.5 hours over a period of 4 hours. The same amount of fresh PBS of same osmolality was returned to the acceptor chamber after sampling in order to maintain sink conditions. At the end of the experiment, samples of the acceptor chamber, content of the donor chamber and membranes were collected for analysis.

The membranes were suspended in 5mL of the respective PBS buffer for 30 min to quantify the amount of drug left on the membranes. The drug concentration in acceptor chamber, donor chamber and on the membranes were quantified using UV-visible spectroscopy in the SpectraMAX® 190 microplate reader with SoftMax® Pro v5 software (Molecular devices) as previously described at the end of chapter 4.4.3.

4.7.2 Drug release study using cellulose hydrate barriers

An *in vitro* release study employing the same Franz diffusion cell setup as chapter 4.7.1 were performed using hydrocortisone and methylprednisolone incorporated LUVs. The liposomal dispersions were tested with cellulose hydrate barriers (Medicell membranes Ltd.) on the Franz diffusion cell setup.

Table 5: Buffer type in the aqueous core of large unilamellar vesicles (LUVs) vs. the buffer type in the environment outside LUVs and calculated relative osmotic pressure (πrel) using Equation 3.

Buffer type		π_{rel} (bar)
Core of LUVs	**Environment outside LUVs**	
PBS300	**PBS65**	-6
PBS300	**PBS190**	-3
PBS300	**PBS300**	0
PBS65	**PBS65**	0
PBS65	**PBS190**	3
PBS65	**PBS300**	6

PBS buffer (5 mL, PBS300, 190 or 65) was added to each acceptor chamber in accordance with Table 5 to obtain the respective osmotic pressures. Cellulose hydrate membranes (Medicell membranes Ltd.) were hydrated in buffer (PBS300 or PBS65) according to which buffer the LUVs were made in. The study began by adding 0.8 mL of LUVs (2 mM total concentration) to the donor chamber on the Franz cells. Sampling (0.5 mL) from the acceptor chamber every 0.5 hours over a period of 4 hours were carried out. The same amount of fresh PBS of same osmolality was returned to the acceptor chamber after sampling in order to maintain sink conditions.

At the end of the experiment, samples of the acceptor chamber, content of the donor chamber and membranes were collected for analysis. The membranes were suspended in 5mL PBS for 30 min to quantify amount of drug left on the membrane. The concentration of drug in acceptor chamber, donor chamber and on the membranes were quantified using UV-visible spectroscopy in the SpectraMAX® 190 microplate reader (Molecular devices) as previously described at the end of chapter 4.7.1.

4.7.3 Drug permeability study of drug solutions using the Permeapad® barrier

The Permeapad® (InnoME GmbH, Espelkamp, Germany) biomimetic barriers were employed in an *in vitro* drug permeability experiment as described in chapter 4.7.1 using suspensions of hydrocortisone. The Permeapad® (InnoME GmbH, Espelkamp, Germany) biomimetic barriers were employed as the barrier between the donor and acceptor chambers instead of the hydrated cellulose membranes. The buffer solutions employed were PBS300 and PBS65 which were used in the acceptor chambers according to Table 5 for osmotic pressures of -6, 0 and 6. This study was performed as previously described in chapter 4.7.1 (n=3). At the end of the study, samples from the donor chamber and acceptor chamber where analysed as previously described at the end of chapter 4.7.1. The drug left on top of and inside the Permeapad® barriers were analysed with high performance liquid chromatography (HPLC) The method used for quantifying HC along with the settings represented in Table 6 where adapted from Adi-Dako *et al.* (2017).

Table 6: Settings for High-performance Liquid chromatography of hydrocortisone left on top of and inside the Permeapad® biomimetic barriers

Parameters	**values**
Flowrate	1 mL/min
Column temperature	25°C
Sample temperature	25°C
Injection volume	20 µL
Runtime	5 min
Detection wavelength	254 nm

In brief, a Waters 2960 separation module (Waters Corporation, Milford, USA) were used in conjuncture with an XTerra™ RP 18^5 µm (3.9x150mm, Waters) separation column and a Waters 996 photodiode array UV-vis detector (Waters). The mobile phase used during the experiment were made from methanol/Milli-Q water/acetic acid with a ratio of 60/30/10 (v/v/v). The standard curve for the HPLC where diluted from a 0.2 mM stock solution (hydrocortisone dissolved in the mobile phase), to concentrations of 0, 12, 25, 50, 75, 88, 100 µM. The Permeapad® barriers were also suspended in the mobile phase prior to measurement.

4.7.4 Drug release study using Permeapad® barriers

The same *in vitro* release experiments as chapter 4.7.2 were conducted on hydrocortisone incorporated LUVs using the Permeapad® (InnoME GmbH) barriers as the membrane between the donor and acceptor chamber on the Franz cells. The buffer solutions employed were PBS300 or PBS65 which were used in the acceptor chamber. This study was performed as previously described in chapter 4.7.2. At the end of experiment, samples from the donor chamber, acceptor chamber were analysed as previously described at the end of chapter 4.7.1.

4.8 Statistical analysis

Student's t-tests were employed to determine if there was a significant difference between the mean apparent permeability of two datasets ($p \leq 0.05$). Confidence interval (95%) was used to evaluate the time point a significance in drug permeability was observed between the environments within the same LUV formulation at different osmotic pressures.

5 Results

5.1 Buffer solutions

Tonicity and pH of the different PBS solutions employed in the experiments. Results are reported in Table 7:

Table 7: Measured pH, tonicity and calculated phosphate concentration of the phosphate buffered saline (PBS) solutions used in the studies *(average ± SD, n=≥4).

Buffer	pH*	Osmolality (mOsm)*	Phosphate concentration (mM)
PBS300	7.40 ± 0.04	300 ± 4	78
PBS190	7.47 ± 0.04	190 ± 7	47
PBS65	7.60 ± 0.05	65 ± 3	16

5.2 Liposomal size analysis and tonicity changes

The influence of tonicity on the sizes of LUVs was investigated by photon correlation spectroscopy (PCS). As shown in (Figure 4 and Figure 5), LUVs with hydrocortisone were exposed to isotonic, hypotonic and hypertonic environments in order to observe how the size distributions of the LUVs would be affected by tonicity changes.

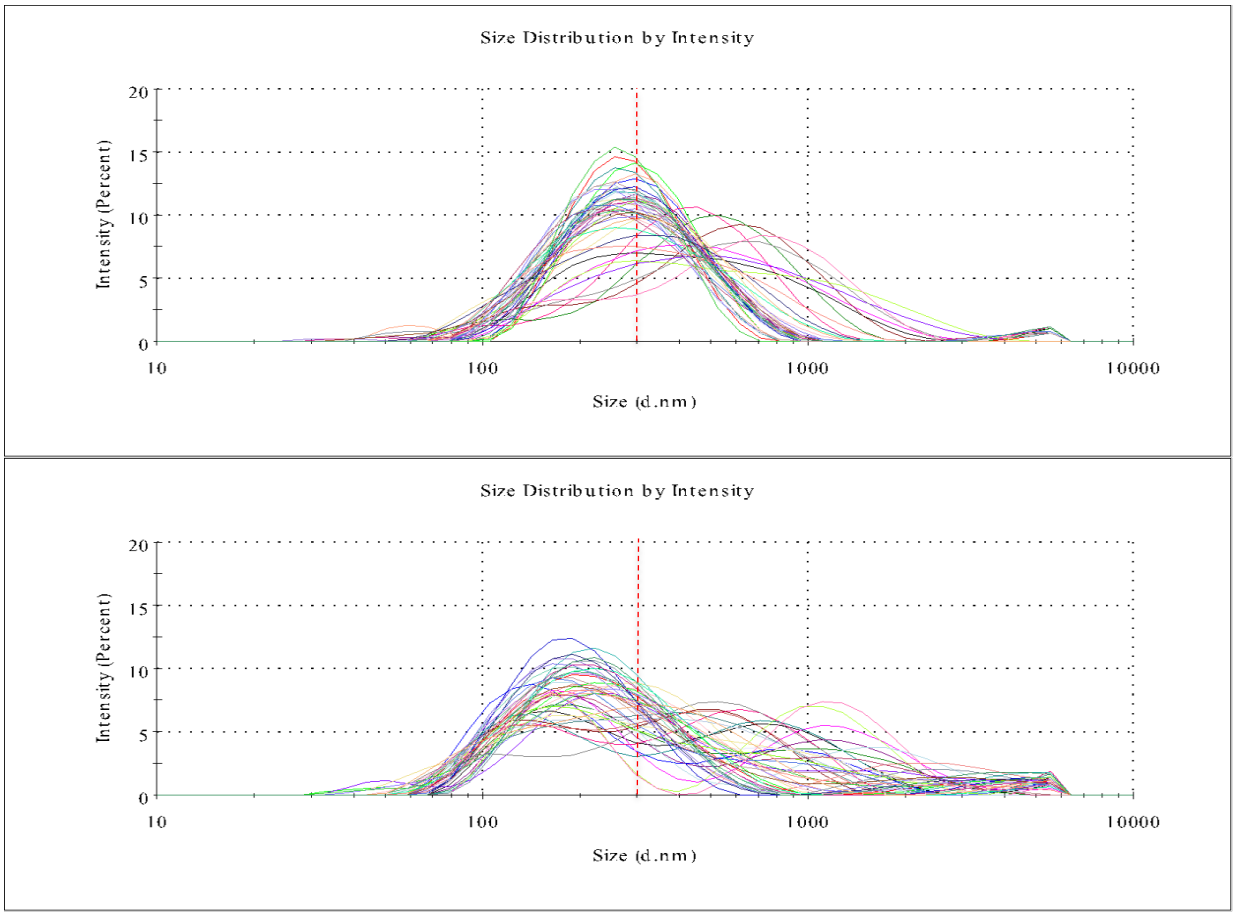

Figure 4: Size and distribution changes of hydrocortisone incorporated large unilamellar vesicles (LUVs) over 1 hour in isotonic environment (top) and when subjected to hypotonic environment (bottom). LUVs were prepared in phosphate buffered saline (PBS) solution of 300 mOsm and exposed to buffer with tonicity of 300 (top) or 65 mOsm (bottom), respectively. The dotted line marks 300 nm size (n=30)

Size distribution changes of HC-LUVs prepared in PBS300 where studied in isotonic environment (PBS300) and in hypotonic environment (PBS65) over a period of 1 hour (Figure 4). When the LUV dispersions prepared in PBS300 were exposed to isotonic environment (top graph), one peak of high intensity was observed at around 290-300 nm. When LUVs were exposed to the hypotonic environment (PBS65, bottom graph) a slight decrease in peak intensity (from 15% to 10 %) was observed at the same dimension (300 nm, red line in Figure 4). Moreover, the formation of peaks at the larger sizes (between 400-2000 nm range) where observed and a small (but noticeable) shift towards smaller size distribution was measured.

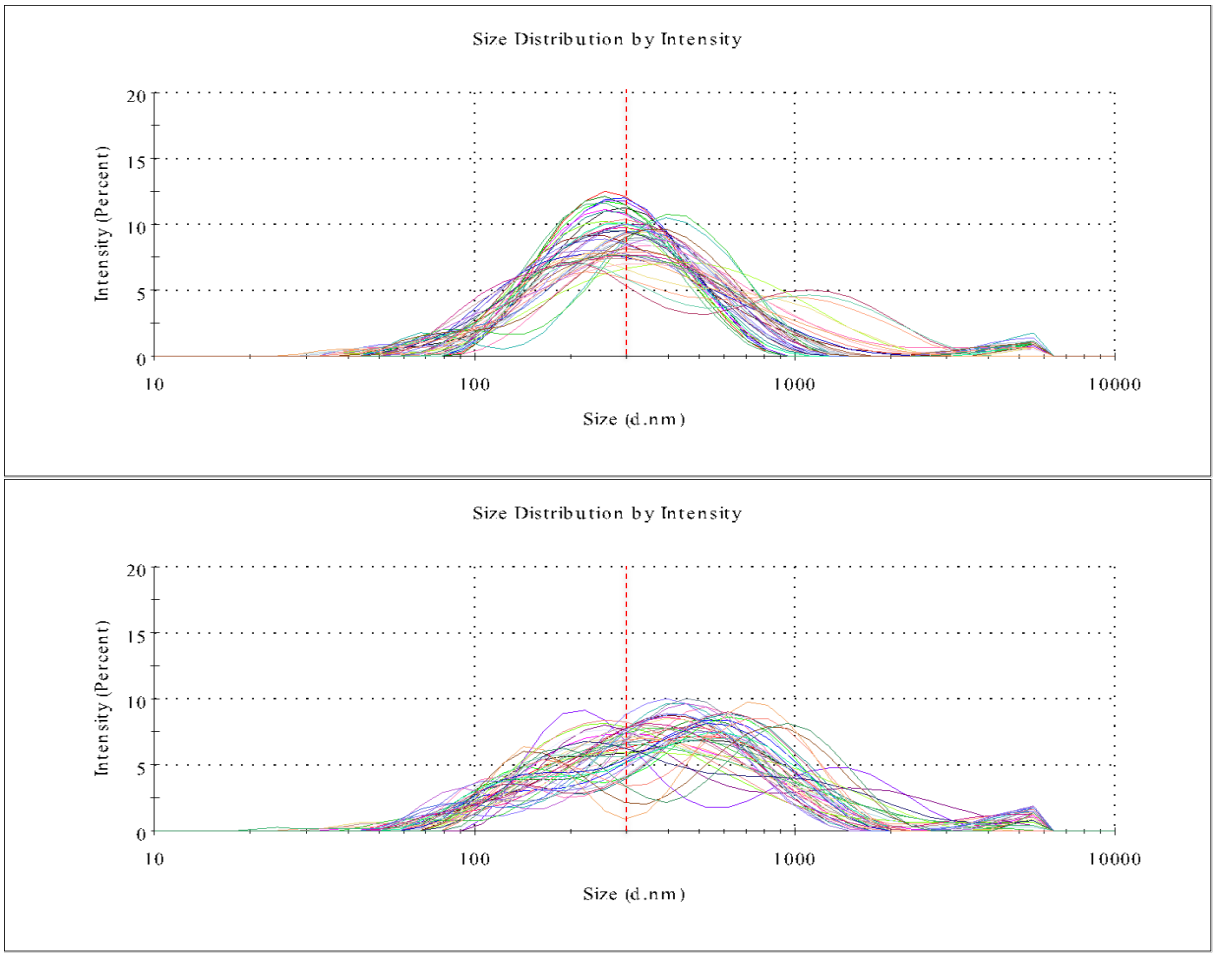

Figure 5: Size and distribution changes of hydrocortisone incorporated large unilamellar vesicles (LUVs) over 1 hour in isotonic environment (top) and when subjected to hypertonic environment (bottom). LUVs were prepared in phosphate buffered saline (PBS) solution of 65 mOsm and exposed to buffer with tonicity of 65 (top) or 300 mOsm (bottom), respectively. The dotted line marks 300 nm size (n=30)

The changes in size distribution of HC-LUVs prepared in PBS65 where studied in isotonic environment (PBS65) and in hypertonic environment (PBS300) over a time period of 1 hour (Figure 5). When LUVs were exposed to isotonic environment (top graph), a single peak (approx. 290 nm) was observed. However, when LUV dispersions where exposed to hypertonic environment, a marked change in size distribution of the LUVs could be observed (bottom graph). The main peak that was observed in isotonic environment decreased in intensity when subjected to hypertonic environment. Additionally, a decreasing in sizes of LUVs could be observed. A general broader size distribution of LUVs sizes (i.e. higher polydispersity index) in hypertonic environment in comparison to the isotonic environment was measured.

5.3 Standard curves

Standard curves measured with UV-vis spectroscopy were used in this work for the quantification of hydrocortisone and methylprednisolone. Calibration curves are based on the Beer-Lambert equation which relates absorbance is to the concentration of a compound in solution as reported by Equation 7 (Clark, 2017):

Equation 7

$$A = \varepsilon * l * c$$

In this equation is A the absorbance of the measured compound, ε molar absorptivity, l the width of the cuvette (1 cm standard) and c the concentration of the compound in solution (Clark, 2017).

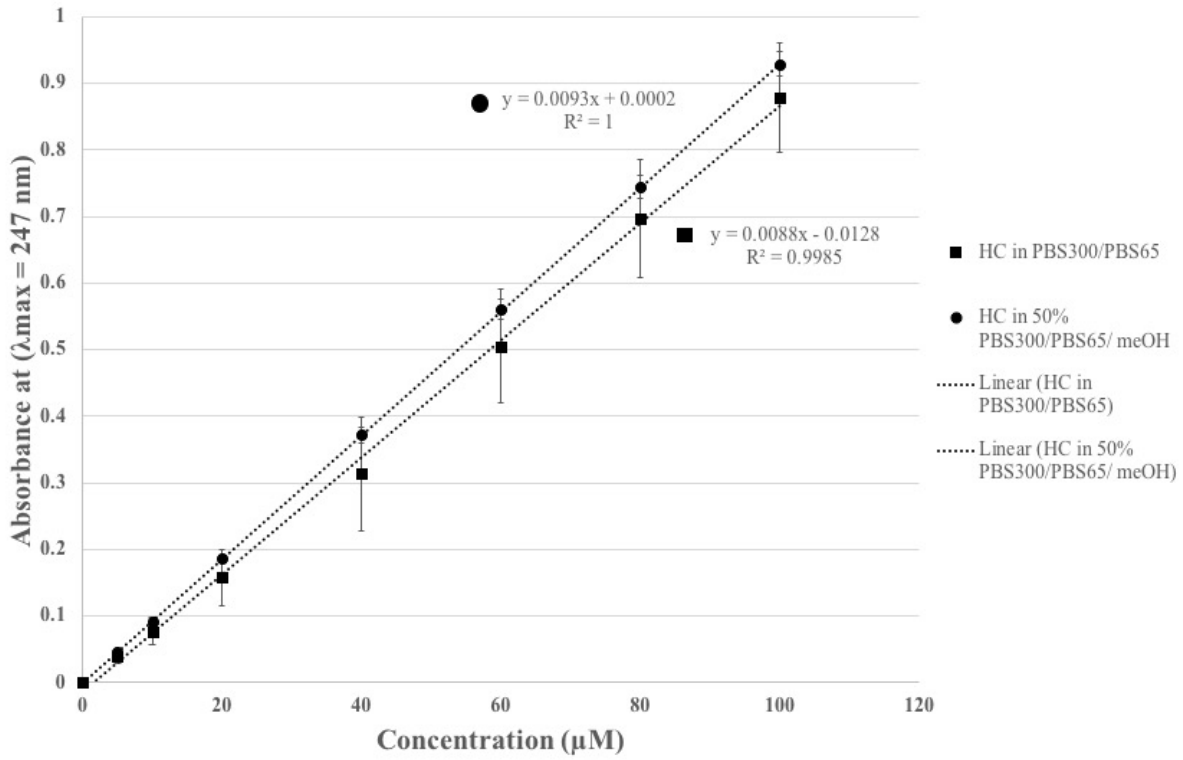

Figure 6: Summary of hydrocortisone (HC) Standard curves plotted as absorbance over concentration (µM). Used to quantify amount of HC entrapped inside large unilamellar vesicles and amount permeated through cellulose hydrate and Permeapad® barrier (mean ± SD, n=36).

As represented in Figure 6, the absorbance of hydrocortisone is plotted as a function of the drug concentration. R^2 (correlation coefficient) was measured to be 0.9985 or above for the standard curves independently on what type of solvent was used. The R^2 close to 1 indicated a linearity of the points on the curves.

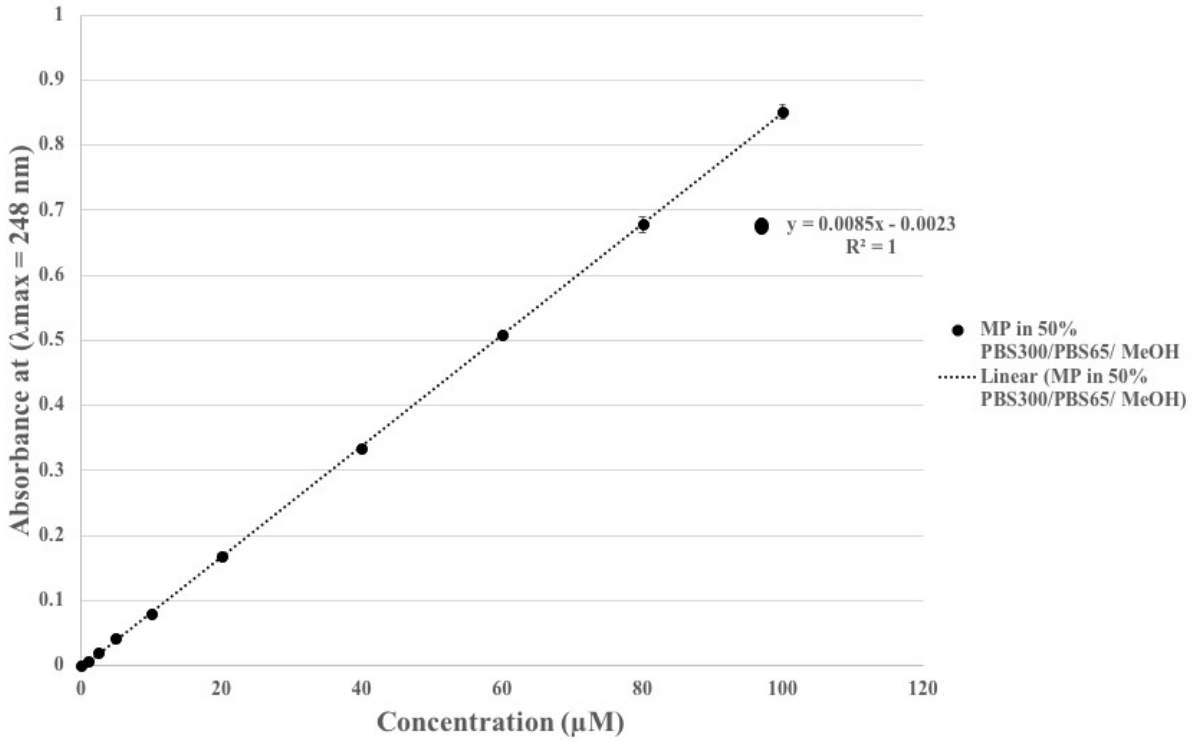

Figure 7: Methylprednisolone (MP) standard curves (summary) plotted as absorbance over the standard concentrations used to quantify amount of MP entrapped inside large unilamellar vesicles and amount permeated through cellulose hydrate barrier (mean ± SD, n= ≥12).

There were found a high correlation between the standard points with a R^2 of 1 was found for methylprednisolone using 50% methanol/PBS (PBS300 or PBS65, respectively) as a solvent (Figure 7).

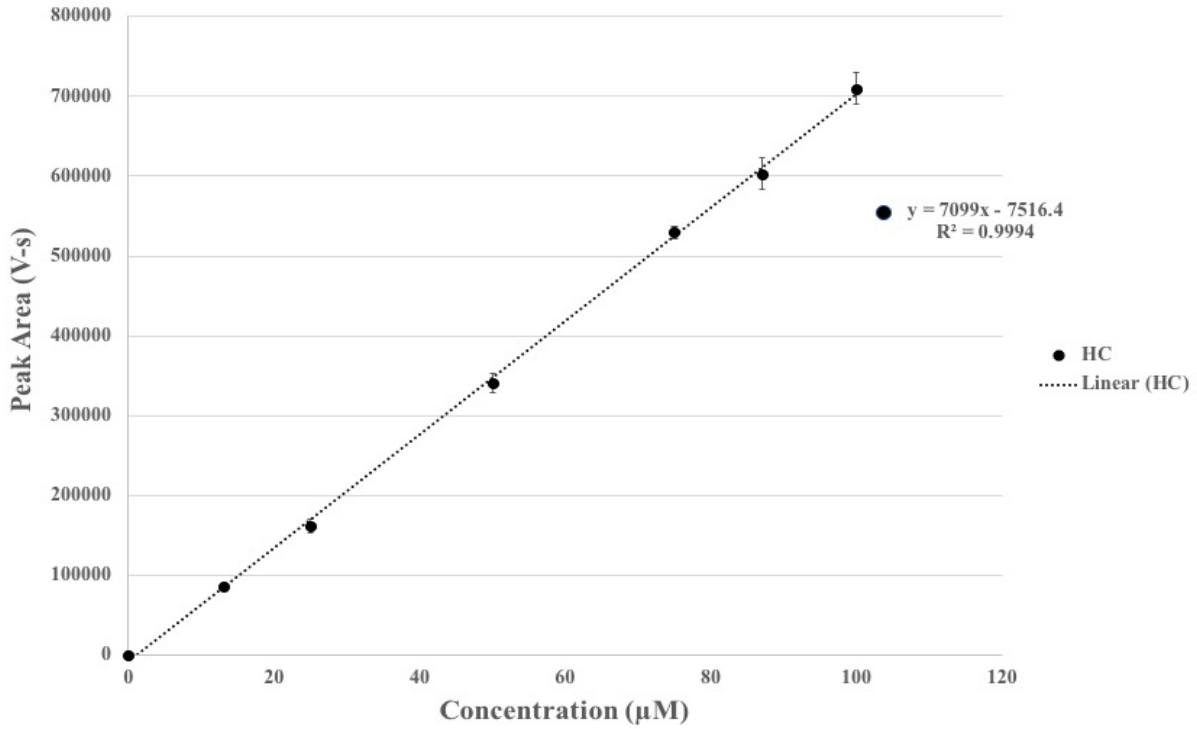

Figure 8: Standard curve of hydrocortisone (HC) for high performance liquid chromatography (HPLC) plotted as peak area under the curve over standard concentration (µM) employed to quantify HC entrapped inside Permeapad® barriers (mean ± SD, n=3).

The standard curve of HC employed for quantification of HC entrapped inside Permeapad® when using HPLC. The R^2 close to 1 shows that there is a linearity between the points on the standard curve (Figure 8).

5.4 Liposomal characterization

The prepared LUVs were characterized according to diameter size, polydispersity, surface charge and drug entrapment. Results are shown in Table 8.

Table 8: Large unilamellar vesicles (LUVs) diameter size (nm), polydispersity index (PDI), zeta potential (ZP) and drug entrapment efficiency into LUVs (EE) and the drug recovery of all the LUVs prepared in this study. The hydrocortisone or methylprednisolone incorporated LUVs were prepared in phosphate buffered saline (PBS) solutions of either 300 mOsm (PBS300) or 65 mOsm (PBS65) (mean ± SD, *n= ≥6, **n≥3).

LUVs with drug (prepared in)	Size (nm)*	PDI*	ZP*	EE (%)**	Recovery (%)**
Hydrocortisone (PBS300)	275 ± 14	0.24 ± 0.02	- 2.78 ± 1.60	74 ± 3	97 ± 2
Hydrocortisone (PBS65)	300 ± 32	0.31 ± 0.08	- 5.86 ± 3.75	78 ± 2	98 ± 2
Methylprednisolone (PBS300)	268 ± 5	0.24 ± 0.01	- 1.29 ± 0.14	87 ± 1	96 ± 2
Methylprednisolone (PBS65)	285 ± 4	0.29 ± 0.03	- 3.12 ± 0.24	87 ± 0	96 ± 1

The formulations of HC-LUVs and MP-LUVs showed smaller sizes and PDI for the LUVs prepared in PBS300 compared to PBS65. Additionally, a slightly more negative ZP was observed for LUVs prepared in PBS65 in comparison to PBS300. The amount of entrapped drug inside the LUVs determined by ultracentrifugation resulted in higher entrapment for the MP-LUVs (~87%), in comparison to HC-LUVs (~75%).

5.5 Thermodynamic solubility

Due to difficulties in finding literature values that gave a good representation of the solubility of the studied drugs, the thermodynamic solubilities of both compounds in aqueous environment (PBS) were experimentally determined (see Table 9).

Table 9: Measured thermodynamic solubilities of hydrocortisone and methylprednisolone in phosphate buffered saline (PBS) solutions of 300 mOsm (PBS300) and 65 mOsm (PBS65) (mean ± SD, *n=≥4).

Buffer solution		µM*	mg/mL
Hydrocortisone	PBS300	1054 ± 29	0.382 ± 0.010
	PBS65	1033 ± 195	0.372 ± 0.077
Methylprednisolone	PBS300	254 ± 7	0.095 ± 0.003
	PBS65	262 ± 5	0.098 ± 0.002

The thermodynamic solubility of hydrocortisone in PBS was measured to be 0.382 ± 0.010 mg/mL for PBS300 and 0.372 ± 0.077 mg/mL for PBS65. The solubility for methylprednisolone in PBS was measured to be 0.095 ± 0.003 mg/mL for PBS300 and 0.098 ± 0.002 mg/mL for PBS65.

5.6 *In vitro* drug permeability and release studies

5.6.1 Drug permeability of saturated drug solutions through cellulose hydrate membranes

Drug fluxes through cellulose hydrate membrane where measured employing saturated drug solutions prepared in PBS, and apparent permeability calculated (Equation 4 and Equation 5). Results are reported in Figure 9.

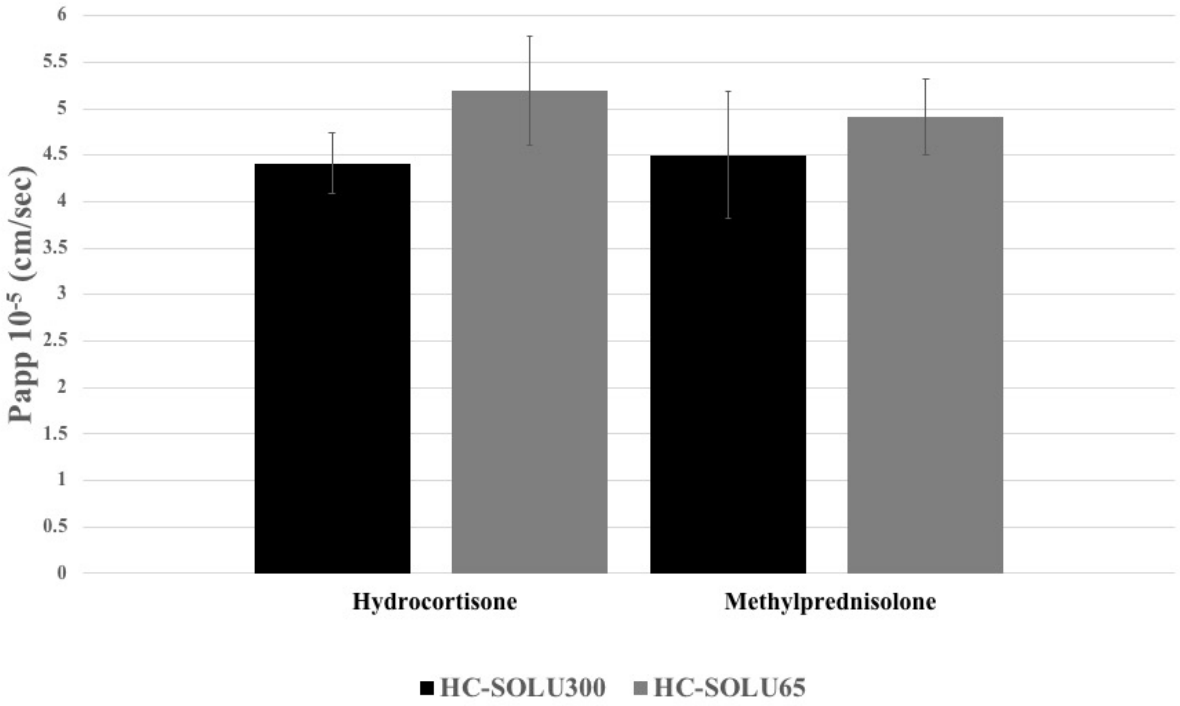

Figure 9: Apparent permeability (P_{app}) of hydrocortisone (0.38 mg/mL*) and methylprednisolone (0.09 mg/mL*) from saturated solutions through cellulose hydrate barriers. Solutions were prepared in phosphate buffered saline (PBS) solutions of 300 mOsm (PBS300) or 65 mOsm (PBS65) (mean ± SD, n= 4, *thermodynamic solubility).

The apparent permeability of HC in PBS300 through cellulose hydrate barriers was found to be $4.41 \pm 0.32 \cdot 10^{-5}$ cm/sec, and $5.19 \pm 0.58 \cdot 10^{-5}$ cm/sec in PBS65. For MP in PBS300, the P_{app} was found to be $4.50 \pm 0.68 \cdot 10^{-5}$ cm/sec and $4.91 \pm 0.68 \cdot 10^{-5}$ cm/sec in PBS65.

5.6.2 Drug permeability and release studies cellulose hydrate membranes

Permeability studies were conducted employing HC-LUV and MP-LUV formulations using cellulose hydrate membranes where changes in tonicity were induced. The apparent permeability of drug permeated from the solutions and LUVs plotted over the absolute osmotic pressures is depicted in Figure 10 (HC) and Figure 12 (MP).

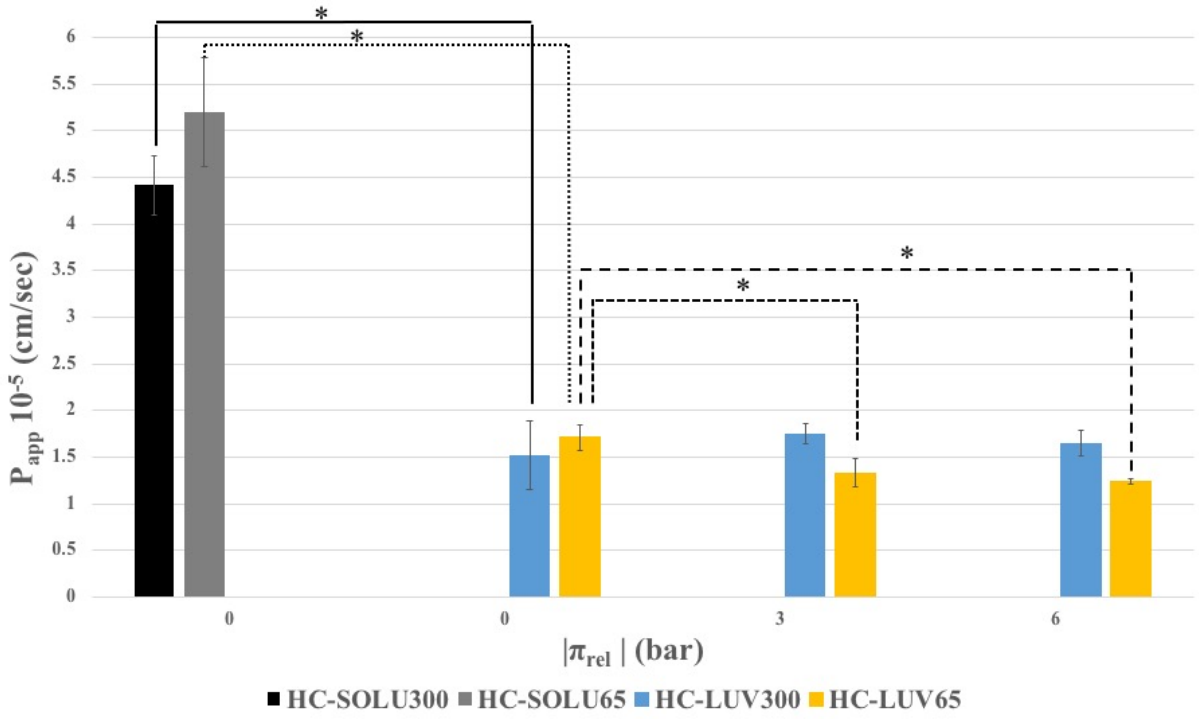

Figure 10: Correlation of absolute osmotic pressures ($|\pi_{rel}|$) and apparent permeability (P_{app}) of hydrocortisone (HC) from large unilamellar vesicles (LUVs, 2 mM total concentration) through cellulose hydrate barriers. Data is compared to P_{app} of HC solutions (HC-SOLU, 0.38 mg/mL). HC-LUVs and HC-SOLU were prepared in phosphate buffered saline (PBS) of 300 mOsm (PBS300) or 65 mOsm (PBS65) in tonicity (mean ± SD, n=4, *$p \leq 0.05$).

The apparent permeability of hydrocortisone through cellulose hydrate membranes were found to be more than two times higher and significantly different (*) for drug solutions compared to hydrocortisone incorporated LUVs in isotonic environments. For HC-LUVs prepared in PBS300, no significant change in permeability were found when LUVs were exposed to hypotonic environments. However, HC-LUVs prepared in PBS65 showed a significant difference in permeability (decreased) when LUVs were exposed to hypertonic environments (*) (Figure 10).

Cumulative µmole of drug released over time (Figure 11) were calculated using Equation 4 to establish at which time point during the studies a significant difference in the release profiles between isotonic LUVs compared to hypertonic LUVs could be found.

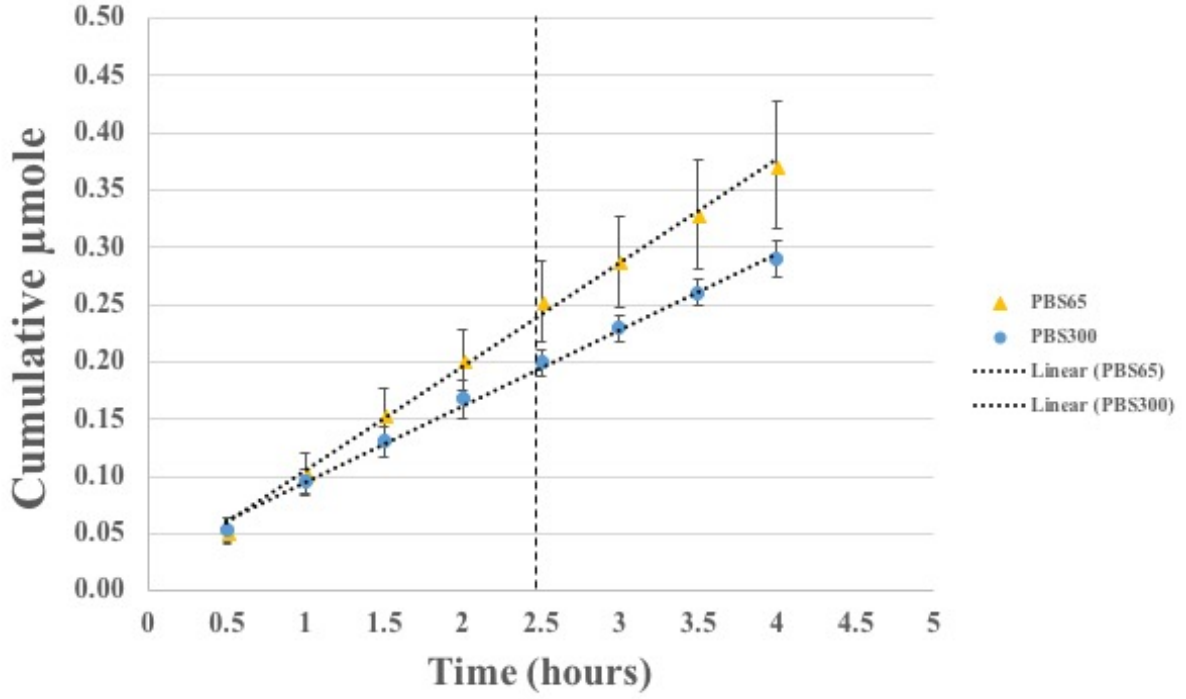

Figure 11: Cumulative µmole of released drug (µmole) plotted over the time (hours) for hydrocortisone incorporated large unilamellar vesicles (HC-LUVs) prepared in phosphate buffered saline (PBS) solution with tonicity of 65 mOsm (PBS65). The LUVs were exposed to isotonic environment (PBS65) and hypertonic environment (buffer of 300 mOsm tonicity, PBS300). Line shows when significant difference between the release profiles occurred (mean ± 95% confidence interval, n=4).

The cumulative amount of drug released (µmole) from HC-LUV65 through the cellulose hydrate barriers in isotonic environment (PBS65) and hypertonic environment (PBS300) showed a significant difference in the release profiles after 2.5 hours.

The apparent permeability for MP through cellulose hydrate barriers were plotted over the absolute osmotic pressure for MP in solution (MP-SOLU) and MP incorporated LUVs (Figure 12).

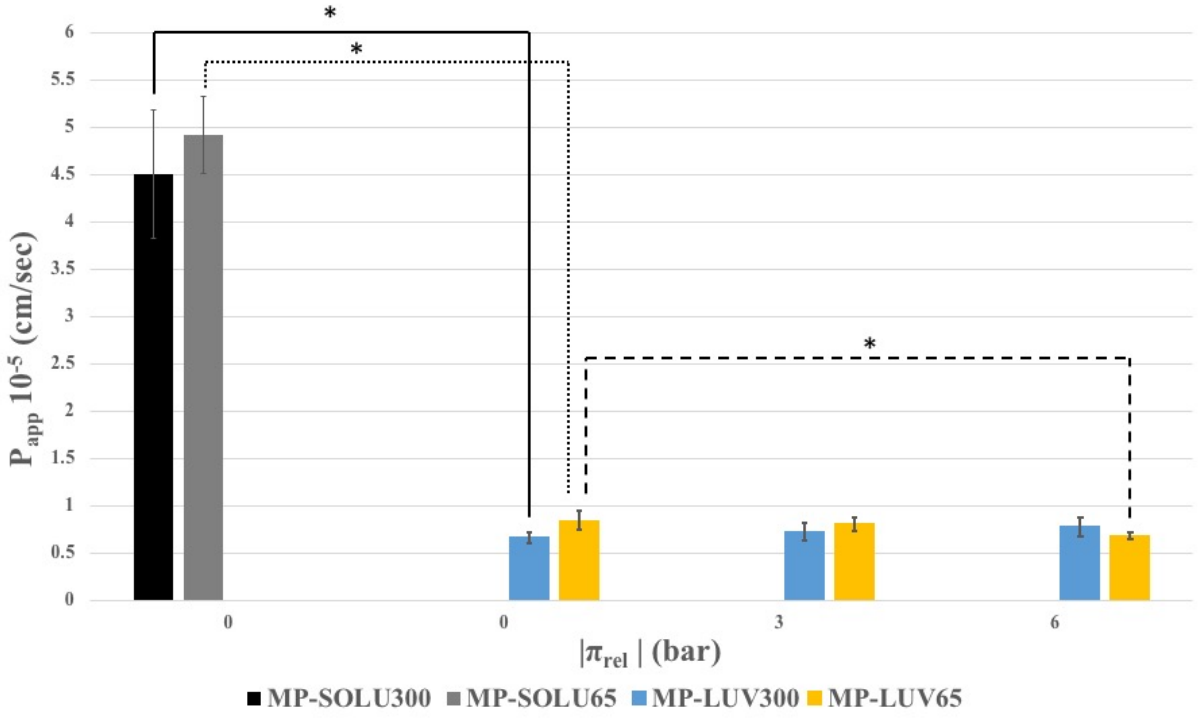

Figure 12: Correlation of absolute osmotic pressures ($|\pi_{rel}|$) and apparent permeability (P_{app}) of methylprednisolone (MP) from large unilamellar vesicles (LUVs, 2 mM total concentration) through the cellulose hydrate barriers. Data is compared to P_{app} of MP solutions (MP-SOLU, 0.09 mg/ml). MP-LUVs or MP-SOLU were prepared in phosphate buffered saline (PBS) of 300 mOsm (PBS300) or 65 mOsm (PBS65) in tonicity (mean ± SD, n=4, *$p \leq 0.05$).

The apparent permeability of MP through cellulose hydrate membranes were found more than four times higher (significant different, *) for drug solutions than for MP-LUVs in isotonic environment. MP-LUVs prepared in PBS300 showed no clear significant change in permeability between isotonic and hypotonic environments, whereas MP-LUVs prepared in PBS65 showed a significant difference (*) in permeability when the LUVs were exposed to hypertonic environment (Figure 12) which were the same as for HC.

The cumulative amount of µmole drug released over time (**Figure 13**) was plotted to establish at which time point a significant difference between release profiles of drug released from MP-LUVs exposed to hypertonic environment differed from isotonic environment.

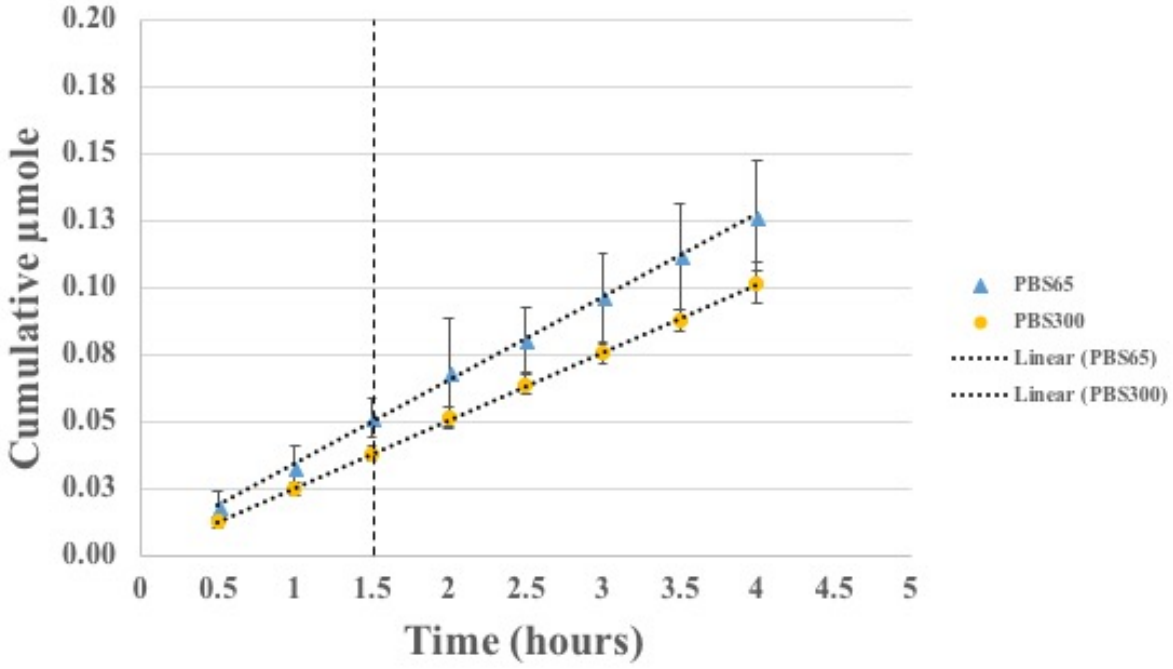

Figure 13: Cumulative amount of released drug (µmole) plotted over the time (hours) for methylprednisolone incorporated large unilamellar vesicles (MP-LUVs) prepared in phosphate buffered saline (PBS) solution with tonicity of 65 mOsm (PBS65). LUVs were exposed to isotonic environment (PBS65) and hypertonic environment (buffer of 300 mOsm tonicity, PBS300). Line shows when significant difference occurred (mean ± 95% confidence interval, n=4)

The release curves showed that the cumulative amount of released drug (µmole) from MP-LUV65 (Figure 13) was significant different between the isotonic environment and hypertonic environment after 1.5 hours.

5.6.3 Drug permeability and release studies using Permeapad® barriers

The *in vitro* drug release of HC-LUVs was also studied employing the Permeapad® biomimetic barriers (to closer mimic biological membranes) The apparent permeability of HC-LUVs exposed to different tonicities are shown in Figure 14.

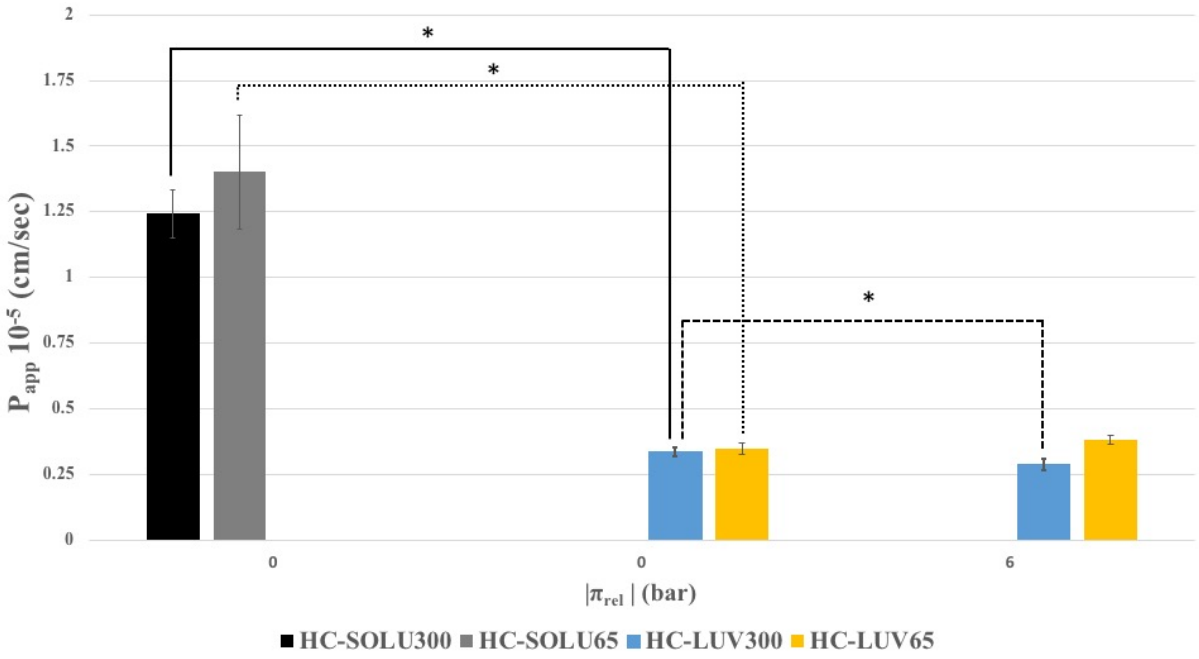

Figure 14: Correlation of absolute osmotic pressures ($|\pi_{rel}|$) and apparent permeability (P_{app}) of hydrocortisone (HC) from large unilamellar vesicles (LUVs, 2 mM total concentration) through the Permeapad® barriers. Data is compared to P_{app} of HC solutions (HC-SOLU, 0.38 mg/ml). HC-LUVs and HC-SOLU were prepared in phosphate buffered saline (PBS) of 300 mOsm (PBS300) or 65 mOsm (PBS65) in tonicity (mean ± SD, n=3, *p≤0.05)

The apparent permeability of hydrocortisone through the Permeapad® barrier were found more than two times higher for drug solutions than for hydrocortisone incorporated LUVs (significant difference, *). HC-LUVs prepared in PBS300 showed a significantly different permeability when exposed to hypotonic environments (*). On the other hand, HC-LUVs prepared in PBS65 showed no significant difference in apparent permeability when exposed to hypertonic environment.

The cumulative amount of drug (µmole) released through the Permeapad® barriers were plotted over the time to establish at which time point a significant difference between the total amount of drug released from HC-LUVs exposed to hypotonic environment differed from isotonic environment. Results are depicted in Figure 15:

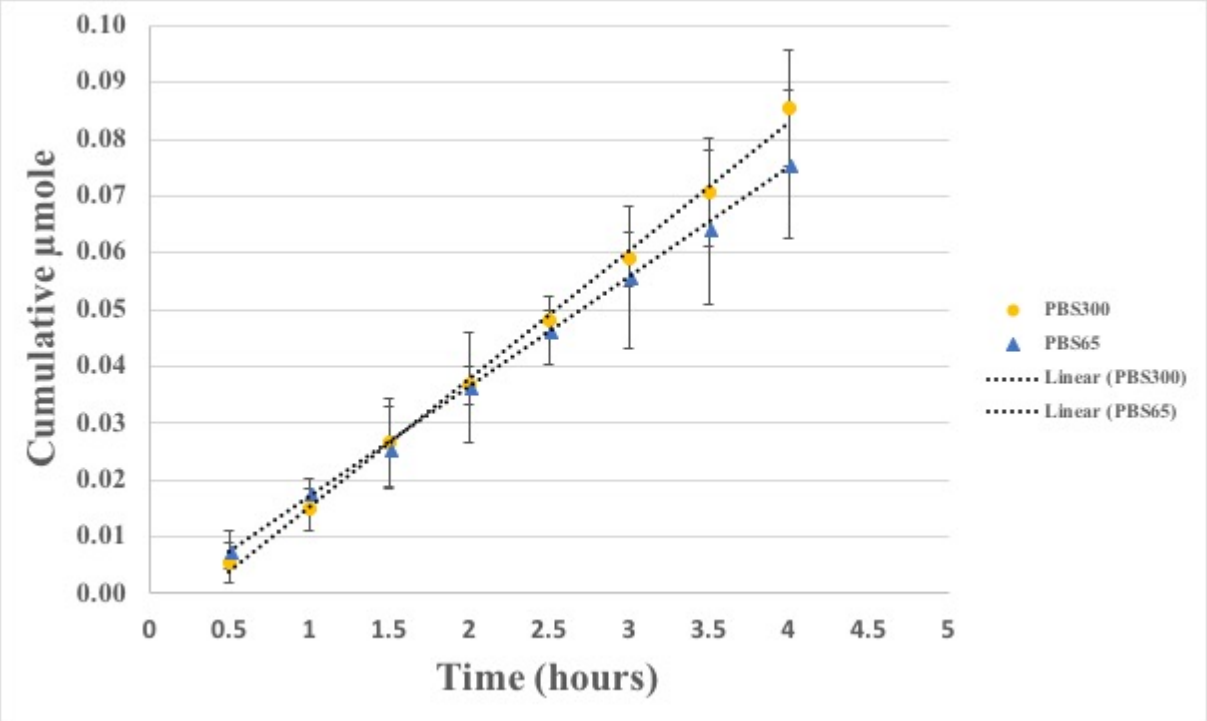

Figure 15: Cumulative amount of released drug (µmole) plotted over the time (hours) for hydrocortisone incorporated large unilamellar vesicles (HC-LUVs) prepared in phosphate buffered saline (PBS) solution with tonicity of 300 mOsm (PBS300) used with the Permeapad® barriers. LUVs were exposed to isotonic environment (PBS300) and hypotonic environment (buffer of 65 mOsm tonicity, PBS65), (mean ± 95% confidence interval, n=3).

The cumulative amount of drug (µmole) released from HC-LUV300 through the Permeapad® barriers in isotonic (PBS300) and hypotonic (PBS65) environments were evaluated using 95% confidence interval. There was not found any time point in which a significant difference in the release profiles of HCLUV300 were detected (Figure 15).

A preliminary study on the transfer of ions (changes in tonicity) from the acceptor chamber to the donor chamber of the Franz diffusion cells were conducted using cellulose hydrate membranes and the Permeapad® barriers. The acceptor chamber contained PBS300 (5 mL) with an initial tonicity of 304 mOsm (measured before the experiment) and the donor chamber contained distilled water (0.8 mL) with an initial tonicity of 2 mOsm. The donor chamber was sampled (150 µL) after 0.5, 1, 2, 3 and 4 hours and fresh distilled water (2 mOsm) were reintroduced. The samples from the donor chamber was measured using the Semi-Micro k-7400 Osmometer (Knauer). Results are reported in Figure 16:

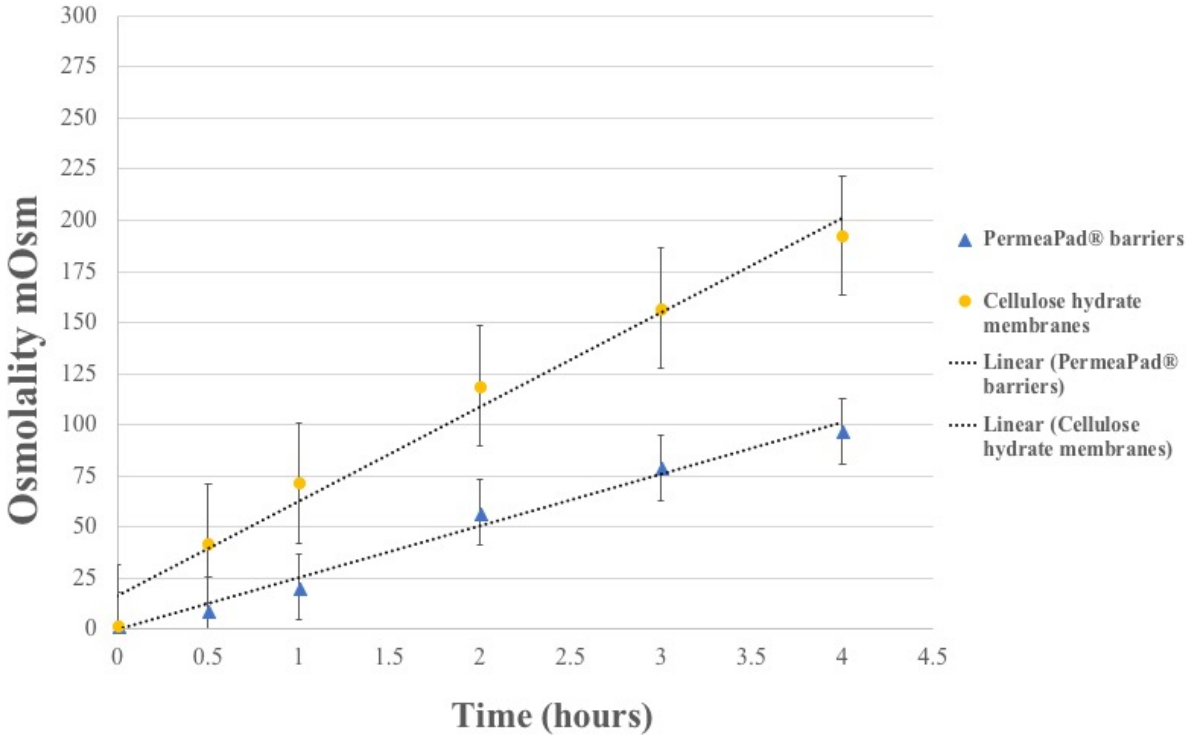

Figure 16: Ion transfer of from acceptor chamber to donor chamber of the Franz cells using cellulose hydrate membranes and the Permeapad® barriers. Results are plotted as osmolality measured over the time (mean ± SD, n=2).

The results show that the Permeapad® had a significantly lower rate of ions transferred across the barriers per time unit when compared to cellulose hydrate membranes.

6 Discussion

6.1 Liposomal characterization

The size distribution of LUVs prepared in PBS300 seems to be slightly lower than LUVs made in PBS65, the polydispersity of the measured LUV formulations gave the same trend with a slightly lower size distribution for LUVs prepared in PBS300. This could be attributed to the ionic content of the dispersing buffers where PBS300 have a higher ionic concentration than PBS65. Liposomal size and distribution have shown to be affected by the charge around them acting both on the electro static repulsion and sedimentation rate between the liposomes (Narenji *et al.*, 2016). A higher ion concentration in the dispersing solution could contribute to more charges particles around the liposomal membranes which would enhance the electrostatic repulsions between individual liposomes reduce aggregation of the liposomal vesicles.

The zeta potential (ZP) of the liposomal formulations where found to have slightly negative surface charges. LUVs prepared in PBS65 showed a slightly more negative ZP than for LUVs prepared in PBS300. The results seem consistent with previous findings of the same liposomal formulations (Wu *et al.*, 2017). Carrión *et al.* (1994) showed that the ZP is affected by a number of variables such as the composition of the liposomal bilayer, the type and concentration of electrolytes in the dispersing buffer among others. A liposomal bilayer consisting of only phosphatidylcholine has shown to give a less charged surface (i.e. more neutral ZP) and a higher concentration of counter-ions in liposomal dispersions have shown to alter the surface charge of liposomes due to electrostatic charge shielding (Carrión *et al.*, 1994; Coday *et al.*, 2015; Sabín *et al.*, 2006) This seems to be consistent with the findings of this study.

The amount of entrapped drug inside the LUVs determined by ultracentrifugation, showed a higher entrapment of drug for MP-LUVs than for HC-LUVs. The higher entrapment of MP could be explained by the partition coefficient values of the two drugs incorporated into the liposomal formulations. LogP is the measurement of the partition coefficient, when adding drugs into a two-phase system like a liposomal dispersion a higher logP indicates that the drug prefers the lipid phase more than the aqueous phase. The logP value of MP = 1.80 (Grabowski *et al.*, 2010) is higher than HC = 1.61 (Benet *et al.*, 2011), this seem to give MP a higher degree of incorporation into the lipid bilayer than hydrocortisone which in turn could account for the higher entrapment of drug in inside the LUV formulation (Bozzuto and Molinari, 2015; Fatouros and Antimisiaris, 2002).

6.2 LUVs and tonicity

The results shown in Figure 4 and Figure 5 confirm that the LUVs are susceptible to changes in the tonicity of the external environment of the liposomes. LUVs do shrink to a certain extent when exposed to hypertonic environment. This seems to be in accordance with literary findings on the subject (Abuin *et al.*, 1995; Disalvo *et al.*, 1996; Fujiwara and Yanagisawa, 2014; Wu *et al.*, 2017). The lack of pronounced reduction in liposomal size can be attributed to the initial size of the liposomes themselves, GUVs have shown to be greatly affected by changes in tonicity and shrink to a higher degree than LUVs, and small unilamellar vesicles (SUVs) to be less affected by changes in tonicity to the extent of being osmotically insensitive (Hallett *et al.*, 1993; Johnson and Buttress, 1973; Polozov *et al.*, 2001) The LUVs are possible too small in size to see real changes in size of the liposomes when subjected to hypertonic environment. When the LUVs were subjected to hypotonic environment there were observed a higher polydispersity and a shift in the LUVs size distribution towards a higher diameter size along with formation of new peaks, this would suggest that there is a limited swelling of the LUVs occurring. This seems consistent with previous findings for the same LUV dispersions (Wu *et al.*, 2017).

It should be noted that studies performed by changing the osmotic pressure of liposomes have shown that LUVs can undergo lysis if there is a great enough influx of water into the liposomes, i.e. changing the tonicity from isotonic to hypotonic environment (Ertel *et al.*, 1993; Mui *et al.*, 1993; Ohno *et al.*, 2009). LUVs have shown to not undergo lysis if there is an osmotic pressure small enough to induce size changes but not so high it would induce lysis (Hallett *et al.*, 1993; Polozov *et al.*, 2001). One of the studies where LUVs underwent lysis, used an osmotic differential of up to 2000 mOsm/kg to make the liposomal membrane stretch beyond its capabilities (Ohno *et al.*, 2009). Whereas in this study the highest osmotic differential used were 235 mOsm/kg

6.3 Thermodynamic solubility

The thermodynamic solubility measured for HC in PBS was compared to the literature values of 0.420 mg/mL in water (Benet *et al.*, 2011). The results are consistent with previous findings of HC solubilized in a similar buffer solution (di Cagno and Luppi, 2013). The lower solubility of HC could be attributed to solutes already present in PBS300 and PBS65, the buffers which had an estimated phosphate concentration of 78 mM (PBS300) and 16 mM (PBS65) could have an impact on the solubility of drugs because the potential energy needed to the break bonds between water molecules and achieve solubilization is much lower for the phosphates (already solubilized) than for HC.

The solubility for methylprednisolone was found to be 3-fold lower in PBS solutions than the reported measured value of 0.323 mg/mL in water (Benet *et al.*, 2011). Some of the lower solubility could probably be attributed to the previously discussed reason of the presents of phosphates in the buffers used for solubilizing the drug. However, hydrocortisone did not show as much decrease in solubility. A possible explanation for the lower solubility of MP in comparison to HC could be attributed to differences in the molecular structure of MP. The molecular structure differs from HC by an extra methyl group on the second left hydrocarbon ring and an extra double bond on the far left of the molecule (Figure 2). Together the extra methyl group and double bond lowers the solubility of the molecule in water and could in turn lower the solubility in buffers further. This might be because of less hydrogen bonding capabilities on the far-left hydrocarbon ring coupled with the presence of already solubilized ion's in the buffer solutions. The electronic potential left on the hydrocarbon ring for breaking hydrogen bonds might not be strong enough to break the hydrogen bonding between water molecules and water molecules to solutes.

6.4 *In vitro* drug permeability and release studies

6.4.1 Drug permeability studies of saturated drug solutions through cellulose hydrate membranes

The apparent permeability between HC and MP dissolved in PBS solutions shown in Figure 9 showed no significant differences in permeability. However, HC and MP showed a remarkably similar permeability through the cellulose hydrate membrane both in PBS300 and PBS65. This could be attributed to the drugs similar molecular weight and partition coefficients (Pade and Stavchansky, 1998).

6.4.2 Drug permeability and release studies using cellulose hydrate membranes

The permeability of HC and MP solutions of through the cellulose hydrate membrane showed a significantly higher permeability than HC-LUV (two times higher) and MP-LUV (4 times higher) in isotonic environments (Figure 10 and Figure 12). This difference in permeability is probably due to the high entrapment of drug inside the liposomes which would lower the permeability of free drug through the cellulose hydrate membrane. The permeability of the drug solutions would be affected by the concentration gradient of drug through the cellulose hydrate membrane, but the permeability of drug from the liposomal formulations would be affected by the concentration gradient through the cellulose hydrate membrane and the liposomal membrane. The results seem consistent with previous findings on the difference in permeability between solutions of fluorescent makers and fluorescent makers entrapped in the same liposomal formulations as used in this study (Wu *et al.*, 2017).

The permeability of drug from HC-LUV showed a significantly lower permeability between HC-LUV65 in hypertonic environment (π_{rel} 3 and 6 bar) compared to HC-LUV65 in isotonic environment (π_{rel} 0 bar, Figure 10). For MP-LUV there were found a significantly lower difference for MP-LUV65 in hypertonic environment (π_{rel} 6 bar) in comparison to MP-LUV65 in isotonic environment (π_{rel} 0 bar, Figure 12). The differences in permeability between the liposomes when subjected to hypertonic environment could be attributed to shrinkage of the liposomes in hypertonic environment. When the liposomes reduce in size the lipid bilayer would contract (i.e. become denser and shrink) hampering the diffusion of drugs out of the liposome. Results are consistent with previous findings of lipophilic markers entrapped inside the same liposomal formulations (Wu *et al.*, 2017). There was no significant difference in permeability for either HC-LUVs or MP-LUVs in hypotonic environment compared to HC-LUVs and MP-LUVs in isotonic environment (Figure 10 and Figure 12). This could mean that swelling of the LUVs is not as pronounced for lipophilic drugs with a high entrapment inside the liposomal bilayer as previously discussed in chapter 6.2. This seems consistent with previously findings for the permeability of lipophilic drugs with high entrapment inside of LUVs (Wu *et al.*, 2017). A reason for the reduced swelling could be that lipophilic drugs trapped inside the liposomal membrane could reduce the liposomes ability to swell when exposed to a hypotonic environment similar to what cholesterol does (Mohammed *et al.*, 2004).

The cumulative release curves of HC-LUV65 and MP-LUV65 in hypertonic environment (π_{rel} 6 bar) compared to isotonic environment (Figure 11 and Figure 13) showed that there were respectively 2.5 hours (HC-LUV65) and 1.5 hours (MP-LUV65) before a significant difference between the release of drug from the liposomal formulations occurred. The time before a significant difference in release were observed could be because drug is not automatically released from the LUVs, but changes in the concentration of drug in the liposomal dispersion could facilitate diffusion of drug out through the liposomal membrane because of difference in the concentration gradient.

6.4.3 Drug permeability and release studies using Permeapad® barriers

Permeapad® biomimetic barriers was employed as a method of trying to discern the relationship between the release of drug from liposomes through biological barriers. Hydrocortisone were selected as the drug used for these studies. Figure 14, shows a significantly higher permeability for HC in solutions compared to HC-LUVs in isotonic environment (approx. 3.5 times higher) which is consistent with previous findings for HC-SOLU vs. HC-LUVs where cellulose hydrate membranes were employed (chapter 5.6.1). The apparent permeability for HC-SOLU through the Permeapad® seems consistent with previous findings (di Cagno *et al.*, 2015). The permeability of drug released from HCLUV300 in in hypotonic environment is shown to be significantly lower than HCLUV300 in isotonic environment and no significant difference between HC-LUV65 in hypertonic environment compared to HCLUV65 in isotonic environment (Figure 14). These results are different in comparison to what was found with cellulose barriers (chapter 5.6.1). This lead to an experiment comparing the ion transfer capabilities of the Permeapad® against the cellulose hydrate membranes.

Initial experiments on the ion transfer capabilities of Permeapad® barrier compared to the cellulose hydrate barriers showed that ions transferred across the barriers where as much as two-fold lower for the Permeapad® during the experiments (Figure 16). This could mean that the transfer of ions is much slower when experiments are conducted with the Permeapad® and the tonicity changes around the liposomes happen at a slower rate than with the cellulose hydrate barriers. This wound then mean that the liposomes swell or contract at a slower rate than with the cellulose hydrate membrane.

Figure 15, shows that the release of drug seems to be lower (non-significant) for HC-LUV300 in hypotonic environment after 2.5 hours and beyond. This could mean that the reduced changes in permeability between HC-LUV65 in hypertonic environment and HC-LUV65 in isotonic environment could be explained by the slow ion transfer of solute molecules between the donor chamber and acceptor chamber on the Franz cells and that the liposomes did not shrink.

The drug recovery for all the studies done were 95-105% for the cellulose hydrate membranes and 90-100% for studies done with the Permeapad® barriers. The difference in recovery where analysed and there were found from 0-5% drug left on the cellulose hydrate barriers compared to 5-10% for the Permeapad® barriers. The higher amount of drug left on the Permeapad® barriers could be explained by the composition of the barriers relating to the cellulose hydrate membranes.

7 Conclusions

The results of this study demonstrate that LUVs were affected by changes in tonicity of the external environment around liposomes. The liposomes shrank when the external environment was changes from isotonic to hypertonic conditions, there were also observed a swelling of the LUVs when subjected to hypotonic conditions. The permeability of hydrocortisone and methylprednisolone incorporated LUVs through cellulose hydrate barriers were studied, there were found a significantly lower permeability for the drugs when subjected to hypertonic environment there were no significant differences found in hypotonic environment. It can then be concluded that the permeability of drugs incorporated into LUVs are affected by changes in tonicity in the external environment of a liposomal formulation. The permeability of hydrocortisone incorporated LUVs through the Permeapad® biomimetic barrier were studied, the findings were significant changes in the permeability of drug through the Permeapad® when subjected to hypotonic environment. There were Non-significant changes in permeability but because the exchange of ions through the Permeapad® where slower than for cellulose hydrate membranes the swelling/shrinking of LUVs where not as pronounced as for the cellulose hydrate.

8 Future perspectives

The application of liposomes has showed great potential in the area of nasal drug delivery to the brain, to which osmotically active liposomes could be of great impact. The nasal pathways has the same tonicity as the body approx. 290 mOsm (Koeppen and Stanton, 2013). It has been shown that the nasal mucosa is susceptible to changes in tonicity, these changes by administering either hypertonic or hypotonic solutions to the nasal epithelium have shown to create shrinkage in the epithelial cells and by that reducing muco-ciliary clearance from the nasal pathways (Appasaheb *et al.*, 2013; Min *et al.*, 2001; Patel *et al.*, 2017). This reduced clearance of the nasal mucosa would be beneficial for prolonging the time a drug formulation would stay in the same place and give the drug a chance to be released from the confines of the formulation. An additional way to ensure prolonged time for release of drug could be the addition of mucoadhesive properties to the liposomal formulation. The Permeapad® has previously shown great promise as a barrier mimicking biological membranes. To conclude that it is a viable option for studies based on the shrinking and swelling of liposomes, further studies and optimisation of the experimental procedures would be needed.

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Appendix

I ABSTRACT Accepted

11th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, March 2018, Granada, Spain

Osmotic environmental changes do affect drug release from phospholipid bilayer vesicles

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INTRODUCTION

Liposomes are spherical vesicles consisting of a single or multiple phospholipid bilayers surrounding an aqueous core. Liposomes are appealing for drug delivery because of their role in solubilizing poorly water-soluble compounds (1, 2). Moreover, drugs incorporated into liposomes avoid early degradation and elimination, helping the improvement of their bioavailability profile. Previous studies have shown that uneven tonicity between the internal aqueous core and external environment of liposomes can influence the release of fluorescent markers from liposomes (3), however this has not been proven yet for actual drugs.

AIM

To investigate if and to which extent the environmental tonicity perturbations (and osmotic pressure) affect the release profiles of hydrophilic (caffeine, CAF) and lipophilic (hydrocortisone, HC) drugs from large unilamellar vesicles (LUV).

METHOD

Preparation of PBS solutions with different tonicity

A 74 mM phosphate buffer saline (PBS) solution (solution A) was prepared by dissolving 22.5 g $NaH_2PO_4 \cdot H_2O$ and 36.8 g $Na_2HPO_4 \cdot 2H_2O$ in 5 L distilled water. The pH was adjusted to 7.40 with NaOH, whereas NaCl was employed to fix tonicity at 300 mOsm (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany). Solution A was diluted with distilled water to obtain two other solutions (B and C, Table 1) with lower tonicities.

Solution	pH	Tonicity (mOsm)
A	7.39 ± 0.02	297 ± 15
B	7.50 ± 0.04	183 ± 1
C	7.60 ± 0.05	64 ± 4

Table 1: The PBS solutions used.

Preparation of LUV

LUV were prepared following a method previously described by Wu et al. (3). In brief, an organic mixture of phosphatidylcholine, methanol, chloroform and PBS was prepared. Organic solvents were gently evaporated by rotary evaporation (Büchi R-124 rotavapor, Büchi vacuum pump V-500, Büchi B-480 water bath, Büchi Labortechnik AG, Flawil, Switzerland), and the obtained liposomal dispersion was extruded at room temperature (23 – 25 °C) through polycarbonate membrane filters (lowest pore-size 400 nm, Nuclepore Track-Etched Membranes, Whatman International Ltd., Buckinghamshire, UK). For loading the drugs in liposomes, caffeine was dissolved in the PBS, whereas hydrocortisone was dissolved in the organic phase together with lipid. For both drugs, LUV dispersions were prepared using solution A (300 mOsm) or, alternatively, solution C (65 mOsm).

Characterization of LUV

Liposomal sizes were determined by photon correlation spectroscopy, and surface charges of liposomes were determined by zeta potential measurement (Zetasizer Nano Zen 2600, Malvern, Worcestershire, UK). Ultracentrifugation method (3) was employed to quantify liposomal drug loading.

In vitro drug release study

Drug release studies were performed employing Franz diffusion cells (standard 0.64 cm^2 diffusional area jacketed flat ground joint, PermeGear Ink, Hellertown, USA) equipped with cellulose hydrate membrane (Visking dialysis tubing MWCO 12 – 14 kDa, Medicell). Experiments were performed at 35 °C (Julabo F12-ED, Julabo Laboratechnik, Seelback, Germany) over a period of 4 hours (n = 4). The acceptor chamber was filled with different PBS solutions (Table 1) with different ion strengths and tonicities. At time zero, LUV dispersion (0.8 mL) containing caffeine (or, alternatively, hydrocortisone (2 mM total drug concentration) was applied to the donor chamber. The drug concentration in the acceptor chamber was detected by UV-visible spectroscopy (Costar® UV 96-well plate, Corning, New York, USA) at wavelengths 273 nm for caffeine and 247 nm for hydrocortisone. The flux of each drug through the cellulose hydrate membrane (J) was determined from Equation 1:

$$J = \frac{dm}{dt} \cdot \frac{1}{A} \quad (1)$$

where *dm/dt* represents the variation of mass over time and *A* is the diffusional area. The relative osmotic pressure (π_{rel}) was estimated by Equation 2:

$$\pi_{rel} = R \cdot T \cdot (Osm_{(out)} - Osm_{(in)}) \quad (2)$$

Where *R* represents the gas constant, *T* is the absolute temperature and $Osm_{(out)}$-$Osm_{(in)}$ is the difference between external and internal osmolality (units of mOsm) of LUV. Based on Equation 2, a negative osmotic pressure is produced when water influx into LUV ($Osm_{(out)}<Osm_{(in)}$) and positive relative osmotic pressure when water efflux out of LUV ($Osm_{(out)}>Osm_{(in)}$).

RESULTS AND DISCUSSION

Characterization of LUV

The general characteristics of the LUV dispersions are reported in Table 2.

LUV	Tonicity (mOsm)	Size (nm)	Zetapotential (mV)	Drug entrapment (%)
Empty	65	298 ± 11	-4.6 ± 0.3	
	300	246 ± 13	-0.7 ± 0.3	
Caffeine	65	333 ± 33	-6.6 ± 1.0	26 ± 0
	300	279 ± 5	-0.2 ± 0.0	22 ± 1
Hydro-cortisone	65	280 ± 24	-9.4 ± 0.5	79 ± 1
	300	281 ± 4	-1.3 ± 0.2	71 ± 1

Table 2: General characteristics of LUV prepared in PBS at different tonicity.

All formulations had a uniform size and surface charge, with some small differences related to the ionic composition of the buffer in which they were dispersed.. LUV were slightly negatively charged in 65 mOsm buffer, and became more neutral at increased ion strength. Drug entrapment was approximately 4-fold times higher for the hydrocortisone-LUV in comparison to caffeine-LUV.

In vitro drug release study

In Figure 1 the correlation between the relative osmotic pressure (generated by uneven tonicity between inner core and external environment of LUV) is plotted against the measured fluxes of caffeine (A) and hydrocortisone (B). As it can be observed in Figure 1, both drugs are highly influenced by a positive osmotic pressure. Specifically, when negative osmotic pressure is applied ($Osm_{(out)}<Osm_{(in)}$), the flux of water (chemical activity driven) is directed inwards, against the direction of the drug molecules. In this case, a small inflection in flux could be observed at the most negative osmotic pressure (i.e. highest inwards flow of water) for both CAF and HC., When positive osmotic pressure was applied ($Osm_{(out)}>Osm_{(in)}$, flux of water directed outwards from LUV), a significant decrease of drug release was observed for both dugs. This interesting phenomenon can be explained by LUV shrinkage that alters the phospholipid bilayer density and rigidity, increasing its resistivity to permeation and therefore reducing drug release. This phenomenon seems to affect hydrophilic compounds (such as CAF) to a higher extent than lipophilic compounds (i.e. HC) that are embedded in the phospholipid bilayer.

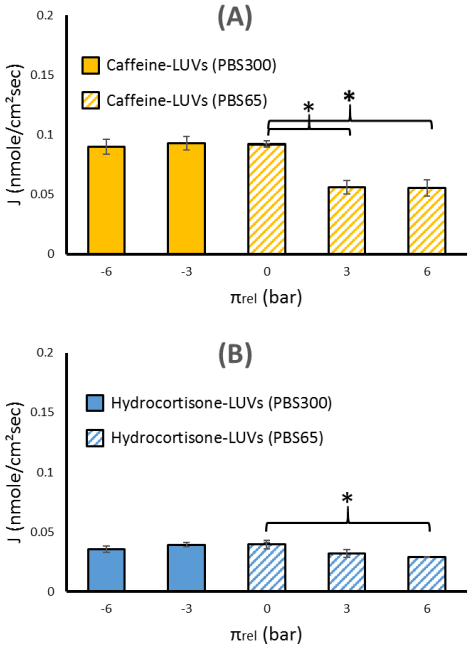

Figure 1: Correlation between the relative osmotic pressure (generated by different tonicities between inner core and external environment of vesicles) and the observed fluxes of caffeine (A) and hydrocortisone (B) in LUV dispersion (* p ≤ 0.05).

CONCLUSION

Perturbation of environmental tonicity seems to play a fundamental role in the release of caffeine and, to a minor extent, hydrocortisone from LUV. This phenomenon could be of crucial importance when designing drug nanocarriers with optimal controlled release properties.

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II POSTER Accepted

11th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, March 2018, Granada, Spain

UiT NORGES ARKTISKE UNIVERSITET

Tonicity environmental changes do affect drug release from large unilamellar vesicles

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/ INTRODUCTION AND AIM

In a recent work we demonstrated that the diffusion of medium-sized hydrophilic and lipophilic markers (MW 600-700 Da) from large unilamellar vesicles (LUVs) was affected by the changes in environmental tonicity to which LUVs were exposed (1). The magnitude of these changes was related to the interplay between the changes in liposome sizes and water molecules flux direction (influx or efflux from LUVs) (2-3). Consequently, the release of a hydrophilic marker form LUV was significantly more affected by the tonicity perturbations in comparison to a lipophilic marker.

The aim of this work was to investigate if the tonicity perturbations can influence the release from LUVs of two medium/small-sized (200-300 Da) drugs (caffeine, CAF and hydrocortisone, HC). In order to have a more realistic evaluation of the magnitude that this effect could have in real biological conditions, the newly introduced assay for drug permeability screening Permeapad® has been employed.

/ CONCLUSIONS

1. LUVs exposed to hypotonic environment swell, whereas, when exposed to hypertonic environment, LUVs shrink;
2. For both CAF and HC, a significant reduction in relative P_{app} was observed when LUVs were exposed to hypertonic environment, whereas no significant difference was observed when LUVs where exposed to a hypotonic environment (cellulose hydrate barrier);
3. For both CAF and HC, the reduction in relative P_{app} in hypertonic environment was less pronounced when LUVs were tested on Permeapad®;
4. Interestingly, using Permeapad® as a barrier, for both CAF and HC, a significant reduction in relative P_{app} was measured when hypertonic LUVs were exposed to hypotonic environments;
5. Permeapad® was proven to be a better tool to predict the tonicity-induced drug diffusional changes from LUVs through biological membranes;

/ RESULTS

LIPOSOMAL CHARACTERIZATION

Table 1: Characteristics of large unilamellar vesicles (LUVs) prepared in neutral PBS at different tonicities. Results are presented as mean ± SD (n ≥ 2).

Drug	PBS (mOsm)	LUVs size (nm)	PI	ZP (mV)	EE (%)
	65	298 ± 11	0.34 ± 0.04	-4.6 ± 0.3	
	300	246 ± 13	0.22 ± 0.01	-0.7 ± 0.3	
Caffeine	65	333 ± 33	0.40 ± 0.06	-6.6 ± 1.0	26 ± 0
	300	279 ± 5	0.30 ± 0.04	-0.2 ± 0.0	22 ± 1
Hydro-cortisone	65	280 ± 24	0.27 ± 0.07	-9.4 ± 0.5	79 ± 1
	300	281 ± 4	0.25 ± 0.01	-1.3 ± 0.2	71 ± 1

TONICITY-INDUCED SIZE CHANGE

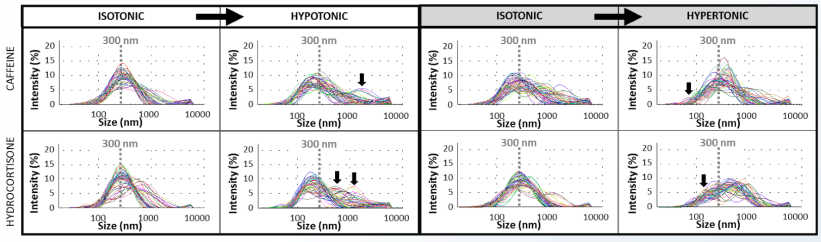

Figure 1: Size distributional changes of large unilamellar vesicles (LUVs) with caffeine or hydrocortisone prepared in neutral PBS (300 or 65 mOsm) exposed to PBS 65 or 300 mOsm, respectively (n=3).

IN VITRO STUDY

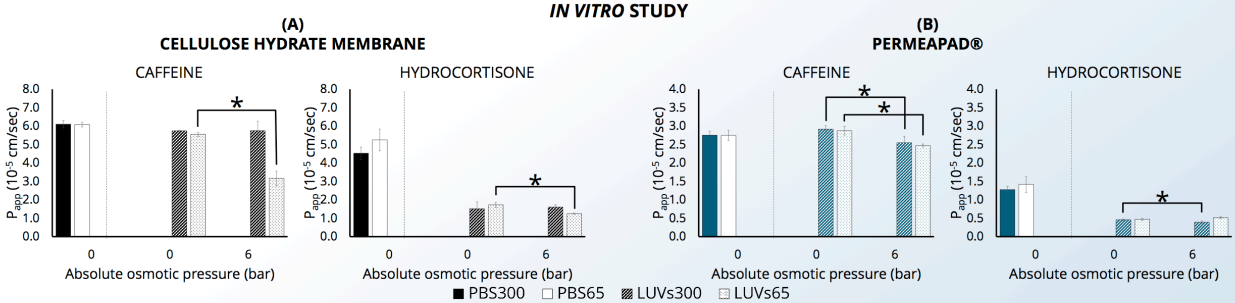

Figure 2: Correlation between osmotic pressures (bar) and the apparent permeability (P_{app}) of caffeine and hydrocortisone for large unilamellar vesicles (LUVs, 2 mM total concentration) through cellulose hydrate membrane (A) and Permeapad® barrier (B). Data are compared with P_{app} of free drug solutions (2 mM for caffeine and 1 mM for HC). Liposomal dispersions were prepared in neutral PBS of 300 or 65 mOsm tonicity and exposed to neutral PBS of different tonicities (65 or 300 mOsm, respectively). Results are presented as mean ± SD (n ≥ 3, *p ≤ 0.05).

/ EXPERIMENTAL

LUVs were prepared following the method previously described (1). In brief, an organic mixture of soy-phosphatidylcholine, methanol, chloroform and phosphate buffered saline (PBS) was prepared. Organic solvents were gently evaporated by rotary evaporation and the obtained liposomal dispersion was extruded with polycarbonate filters (minimum pore size of 400 nm). CAF was solubilized in the PBS whereas HC was dissolved in the organic phase together with the lipid. LUVs were exposed to PBS with different tonicities to induce an osmotic stress on the vesicle membranes. Drug permeability studies were performed employing Franz diffusion cells equipped with cellulose hydrate membrane or with the biomimetic barrier Permeapad®. The cumulative amount of diffused drug over time was calculated, and the linear part of the slope (representing steady state condition) was used to determine the flux. The relative apparent permeability (P_{app}) was calculated by normalizing the flux over the total initial drug concentration. Quantitative determination of the drugs was performed employing UV-visible spectroscopy at wavelengths 273 nm (CAF) and 247 nm (HC). Size and surface charge of LUVs were measured employing the Zetasizer Nano Zen 2600 (Malvern).

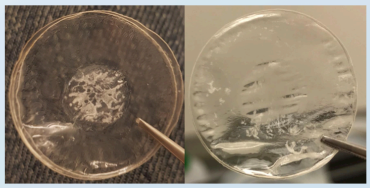

Figure 3: Pictures of Permeapad® barriers collected at the end of the *in vitro* diffusion experiments with large unilamellar vesicle dispersions with CAF (left) or HC (right).

/ ACKNOWLEDGEMENT

The authors are grateful to InnoME for providing Permeapad® barriers and Lipoid for providing the lipids

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