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Development of biomimetic phospholipid-vesicle based permeation assays (PVPA) as screening tool in drug development

Elenaz Naderkhani

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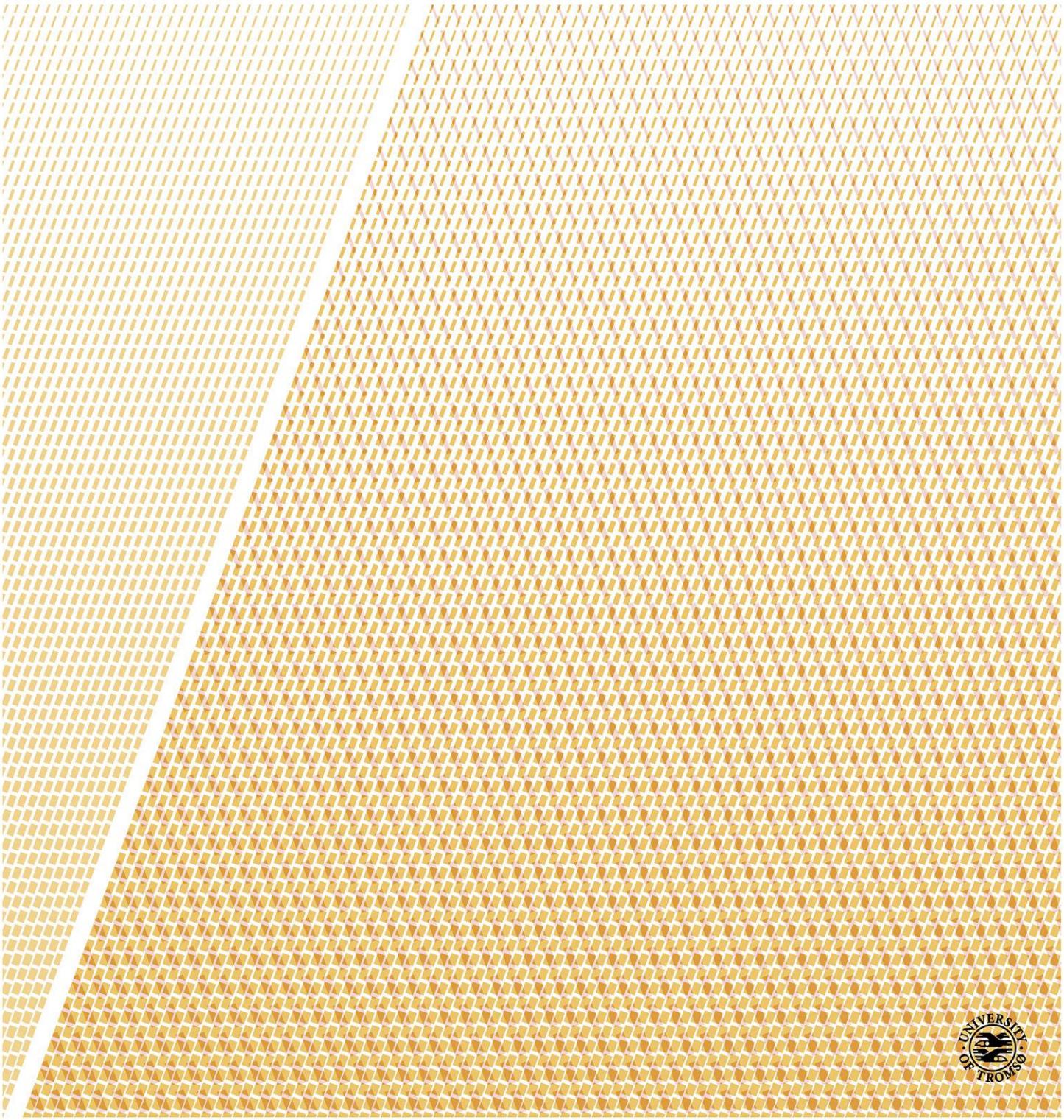

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Development of biomimetic phospholipid vesicle-based permeation assays (PVPA) as screening tool in drug development

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LIST OF ABBREVIATIONS

ACV	Acyclovir
BCS	Biopharmaceutics Classification System
BSA	bovine serum albumin
Caco-2	adenocarcinoma cell line from human colon
Chol	cholesterol
CMC	critical micelle concentration
CYP	cytochrome P
E-80	egg phospholipids
E-PC	egg phosphatidylcholine
E-PG	egg phosphatidylglycerol
ER	electrical resistance
GIT	gastrointestinal tract
FaB	fasted state buffer
FaSSIF	fasted state simulated intestinal fluid
FeB	fed state buffer
FeSSIF	fed state simulated intestinal fluid
ft.	freeze-thaw
HPLC	high performance liquid chromatography
P_{app}	apparent permeability coefficient
PAMPA	parallel artificial membrane permeability assay
PB	phosphate buffer
PE	phosphatidylethanolamine
P-gp	P-glycoprotein
PI	phosphatidylinositol
PS	phosphatidylserine
PSA	polar surface area

PVPA	phospholipid vesicle-based permeation assay
$PVPA_{biomimetic}$	PVPA with phosphatidylcholine, phophatidylethanolamine, phophatidylserine, phosphatidylinositol and cholesterol in the barrier
$PVPA_{o}$	PVPA with E-80 in the barrier
UWL	unstirred water layer

ABSTRACT

Although several routes of administration can be utilized to bring a drug to the desired site of action, oral administration is still the most important and prevalent route of administration, due to its cost efficiency, convenience and patient compliance. A prerequisite for successful oral therapy is the ability of a drug to cross the gastrointestinal barrier. Over the past two decades, the number of new biological active chemical entities has increased due to the modern discovery programs, often based on combinatorial chemistry and high-throughput screening. Consequently, appropriate and reliable high-throughput *in vitro* models to assess the permeability of new drug candidates and drug formulations are required to increase the success rate and to reduce the time and cost for development. The phospholipid vesicle-based permeation assay (PVPA) is an *in vitro* permeability model consisting of a tight layer of liposomes immobilized on a filter that successfully has been used to test novel active substances and formulations. The first part of this thesis was to employ the PVPA for the first time as a screening tool to assess and improve the permeability of acyclovir (ACV), a poorly permeable model drug, by designing mucoadhesive liposomal formulations. The incorporation of ACV into liposomes resulted in a significant increase in the *in vitro* permeability of ACV, and mucoadhesive coating further enhanced the permeability for some of the formulations. The next part of this thesis was to develop a more robust, biomimetic PVPA with a lipid composition mimicking that of the intestinal barrier. The permeability values obtained showed that the positively charged basic compounds showed increased permeability through the negatively charged biomimetic PVPA compared to the original PVPA. The results from the model drugs also correlated well with *in vivo* on fractions absorbed in humans. Further, the charge in lipid composition resulted in a tremendously increase in barrier robustness in the presence of tensides compared with the original PVPA as well as improved storage stability for up to 6 months at -70^{o}C. The biorelevance of the model was further improved by using biorelevant media. The biomimetic barrier was found to be compatible with fasted state and fed state simulated intestinal fluids (FaSSIF and FeSSIF). Four model drugs exhibited changes in permeability in the presence of the different simulated intestinal fluids in agreement with previous reports. Collectively, these findings moved the biomimetic PVPA an important step forward toward use as a better *in vitro* permeability model in drug development.

LIST OF PUBLICATIONS

The present thesis is based on the following publications and manuscript and will be referred to in the text by their Roman numerals.*

Paper I

Naderkhani, E., Erber, A., Škalko-Basnet, N., Flaten, G.E. (2014).

Improved permeability of acyclovir: optimization of mucoadhesive liposomes using the phospholipid vesicle-based permeation assay. *Journal of Pharmaceutical Sciences*, 103, 661-668.

Paper II

Naderkhani, E., Isaksson, J., Ryzhakov, A., Flaten, G.E. (2014).

Development of a biomimetic phospholipid vesicle-based permeation assay for the estimation of intestinal drug permeability. *Journal of Pharmaceutical Sciences*, 103, 1882-1890.

Paper III

Naderkhani, E., Vasskog, T., Flaten, G.E.

Biomimetic PVPA *in vitro* model for estimation of the intestinal drug permeability using fasted and fed state simulated intestinal fluids. *Resubmitted Manuscript (February 2015).*

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1. INTRODUCTION

The first prerequisite for successful drug therapy is the ability of a drug to cross the biological barrier. Different routes of administration are utilized to bring the drug to the desired site of action. Oral administration is the most important and most frequently used route, but drug administration to mucus membranes such as the vaginal barrier, is gaining increasing interest due to its advantages, and is expected to be more utilized with development of drug therapy (Berginc et al., 2014, Kanzer et al., 2010). Therefore, appropriate and reliable high-throughput *in vitro* models to assess the permeability of new drug candidates and drug formulations at the desired site of action are required.

1.1. The gastrointestinal tract

The understanding of functions of the human gastrointestinal tract (GIT) is important in the application of biopharmaceutics for the development of orally administered drugs. The human (GIT) is a collection of organs responsible for food digestion, nutrient absorption and waste expulsion. The GIT is divided anatomically into 3 organs: the stomach, the small intestine, and the large intestine. The stomach is a J-shaped, hollow, muscular bag designed for the digestion of dietary intake with a volume capacity greater than 3 L. The stomach contributes little to the absorptive function of the GIT (Kurana, 2008). The main site for the absorption of nutrients, water, and electrolytes is the small intestine, due to its specialized anatomy. The human small intestine is approximately 6 meters long and is divided into 3 segments: the duodenum, the jejunum and the ileum. A cross-section of the digestive tube reveals 4 major tissue layers. From the innermost layer outward, these are the mucosa, the submucosa, the *muscularis externa* (muscle coat), and the serosa. The mucosa forms the main barrier for drug and nutrient uptake, and is divided into 4 layers: a surface monolayer, the basal membrane, the *lamina propria* (a connective tissue framework), and the *muscularis mucosa* (a thin layer of smooth muscle) (Deferme et al., 2008). The surface layer comprises a monolayer of columnar epithelial cells (enterocytes), mucus-secreting goblet cells, microfold cells (M-cells) and lymphocytes. Epithelial cells including enterocytes are polarized and contain two functionally distinct membrane domains at either pole of the cell. The apical membrane contains specific constituents that are structurally and functionally different to those on the basal (serosal-blood) side at the opposite pole of the cell (Sjögren et al., 2014). In addition, the epithelial cells are linked together by tight junctions, thus

preventing undesirable leaks within adjacent cells/epithelial sheets. These tight junctions are made up of protein subunits and are responsible for the regulation of water, ions and molecules through the paracellular pathway (discussed in section 1.1.2) and are also involved in the control of cell growth and differentiation (González-Mariscal et al., 2008).

The epithelial monolayer, which is covered with mucus, primarily acts as a protecting barrier against harmful microorganisms and chemicals. Mucus is a viscous coating on many epithelial surfaces and consists primarily of water (up to 95% weight), carbohydrates, inorganic salts, lipids and glycoproteins (termed mucins). Mucins are responsible for the gel-like properties of mucus (Serra et al., 2009). In addition to serving as a barrier, the epithelium has specialized functions that support nutrient digestion and absorption. The large absorptive area is greater than 250 square meters and is created by *plicae circulares* (circular folds), villi and microvilli, which facilitates nutrient and drug absorption (Kurana, 2008). The circular folds, which are macroscopic valve-like folds surrounding the inside of the intestinal lumen, are estimated to increase the surface area of the small intestine by 3-fold. Moreover, the intestinal villi increase the surface area by approximately 30- fold and microvilli approximately 600-fold, leading to increased surface area available for both active and passive absorption (Balimane et al., 2001). Further, the large intestine consists of the colon, cecum, appendix and rectum. As most of digestion and absorption takes place in the small intestine, the large intestine absorbs the remaining water and electrolytes (Kurana, 2008).

The pH along the GIT varies greatly from acidic to basic and might impact the drug solubility and/or stability. In the stomach, pH value of 2.5 has been reported, whereas pH of the duodenum has been found to be approximately 6.3. Further, the median pH of the jejunum has been reported to be 6.9. Between the jejunum and the distal ileum the pH gradually increases from approximately 6 to about 8. However, it should be noted that meal intake influence the pH of the GIT to various degrees (Avdeef, 2003, Bergström et al., 2014).

1.1.1. Requirements for drug absorption

To achieve systemic circulation, orally administered drugs must withstand the physiological environment in the GIT as well as to be absorbed (Sarmento et al., 2012). Other factors such as physicochemical properties of the drug also play a crucial role for adequate drug absorption from

the GIT. These properties may impact dissolution, permeability, precipitation and interaction with food in the GIT, further influencing the performance of the drug *in vivo* (Bergström et al., 2014).

One of the rule-based systems for estimating intestinal drug absorption is the well-accepted 'rule of five', introduced by Lipinski and co-workers in 1997. The 'rule of five' states that poor absorption/permeation is more likely to occur if the drug has more than 5 H-bond donors, more than 10 H-bond acceptors, a high molecular weight > 500 and a log P > 5 (Lipinski et al., 1997). A compound violates Lipinski's rule of five if at least two of these sub rules are violated. It should be noted that this rule, only applies to absorption by passive diffusion. Drugs that are absorbed by transporter proteins are exceptions to the rule (see section 1.1.2). Orally administered drugs should possess a certain degree of hydophilicity to dissolve in the GI fluid, and meanwhile also have adequate lipophilicity to traverse the intestinal membrane. Another important factor that influences drug performance *in vivo* is the relationship between the acidic or basic properties of the drug and the pH of the GI environment. As most of drugs are either weakly acidic or basic, the varying pH of the small intestine may have an impact on the drug's ionization behavior, and thereby influence the intestinal drug absorption. Accordingly, the drug should exist in its non-ionized form in order to be able to cross the intestinal membrane (Dahan et al., 2010, Lennernäs, 2014). In the upper small intestine, where the pH is likely to be more acidic, weakly acidic drugs exist primarily in their non-ionized form, and the passive transcellular pathway is the dominant permeation route. Conversely, weakly basic drugs exist mostly in the form of ionized species, and consequently the passive transcellular route in the upper small intestine plays a minor role in their absorption.

Another important system that has gained considerable attention due to its ability to account for the solubility and permeability of a drug and its effects on intestinal absorption is the Biopharmaceutics Classification System (BCS). BCS was first introduced in 1995 by Amidon and co-workers and this system classifies drugs into four groups based on their aqueous solubility and intestinal permeability characteristics (Amidon et al., 1995). As shown in Figure 1.1, BCS classes consist of the following groups: Class I (high solubility-high permeability), Class II (low solubility-high permeability), Class III (high solubility-low permeability) and Class IV (low solubility-low permeability). This system is also utilized by many drug regulatory agencies

worldwide for the assurance of waivers of clinical bioequivalence studies for a number of Class I drugs (Ozawa et al., 2014). In the past few years, the BCS has been further developed in the context of drug metabolism, the effects of transporters on drug absorption, dissolution behavior, and pH considerations to better predict the drug's *in vivo* performance (Butler and Dressman, 2010, Wu and Benet, 2005, Zaki et al., 2010).

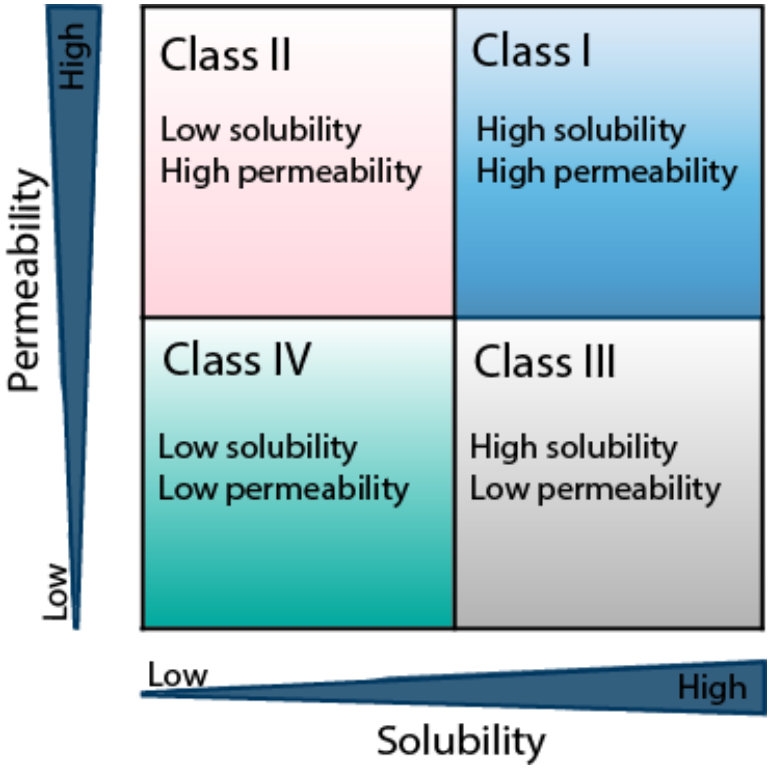

Figure 1.1: Illustration of the Biopharmaceutics Classification System.

1.1.2. Routes of intestinal drug absorption

Drug absorption primarily occurs in the small intestine and is a complex multi-pathway process (Sarmento et al., 2012). There are different pathways of drug absorption through the intestinal membrane (summarized in Figure 1.2): passive transport (panels A-B), which involves paracellular and transcellular transport, active/carrier-mediated transport (panels C-F) and transcytosis/endocytosis (panels G-H).

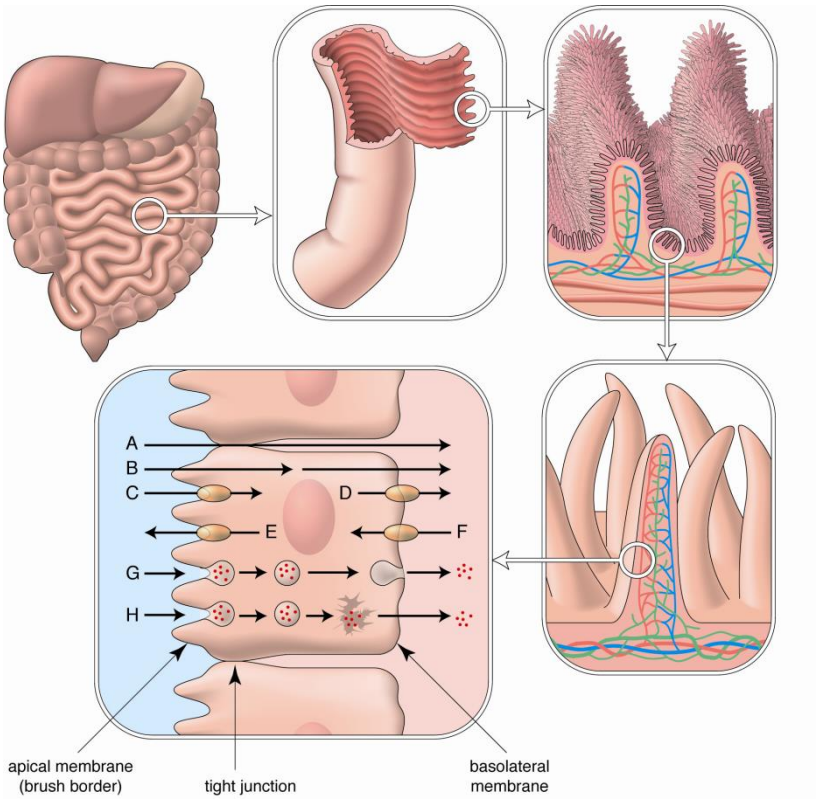

Figure 1.2: Different routes of intestinal drug absorption: (A) paracellular passive diffusion, (B) transcellular passive diffusion, (C-F) influx/efflux facilitated transport by membrane proteins, (G) transcytosis, and (H) endocytocis (reprinted from Våbenø 2004, with kind permission from Dr. Jon Våbenø).

The permeation rate for passive diffusion through the membrane relies on the concentration gradient. The majority of drugs in the marked are primarily absorbed through the passive transcellular pathway, permeating through the intestinal membrane in the direction of the concentration gradient (Sjögren et al., 2014). The transcellular pathway (B, Figure 1.2) is the predominant transport route for lipophilic drugs. Drug transport can also occur through the passive paracellular pathway, which is another passive route. Drug transport through this pathway occurs via the tight junctions between enterocytes. Due to the small paracellular pores (ranging between 6-10 Å), this pathway is primarily relevant for small hydrophilic compounds (Anderson, 2001). Moreover, the negative characteristics of the tight junctions facilitate the transport of cationic compounds more easily than the transport of anionic and neutral compounds (Linnankoski et al., 2010).

Active transport is an energy dependent process where a drug molecule is transported through the intestinal membrane by different carrier proteins expressed at the basolateral and/or apical side of the membrane. It should be noted that carrier proteins can function as efflux or influx pumps. One of the most studied efflux transporters is the ATP-binding cassette transporter, a P-glycoprotein (P-gp) expressed in the gut wall. Efflux transporters function to expel a drug molecule out of the cell and back into the intestinal lumen, resulting in limited absorption, whereas influx transporters actively transfer the drug through the basolateral side to the inner membrane leaflet (Sjögren et al., 2014).

Transcytosis and endocytosis (panels G-H, Figure 1.2) are particularly important transport pathways for transporting certain macromolecules. In these pathways, the macromolecules are captured in vesicles on the apical side of the cell and drawn across the cell membrane and released on the basolateral side. Macromolecules that are captured in vesicles can also be released onto the cell interior (Daugherty and Mrsny, 1999).

1.2. Screening of drug permeability properties

As the modern drug discovery programs are producing large amounts of potential drug candidates and drug formulations, appropriate and reliable models to assess drug absorption early in the development process are required to increase the success rates of drugs and reduce the time and costs associated with their development. In addition, these models can help to avoid the excessive use of animal studies in the early developmental stages can (Lennernäs, 2014).

1.2.1. *In silico* models for the prediction of drug permeability

During drug discovery, it is important to accurately determine whether a drug candidate offers satisfactory pharmaceutical properties, such as adequate solubility and high intestinal permeability. In the last few years, interest in linking calculated molecular properties to *in vivo* drug performance has significantly increased. There are currently several computational methods available to forecast passive intestinal drug absorption (Bergström et al., 2014).

The complexity of the theoretical methods varies to a high degree; ranging from the simple counting of atoms (e.g., Lipinski rule of five) to time consuming approaches based on quantum mechanics calculations (Lipinski et al., 1997, Stenberg et al., 2000). Common to all theoretical methods is that the estimation of drug permeability is achieved based on calculated molecular

descriptors, such as lipophilicity, molecular weight and hydrogen bonding. Some models also take into account the effect of polar surface area (PSA), which is defined as the part of the molecular surface to which nitrogen, oxygen and their connected hydrogen atoms contribute (Palm et al., 1997). Other models have introduced a modification of the Lipinski rules, suggesting that compounds with PSAs equal to or less than 140 $Å^2$ (or 12 or fewer hydrogen bond donors and acceptors) and 10 or fewer rotatable bonds will have high oral bioavailability (Veber et al., 2002). A similar rule-based system to Lipinski's rule of five, known as the 'Oral PhysChem Score', has also been introduced by Lobell and co-workers (Lobell et al., 2006). By applying a traffic light color scheme and a scoring system with a range of 0-10, this system is able to predict the biopharmaceutical properties of a compound. The compound's molecular weight, lipophilicity, number of rotatable bonds, PSA and calculated solubility are each assigned a traffic light color (green, 0; yellow, 1; red, 2) and the values are summed to forecast the biopharmaceutical properties (Bergström et al., 2014, Lobell et al., 2006). Other researchers have also investigated the impact of aromatic rings within a molecule on its developability properties regarding the oral absorption and oral bioavailability (Ritchie and MacDonald, 2009). Ritchie and MacDonald noted that the developability of molecules significantly increases when the number of aromatic rings is fewer than 3. Their conclusion was based on changes in various parameters, such as solubility, which significantly decreased with the number of aromatic rings, and lipopophilicity, CYP3A4 inhibition and serum albumin binding, which all significantly increased with an increased number of aromatic rings (Ritchie and MacDonald, 2009).

To summarize, the application of *in silico* modeling has already been shown to offer great potential for predicting intestinal permeability before synthesis of a compound. Furthermore, *in silico* modeling reduces the number of experimental permeability studies at the early stages of drug development and can be conducted pre-synthesis. However, due to the complex multi-pathway utilized for drug absorption, the currently available *in silico* methods are not able to accurately predict drug performance *in vivo*. Hence, more advanced experimental models performed under physiologically relevant conditions might be required later, thereby facilitating the discovery and development process.

1.2.2. Caco-2 model

To date, a number of cell culture models mimicking *in vivo* barriers have been developed and utilized to predict the permeability of drugs. As normal human enterocytes fail to form a polarized epithelial monolayer consisting of an apical and basolateral surface, continuously growing tumor cell cultures are used, which are able to spontaneously differentiate and form a polarized monolayer (Deferme et al., 2008).

The current gold standard in *in vitro* permeability screening is the cell-based Caco-2 model, which is widely established and used in both industry and academia (Artursson, 1990, Artursson, 1991). The Caco-2 model is derived from human colon adenocarcinoma and differentiates spontaneously to form a polarized epithelial monolayer (i.e., consisting of an apical and basolateral surface) as well as tight junctions. To resemble the intestinal epithelium in humans, the Caco-2 cell lines are cultured for at least 20 days on a porous filter support. However, attempts have been made to reduce the culture time by altering the filter support, the filter coating, the cell culture medium and the seeding density (Balimane et al., 2006, Chong et al., 1997). Due to the expression of transport proteins such as P-gp, metabolic enzymes such a cytochrome P isoenzymes (CYP) (except from CYP3A), and the well-established tight junctions, this cell-based model is capable to predict passive diffusion as well as active transport and facilitated diffusion of drug compounds. To confirm the epithelial integrity of the monolayer, transepithelial electrical resistance (TEER) is measured (Buckley et al., 2012, Deferme et al., 2008). Moreover, additional information on the monolayer integrity can be obtained by employing hydrophilic markers that pass the monolayer via the paracellular pathway (e.g., mannitol, sodiumfluorescein or atenolol) (Deferme et al., 2008).

One of the advantages of the Caco-2 model is its human cell-derived origin. Thus, this model shares similarities in cell morphology with human enterocytes to better mimic *in vivo* intestinal permeability. It is also amenable to automation.

Despite the advantages of the cell-based monolayers discussed above, there are still major drawbacks associated with their use. These major drawbacks concern the intrinsic variability of transport properties and permeability data obtained from different laboratories (Turco et al., 2011, Balimane et al., 2006). Factors that are reported to influence outcomes include minor variability

in cell culture conditions, experimental protocols, and the age of the cells. Another drawback of this model is the smaller size of the paracellular channels (controlled by tight junctions) compared with those of the human small intestine. This may contribute to underestimation of the permeability of low molecular weight hydrophilic compounds that are absorbed primarily via the paracellular pathway (Balimane et al., 2006, Deferme et al., 2008). Furthermore, the unstirred water layer (UWL) adjacent to the intestinal membrane is also reported to be thicker than that found *in vivo* (Artursson and Karlsson, 1991, Adson et al., 1995). According to the literature, UWL of approximately 100 µm has been estimated for the human intestine while for the Caco-2 monolayers 1000 µm has been reported (Hilgers et al., 1990, Lennernäs, 1998). Consequently, the permeability of lipophilic compounds can be diminished in the cell model due to the additional permeation barrier created by the UWL (Artursson and Karlsson, 1991, Katneni et al., 2008). In addition, this model is less compatible with the typical cosolvents (e.g., DMSO and PEG) compared to other artificial *in vitro* models, reducing the applicability of the model to poorly soluble compounds (Balimane et al., 2006, Buckley et al., 2012). Finally, this model is known to be relatively costly and time consuming compared to the non-cell-based *in vitro* models that will be later discussed.

1.2.3. Alternative cell lines to Caco-2

One of the cell-based models that has received a great deal of attention as an alternative to the Caco-2 model for permeability studies is a model utilizing Madin-Darby canine kidney (MDCK) cells. Similar to Caco-2 cell lines, MDCK cells differentiate and form a polarized monolayer consisting of tight junctions. The short culture time of 3-5 days increases the throughput of absorption studies (Irvine et al., 1999, Raub et al., 1993). The TEER of the MDCK cells is closer to the TEER of the small intestine, offering good apparent permeability (P_{app}) comparability with the *in vivo* situation for hydrophilic compounds (Deferme et al., 2008). MDCK cell monolayers exhibit good applicability in permeability screening for lipophilic drugs that cross the membrane via the passive transcellular pathway (Irvine et al., 1999, Raub et al., 1993). In addition, these cells exhibit good compatibility with a range of solubilizing excipients (Taub et al., 2002). Although MDCK cells offer many advantages, their canine origin is considered to be a disadvantage. Their relatively low expression levels of transporter proteins and minimal

metabolic activity should also be taken into consideration when using drug compounds that are actively transported through the membrane (Buckley et al., 2012).

Another *in vitro* cell-based model developed to study the intestinal absorption of drug compounds consists of TC7 cells, a clonal selection isolated from a late passage of the parental Caco-2 cell line (Carriere et al., 1995). As the population of Caco-2 cell lines has been reported to be heterogeneous and highly dependent on culture conditions, the TC7 cells were developed to increase the homogeneity (Zucco et al., 2005). Although previous literature indicated the increased homogeneity of these cells, a more recent study suggests that TC7 cells are not suited in predicting the intestinal absorption of highly lipophilic compounds particularly when transport-mediated routes and/or first-pass metabolism are involved (Turco et al., 2011).

Another interesting cell-based model consists of 2/4/A1 cells derived from fetal rat intestine. This model has been shown to form leakier monolayers than the Caco-2 cell line, thus mimicking the intestinal paracellular pathway more closely. Furthermore, the lack of transporters and efflux systems make this cell line suitable to study passive drug permeability (Tavelin et al., 2003).

Intestinal co-culture cell lines such as HT29-H and HT29-MTX are also utilized in permeability studies. These cell lines are capable of forming mucus-secreting goblet cells and thus more closely mimic the absorptive and secretive components of the intestinal epithelia (Walter et al., 1996, Wikman-Larhed and Artursson, 1995). The P_{app} values of passively absorbed drugs exhibit good correlations with the fractions absorbed *in vivo*. However, the absorption rates for actively transported drug compounds have been reported to be underestimated compared to the *in vivo* situation (Walter et al., 1996).

The LLC-PK1 cell line is also an alternative to the Caco-2 model to predict the intestinal permeability of drug compounds. The LLC-PK1 cells are derived from porcine kidney epithelial cells and have been shown to be easily transfected compared to Caco-2 cells (Deferme et al., 2008).

1.2.4. Parallel artificial membrane permeability assay (PAMPA)

The parallel artificial membrane permeability assay (PAMPA) is an artificial *in vitro* model first introduced by Kansy and co-workers to predict the rapid transcellular permeation of drugs in the intestine (Kansy et al., 1998). PAMPA consists of a hydrophobic filter support soaked with lecithin in an organic solvent, which mainly consists of *n*-dodecane. The created artificial lipid membrane separates the donor and the acceptor compartments in the assay, as shown in the schematic illustration Figure 1.3. In a permeability experiment, the concentration of the drug dispensed onto the lipid membrane can be measured from the acceptor compartment at certain time intervals (Kansy et al., 2004).

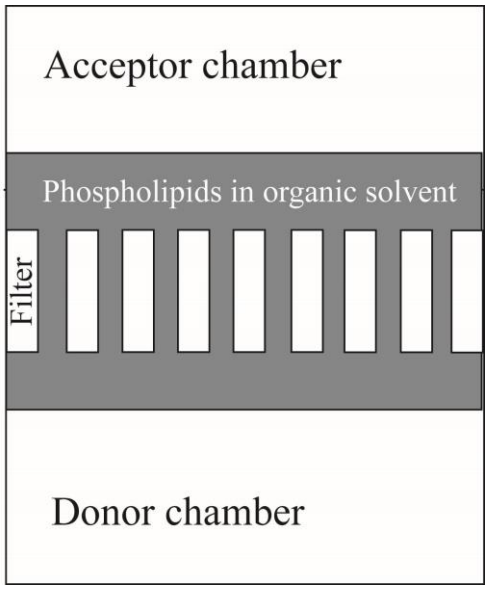

Figure 1.3: Schematic illustration of the PAMPA permeation cell (reprinted from Flaten 2007, with kind permission from Dr.Gøril Eide Flaten).

Several recent attempts to alter the composition of the PAMPA have been made to optimize and increase the predictability of oral absorption. Numerous variants of this model have been reported with different lipid compositions, including the *n*-hexadecane (HDM)-PAMPA (Wohnsland and Faller, 2001), the biomimetic (BM)-PAMPA (Sugano et al., 2001b) and the double sink (DS)-PAMPA models (Avdeef, 2003, Avdeef and Tsinman, 2006). The latest development of this model is known as the double artificial membrane permeation assay (DAMPA), which consists of a double artificial membrane with an intracellular compartment (Kataoka et al., 2014). As the

intracellular pH (physiological pH 7.4) is considered to impact permeability of ionic compounds, this model was developed to predict the permeation of these compounds from the apical side to the intracellular compartment and from the intracellular compartment to the basal side (Kataoka et al., 2014).

PAMPA is widely used in pharmaceutical research as an *in vitro* model to assess the passive permeability of drugs. Compared to cell-based models, this assay allows access to a wider pH range to provide insight into human gastrointestinal absorption (Avdeef, 2003, Avdeef et al., 2005). Moreover, the setup consists of 96-well microtiter plates coupled with analysis using a spectrophotometric plate reader to allow screening of a large number of compounds per day (Balimane et al., 2006, Kansy et al., 1998). PAPMA appears to be compatible with a range of cosolvents, thus enhancing the applicability of this model for poorly soluble compounds (Buckley et al., 2012, Sugano et al., 2001b).

Although PAMPA is gathering increasing interest in pharmaceutical research, several disadvantages are associated with the model. One disadvantage is that PAMPA underestimates the permeability of actively transported compounds as well as low molecular weight hydrophilic compounds absorbed by the paracellular pathway (Balimane et al., 2006). The use of biorelevant media is also lacking in PMAPA. In addition, similar to cell-based models, a significant UWL exists in the PAMPA model. The presence of the UWL in PAMPA has been estimated to range between 1900-3800 μm depending on the experimental setup used, creating a rate-limiting barrier for the permeation of highly lipophilic compounds (Buckley et al., 2012, Nielsen and Avdeef, 2004). It should also be noted that, to date, PAMPA comprises numerous methods applied in various laboratories. Use of different membrane constituents, sink conditions, and permeation times, makes thus the inter-laboratory comparison extremely difficult. Therefore, standardization and validation methods for this model should be introduced (Deferme et al., 2008).

1.2.5. Phospholipid vesicle-based permeation assay

The original phospholipid vesicle-based permeation assay ($PVPA_o$) is a novel screening model for passive drug permeability, that was first introduced in 2006 by Flaten and co-workers to mimic the intestinal epithelia (Flaten et al., 2006b). The novelty of the concept is the utilization of liposomes that are similar to the cells, which more closely facilitates the submicron

morphology of the epithelium than other currently used artificial models. The $PVPA_o$ is prepared by depositing two populations of liposomes of different sizes prepared from egg phosphatidylcholine (E-PC) on a filter support via centrifugation, followed by a freeze-thaw cycle to promote liposome fusion, which finally results in a tight barrier. The schematic illustration of the PVPA barrier provided in Figure 1.4, shows the large multilamellar vesicles on top of the filter support and the smaller unilamellar liposomes inside the pores.

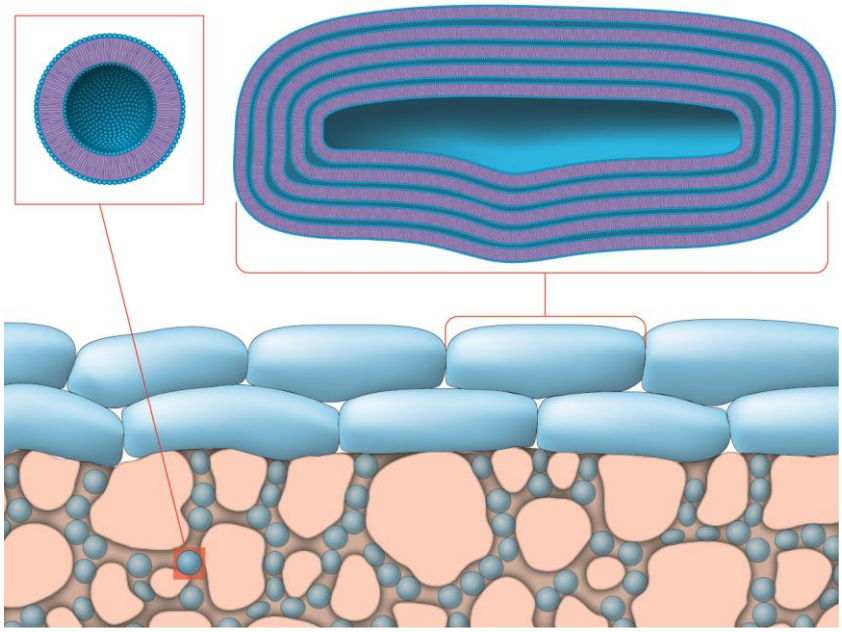

Figure 1.4: Schematic illustration of the phospholipid vesicle- based barrier (reprinted from Flaten 2007, with kind permission from Dr. Gøril Eide Flaten)

The $PVPA_o$ has been successfully used for permeability testing of novel active substances, marketed drug substances as well as drug in complex formulations, and has shown potential for automation using a robotic system connected to a plate reader (Flaten et al., 2009, Flaten et al., 2011, Hansen et al., 2011, Kanzer et al., 2010, Svenson et al., 2009). The $PVPA_o$ has been shown to correlate well with the extent of drug absorption *in vivo*, comparable to the Caco-2 model and better than the PAMPA model. The $PVPA_o$ barrier has further been shown to be stable in a pH range from 2.0 to 8.0 and is suitable for obtaining information on segmental intestinal absorption. Moreover, this assay has the potential to work as permeability model for absorption sites with a

broad environmental pH range (Flaten et al., 2006a). In contrast to other *in vitro* models, the PVPA model appears to have negligible UWL, with the permeability of the highly lipophilic drug, testosterone being unaffected by shaking or stirring (Flaten et al., 2007).

The $PVPA_o$ barrier mainly consists of PC, a component found in many absorption barriers. Recently, a modified PVPA mimicking healthy and compromised skin barriers has been introduced (Engesland et al., 2013). This model could have the potential for use as a screening tool in both pharmaceutical and cosmeceuticals manufacturing.

Although the PVPA models offer several advantages in terms of being less expensive and more efficient than the cell-based counterparts, they underestimate the permeability of actively transported compounds. Other disadvantage associated with the model is the lack of morphological features such as villi that make up the brush border of epithelial cells (Flaten et al., 2006b).

Similar to cell-based models, the integrity of the PVPA barrier is assessed by measuring the permeability of a hydrophilic marker calcein as well as measuring the electrical resistance (ER) across the barrier. The hydrophilic marker, calcein is used as it is expected to demonstrate low permeability through lipid bilayers (Flaten et al., 2006b).

1.3. The solubility-permeability interplay of drugs

In the last decade, the number of poorly water soluble drug candidates has increased, due to modern drug discovery programs based on combinatorial chemistry and high-throughput screening (Augustijns et al., 2014). An important prerequisite for successful oral drug therapy is the ability of the drug to cross the GI barrier. Adequate solubility and permeability are therefore important properties of all chemical compounds to ensure compound solvation and distribution at the site of absorption. Furthermore, poor water solubility of a compound can contribute to low and variable bioavailability. As a consequence, frequent high-dose administration is necessary to achieve the desired drug concentration in systemic circulation, again leading to GI mucosal toxicity.

The improvement of drug solubility and oral bioavailability remains one of the most challenging aspects of the drug development process. There are several approaches reported in the literature

for the enhancement of poorly water soluble drugs. Lipid-based formulations (Allen and Cullis, 2013), self-emulsifying systems (Yano et al., 2010) cyclodextrin complexation (Loftsson and Brewster, 2011) and cosolvents (Beig et al., 2012) are some of the approaches that are used to handle the problem of poor aqueous solubility. Although the impact on solubility for these approaches is well understood, their influence on the intestinal membrane permeability of a lipophilic drug has been overlooked and poorly understood. Lately, several studies reports that only unbound drug molecules are able to permeate across the GI barrier and eventually reach systemic circulation (Beig et al., 2012, Dahan and Miller, 2012). Hence, care is required to understand the impact of increased solubility and consequently decreased permeability on formulation development (Kostewicz et al., 2014).

In terms of assessing the permeability of poorly soluble drugs by models, incomplete solubilization most likely lead to unreliable permeability values because the actual concentration in the donor compartment is unknown. When working with poorly soluble drugs in conventional buffer solutions, another frequent occurrence is the possibility of drug precipitation in the test setup. Furthermore, highly lipophilic drugs have been shown to accumulate in the lipophilic barrier and/or adsorb onto the experimental material resulting in poor recovery (Ingels et al., 2004). The application of solubilizing excipients, i.e., surfactants or cosolvents in the test setup might help to prevent this action. Lipophilic drugs, in particular, are known to diffuse back from the receiver compartment through the permeation barrier to donor compartment (Ingels and Augustijns, 2003). Attempts have therefore been made to reduce drug accumulation in the barrier by applying surfactants and/or bovine serum albumin (BSA) in the receiver compartment to achieve better sink conditions (Fischer et al., 2012, Katneni et al., 2008).

1.3.1. Biorelevant media

Besides the conventional solubilizing agents, the biorelevant media exhibit suitable solubilizing capacities for lipophilic drugs. It has been recognized that presence of certain components of the intestinal lumen such as lecithin and the endogenous surfactant, sodium taurocholate could influence the solubility and permeability characteristics of poorly soluble compounds. The bile salt, sodium taurocholate is known to form micelles above its critical micelle concentration (CMC) and thus incorporate lipophilic compounds into micelles. Additionally, both sodium taurocholate and lecithin in the GI fluid are known to interact and form mixed micelles. This

generally results in enhanced solubility of poorly soluble drugs, but on the other hand might decrease the free fraction of the drug, thereby resulting in decreased apparent permeability (Augustijns et al., 2014, Dahan and Miller, 2012).

Among the biorelevant media, fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) are the most utilized fluids applied first as dissolution medium and later as transport medium in permeability screening studies (Galia et al., 1998). In the literature there are several permeability studies performed on cell-based models employing these fluids (Fossati et al., 2008, Ingels et al., 2004, Kataoka et al., 2006, Lind et al., 2007) The Caco-2 model has been shown good compatibility in presence of biorelevant media. Simulated intestinal fluids are also reported to reduce the drug adsorption to surfaces in the apical compartment compared with the saline buffers, thus increasing the recovery of poor water soluble drugs (Fossati et al., 2008, Ingels et al., 2004). However, simulated intestinal fluids' influence on drug permeation is more controversially discussed. Enhanced, unchanged and diminished drug permeability results are reported in different studies applying these fluids as transport medium. These results are assumed to occur due to different absorption mechanisms of the drugs used in the studies (Fossati et al., 2008, Ingels et al., 2004, Kataoka et al., 2006, Lind et al., 2007, Markopoulos et al., 2014).

1.4. Mucoadhesive liposomes as drug delivery systems

Since the introduction of liposomes in the 1960s, impressive amounts of literature have been published showing the broad spectrum of applications of these colloidal systems (Allen and Cullis, 2013, Bangham et al., 1965). Liposomes consist of an aqueous core enclosed by one or several lipid bilayers, and have the advantage to incorporate both lipophilic and hydrophilic drugs. The bioavailability of a drug suffering from limited solubility and permeability could be optimized and improved by controlling the drug carrier's properties such as size and surface characteristics. Additionally, encapsulation of drugs in liposomes represents a feasible and efficient approach to control its release, reduce drug toxicity, and increase its physical stability (Vanic and Skalko-Basnet, 2014). However, due to their liquid nature and low viscosity, conventional liposomes show low retention time at the specific site of delivery. The interest in developing mucoadhesive drug delivery systems has therefore gained increased attention to enhance the contact time between the delivery system and the absorbing membrane (Figure 1.4).

To date a wide variety of biodegradable mucoadhesive polymers have been used for the development of pharmaceutical drug delivery systems. Among mucoadhesive polymers, chitosan and Carbopol® has been frequently used in designing mucoadhesive nanosystems (Serra et al., 2009).

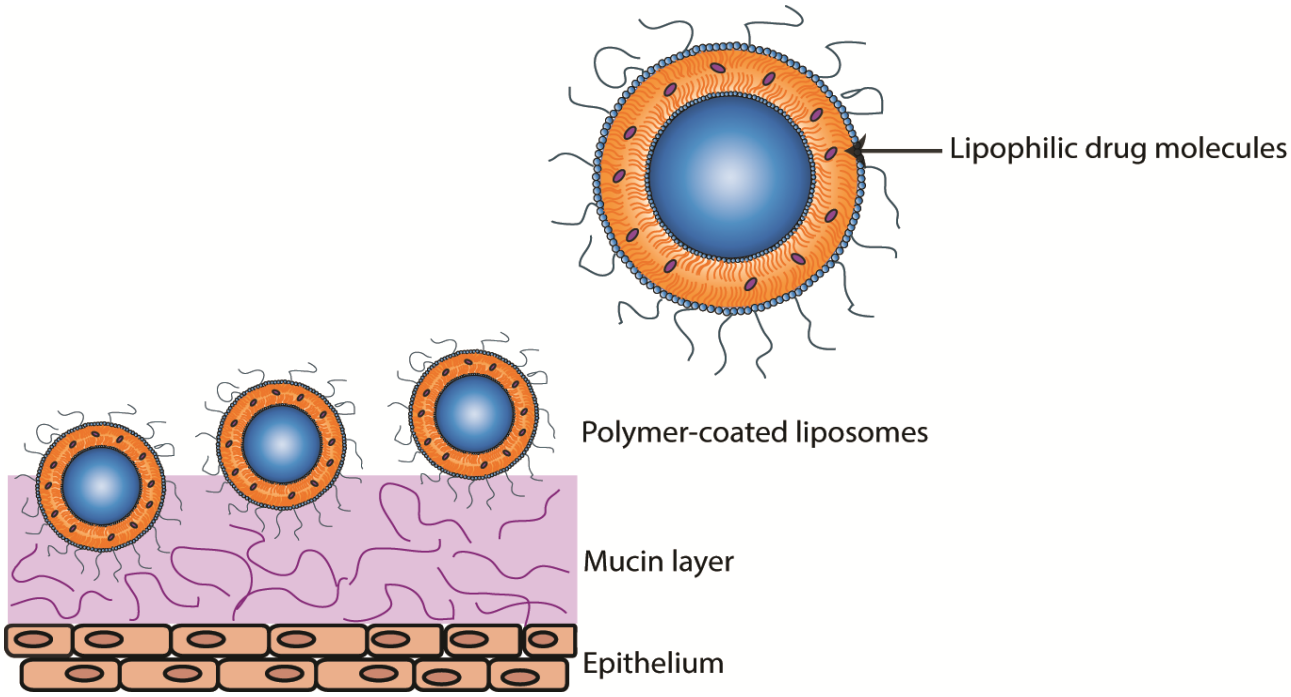

Figure 1.4: Illustration of muoadhesive liposomes on mucosal surface.

1.4.1. Carbopol

Carbomers, also referred to as Carbopol® polymers, are polymers of acrylic acid that are cross-linked with polyalkenyl ethers or divinyl glycol. The anionic polymers have the ability to swell and form gel networks upon neutralization in aqueous medium (e.g., water). The swelling property is due to their Carboxylic groups on the backbone (see Figure 1.5) which become ionized and further result in repulsion between the negative charges (Islam et al., 2004).

Carbopol® polymers are used in diverse range of pharmaceutical applications and also in the dermocosmetic field. These polymers offer variety of advantages, such as high viscosity at low

concentrations, wide viscosity interval, bioadhesive properties, temperature stability compatibility with many active ingredients and good patient acceptance (Islam et al., 2004, Romanko et al., 2009). These properties make Carbopol® potentially valuable as pharmaceutical excipients in diverse range of applications such as in controlled-released in solid dosage forms, as thickener to produce a wide range of viscosities in topical and oral suspensions and in drug delivery systems for buccal, ocular, nasal, topical, intestinal, rectal and vaginal applications (Islam et al., 2004).

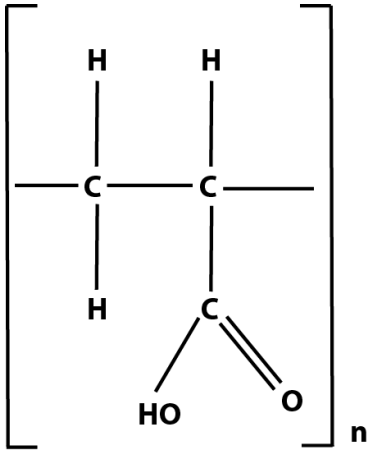

Figure 1.5: Chemical structure of carbomers consisting of monomeric units.

1.4.2. Chitosan

Chitosan is a polymer derived by deacetylation of chitin, one of the most abundant polysaccharides found in exoskeleton of crustacea, insects and some fungi (Dash et al., 2011). The main parameters influencing the physical properties of chitosan are its molecular weight and degree of deacetylation. These parameters have significant effects on its solubility and rheological properties. At low pH (below 6.0), chitosan is readily soluble due to its amine groups with a pKa value of 6.3. The viscosity of chitosan solution is reported to increase with increasing degree of deacetylation, increase in chitosan concentration and decrease in temperature (Dash et al., 2011, Singla and Chawla, 2001). The chemical structure of chitosan is presented in Figure 1.6.

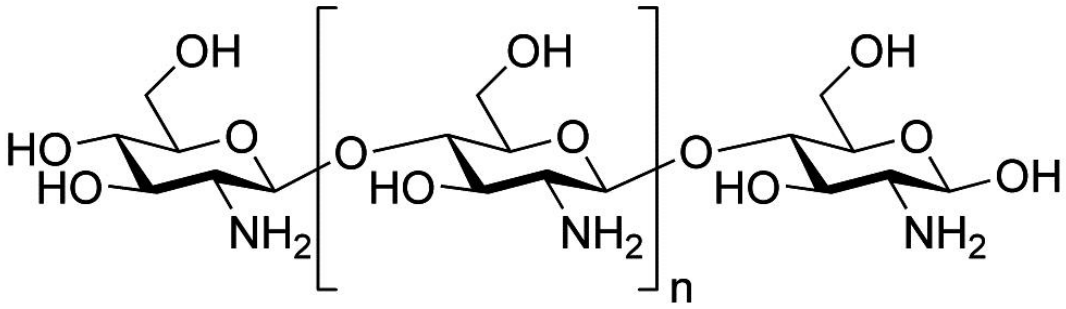

Figure 1.6: Chemical structure of chitosan.

Over the last decades, chitosan has gained increased attention as excipients in pharmaceutical formulations due to its various advantages such as low toxicity, biocompatibility, antimicrobial and excellent mucoadhesive properties (Singla and Chawla, 2001). The mucoadhesiveness of chitosan is reported to be due to its charge at physiological pH. Mucus is known to be negatively charged and chitosan, being a cationic polymer, can bind electrostatically to mucus and assure closer contact of the delivery system (Ribeiro et al., 2009).

2. AIMS OF THE STUDY

The overall aims of the thesis were to develop a biomimetic *in vitro* PVPA for better estimation of intestinal permeability, including evaluation of mucoadhesive drug delivery systems to investigate the $PVPA_o$ model's potential as a tool in formulation optimization.

The specific aims were as follows:

- Improve the permeability of acyclovir (ACV) by developing and optimizing mucoadhesive liposome formulations (paper I).

- Design a biomimetic PVPA barrier ($PVPA_{biomimetic}$) to better mimic the intestinal barrier by altering the lipid composition of the barrier (paper II).

- Investigate whether the use of more biologically relevant pH conditions in permeability experiments leads to improved prediction of *in vivo* absorption (paper II).

- Investigate the stability of the $PVPA_{biomimetic}$ in the presence of surfactants and cosolvents (paper II).

- Improve the biorelevance of $PVPA_{biomimetic}$ by employing the biorelevant media (FaSSIF and FeSSIF) for better prediction of *in vivo* absorption of a selection of model drugs with diverse sets of physicochemical properties (paper III).

- Investigate the storage stability of the $PVPA_{biomimetic}$ to elucidate the applicability of the model in increased throughput strategies (Paper III).

3. SUMMARY OF PAPERS

3.1. Paper I

The aim of the first paper was to improve the permeability of the model drug ACV by developing and optimizing different liposomal formulations with mucoadhesive properties.

Liposomes were prepared from E-PC and E-PC/egg phosphatidylglycerol (E-PC/E-PG). To find a suitable liposomal formulation and to assure liposomal retention at the mucosal site, the liposomes were coated with mucoadhesive polymers (Carbopol® or chitosan). Furthermore, the liposomes were characterized for their size, both before and after probe sonication, as well as for their entrapment efficiency, surface charge and mucoadhesion activities.

Given that ACV can be applied via various drug administration routes, we used $PVPA_o$, a well-established *in vitro* permeability model, as a tool for optimizing the characteristics of the liposomes and their drug permeability profile. As the $PVPA_o$ mainly consist of PC, it has the potential to serve a general model mimicking various absorption barriers.

Sonication reduced the size of the ACV-containing liposomes and led to a more homogeneous size distribution. However, it also resulted in a significant loss of the entrapped drug. Therefore, we applied the polymer coating in the presence of unentrapped ACV to increase the entrapment efficiency. However, the chitosan polymer coating resulted in low entrapment efficiency (approximately 3%) of ACV after sonication. Carbopol was therefore used in all further studies.

Regarding the entrapment efficiency, the negatively charged E-PC/E-PG liposomes demonstrated a higher entrapment efficiency of ACV than the neutral E-PC liposomes. However, coating with Carbopol influenced the entrapment of ACV into the neutral E-PC liposomes. The entrapment efficiency of ACV increased from 15.24 ± 1.85% in the non-coated E-PC to 20.34 ± 5.43% in the coated E-PC.

The results of the surface characterization provided us with valuable information regarding the inclusion of the negatively charged E-PG in the lipid bilayer and the formation of a coating layer comprised of an anionic polymer. The zeta potential values for liposomes containing E-PG were negative, and coating the liposomes with Carbopol further increased the negative values relative to the non-coated liposomes.

The mucoadhesive properties of the formulations were tested, and the results clearly showed that the coated E-PC formulations demonstrated a higher degree of mucoadhesion than the non-coated E-PC liposomes. In the case of the E-PC/E-PG liposomes, the mucoadhesion trend was not as clear.

In the *in vitro* permeability testing, we found that the incorporation of ACV into liposomes resulted in significantly increased permeability compared with an aqueous solution of ACV. Furthermore, coating with Carbopol enhanced the permeability from both the E-PC/E-PG liposomes and the sonicated E-PC liposomes.

3.2. Paper II

The focus of the second paper was to develop a more biomimetic PVPA for the estimation of intestinal drug permeability.

To improve the biomimetic properties of the $PVPA_o$ model, we first utilized a more biologically relevant pH condition (pH 6.2) in the permeability setup to see if pH would lead to improved prediction of *in vivo* absorption. Furthermore, we changed the lipid composition of the PVPA barrier to one that more closely mimics the intestinal barrier. This approach was also intended to better mimic the *in vivo* conditions. Finally, as the lipid composition was altered, we wanted to evaluate the stability of the biomimetic PVPA ($PVPA_{biomimetic}$) in the presence of tensides and cosolvents.

Utilization of a new lipid composition comprised of PC/PE/PS/PI/Chol in the $PVPA_{biomimetic}$, required some alterations in the original preparation procedure to achieve a tight barrier. To evaluate barrier integrity, we used the hydrophilic marker calcein, which exhibited a permeability of 0.053×10^{-6} cm/s and an ER of 1532 ohm. The low permeability of calcein and an ER above 1000 ohm indicated a tight barrier.

Furthermore, the $PVPA_{biomimetic}$ was evaluated by performing permeability experiments at pH 6.2 and 7.4 using 19 drugs with various physicochemical properties with a wide range of fractions absorbed in humans. When comparing the drugs in different pH conditions, we observed an increased permeability of the acidic drugs and a decreased permeability of the basic drugs at pH 6.2 compared with pH 7.4. These results were consistent with the pH partition hypothesis. The same observations in terms of permeability were also observed with the $PVPA_o$ barrier. Furthermore, the degree of correct classification according to the *in vivo* conditions was comparable for the $PVPA_o$ and the $PVPA_{biomimetic}$.

Compared to the $PVPA_o$, the $PVPA_{biomimetic}$ appeared to be significantly more robust towards the tensides. This is a promising finding that makes the $PVPA_{biomimetic}$ a suitable *in vitro* model for screening the permeability of poorly soluble drugs.

3.3. Paper III

The aim of the third paper was to improve the biorelevance of our previously developed $PVPA_{biomimetic}$ model by investigating the applicability of biorelevant media, namely FaSSIF and FeSSIF.

The hydrophilic marker calcein was used to evaluate the compatibility of the $PVPA_{biomimetic}$ barrier with FaSSIF and FeSSIF. It is well known that bile salt concentration and the amount of lecithin in the biorelevant media are important factors that affect the solubility of a drug and thus its permeability. Drugs may therefore interact uniquely in the presence of biorelevant media, depending on their physicochemical properties. To assess the permeability behavior of drugs in the presence of FaSSIF and FeSSIF, four model drugs with various physicochemical properties were selected. Finally, the storage stability of the $PVPA_{biomimetic}$ barrier was determined during storage at -70°C.

Regarding the compatibility testing, we found that the barrier maintained its integrity and was compatible with the presence of FaSSIF and FeSSIF based on its ER and calcein permeability values. To the best of our knowledge, this was the first time that FeSSIF was used in a PVPA model. Furthermore, the model drugs exhibited changes in permeability in the presence of the different simulated fluids, which is in agreement with previous reports.

Regarding the storage stability of the barrier, we observed that $PVPA_{biomimetic}$ remained stable under storage conditions for a period of 6 months. This was thus a tremendous increase in stability compared with the $PVPA_o$ barrier, which is only stable for up to 2 weeks. These findings demonstrated that the $PVPA_{biomimetic}$ developed in paper II has the potential to be a better *in vitro* model compared to $PVPA_o$, which represents an important step forward.

4. RESULTS AND DISCUSSION

4.1. Optimization of mucoadhesive, ACV-containing liposomes using the $PVPA_o$ (Paper I)

In light of the increasing number of new poorly-water soluble drug candidates, various nano-sized drug carriers have gained attention as a means of improving and enhancing the drugs' therapeutic efficacy. Among the different drug carriers, mucoadhesive pharmaceuticals have gained considerable interest in pharmaceutical technology (Alsarra et al., 2008, Merzlikine et al., 2009, Pavelic et al., 2005, Ruiz-Caro et al., 2012). They are expected to interact with the mucus layer to enhance the contact time between the delivery system and the absorbing membrane, thereby providing improved delivery of the drug to the underlying tissue (Serra et al., 2009).

One drug in the current pharmaceutical market that poses a notable challenge is the antiviral agent ACV, a class III drug according to the BCS. ACV is characterized by its hydrophobicity and low solubility both in water and in lipid bilayers. As discussed earlier, liposomes offer increased solubility of poorly-water soluble drugs, prevent the degradation of the drug in the presence of mucosal fluids, and permit the controlled release of the entrapped drug at the target site (Vanic and Skalko-Basnet, 2014). To overcome the limitations of ACV and thus improve its permeability, we developed and optimized mucoadhesive ACV-containing liposome formulations. The well-established $PVPA_o$ model was applied as a tool to study the permeability profile of the drug. To our knowledge, this was the first time that $PVPA_o$ was utilized as an *in vitro* model to assess the permeability of mucoadhesive drug formulations.

4.1.1. Characterization of ACV-containing liposomes

In this study, the effects of the lipid composition and the type of polymer coating used were investigated to optimize the mucoadhesive, ACV-containing liposomes. It is known from the literature that the surface characteristics of liposomes may influence both the entrapment efficiency as well as the permeability of drugs (Fresta et al., 1999, Pavelic et al., 2005). Accordingly, two lipid compositions (E-PC and E-PC/E-PG) were employed for the preparation of liposomes, and their surfaces were further coated with polymers (Carbopol® or chitosan).

Size distribution is an important characteristic of liposomes destined for topical skin and oral drug delivery (Cevc, 2004, Takeuchi et al., 2005); however, little is known about the effects of

vesicle size on drug delivery intended for mucosal targeting at vaginal site. As ACV can be applied via various routes of drug administration, we applied both multilamellar and small unilamellar vesicles in all studies.

Size reduction of liposomes by sonication is known to cause significant loss of an entrapped drug. As polymer coating in the presence of unentrapped drug has been shown to increase the entrapment efficiency, this approach was applied for our formulations (Karn et al., 2011). Unfortunately, coating with chitosan failed to improve the entrapment efficiency of ACV and resulted in approximately 3% entrapment after liposome sonication. An attempt was therefore made to increase the chitosan concentration from 0.1% to 0.6% (w/v) to improve the entrapment. However, this effort also failed to enhance the entrapment efficiency. Although the mechanism behind this observation is unknown, one can hypothesize that this unexpected low entrapment efficiency occurred due to an interaction between the negatively charged lipid and the positively charged chitosan, forcing ACV out of the liposome bilayer. However, further investigation is necessary before any conclusions can be drawn. Furthermore, as Carbopol coating resulted in enhanced entrapment efficiency (Table 4.1) in the presence of unentrapped ACV, this polymer was used in all further studies.

Regarding the ACV entrapment efficiency, the negatively charged E-PC/E-PG liposomes appeared to encapsulate more of the drug than the neutral E-PC liposomes (Table 4.1). PG is known from the literature to act as a stabilizing lipid in the bilayer and thus exhibits a positive effect on the entrapment of drugs within liposomal formulations (Pavelic et al., 2005). The increased entrapment within negatively charged liposomes could be attributable to an interaction between the drug and the negatively charged bilayer. Our findings were thus in accordance with several earlier studies (Fresta et al., 1999, Law and Hung, 1998, Pavelic et al., 2005). Furthermore, coating with Carbopol exhibited enhanced entrapment efficiency only in the case of neutral E-PC liposomes. Coating with the negatively charged E-PC/E-PG liposomes did not improve ACV entrapment efficiency. This observation may be attributable to the negatively charged lipid bilayer interacting differently in the presence of the negatively charged coating material, thus influencing the entrapment efficiency of the drug. However, further investigation is necessary to elucidate the mechanisms behind this observation.

Table 4.1: Entrapment efficiency and zeta potential of ACV in the different liposome formulations. Values are given as mean ± S.D.(n=3).

Type of Formulation	Entrapment Efficiency ± S.D. (%)	Zeta Potential ± S.D. (mV)
PC-non-coated	15.24 ± 1.85	-6.05 ± 0.74
PC-coated	20.34 ± 5.43	-10.34 ± 1.65
PC-non-coated-sonicated	7.30 ± 1.63	-1.71 ± 0.27
PC-coated-sonicated	9.63 ± 6.39	-7.65 ± 1.63
PC/PG-non-coated	22.12 ± 4.98	-30.00 ± 2.81
PC/PG-coated	17.92 ± 1.58	-35.80 ± 2.68
PC/PG-non-coated-sonicated	11.48 ± 7.41	-23.20 ± 1.64
PC/PG-coated-sonicated	5.33 ± 0.57	-31.00 ± 1.48

4.1.2. Surface characteristics influencing *in vitro* mucin-binding

In order to confirm the inclusion of the negatively charged lipid (E-PG) and the formation of the anionic polymer (Carbopol) on the surface of liposomes, the zeta potentials of the different formulations were measured. When the surface characteristics of the E-PC and E-PC/E-PG liposomes were compared, a clear trend towards higher negative zeta potentials was observed for E-PC/E-PG liposomes (Table 4.1). These results thus confirmed the successful introduction of the E-PG lipid into the liposome bilayer. Furthermore, coating with Carbopol exhibited an increasingly negative zeta potential as expected and thus supported the application of a coating layer of Carbopol on the surface of the liposomes.

As the surface characterization confirmed the formation of a coating layer, the next approach was to assess the mucoadhesive properties of the different formulations in the presence of pig mucin. This method has earlier been utilized to determine the mucoadhesive properties of chitosan containing nanoparticles (Pawar et al., 2012). The results (Figure 4.1) clearly indicated that the coated E-PC formulations demonstrated superior interaction with mucin compared to the non-

coated E-PC liposomes, as expected. Jøraholmen et al., observed the same findings for liposomes coated with 0.1% chitosan (Jøraholmen et al., 2014). Furthermore, the small sonicated liposomes also exhibited superior mucoadhesiveness compared to the non-sonicated multilamellar liposomes. It is known that smaller particles can be expected to provide a larger surface area and thus provide enhanced contact surfaces for possible mucoadhesion. Furthermore, in the case of E-PC/E-PG liposomes, the trend in mucin binding was not as clear, as the non-coated formulations exhibited quite high interactions with mucin. A possible explanation for this observation could be electrostatic interactions occurring between the E-PC/E-PG formulations and mucin. However this should be investigated in further studies.

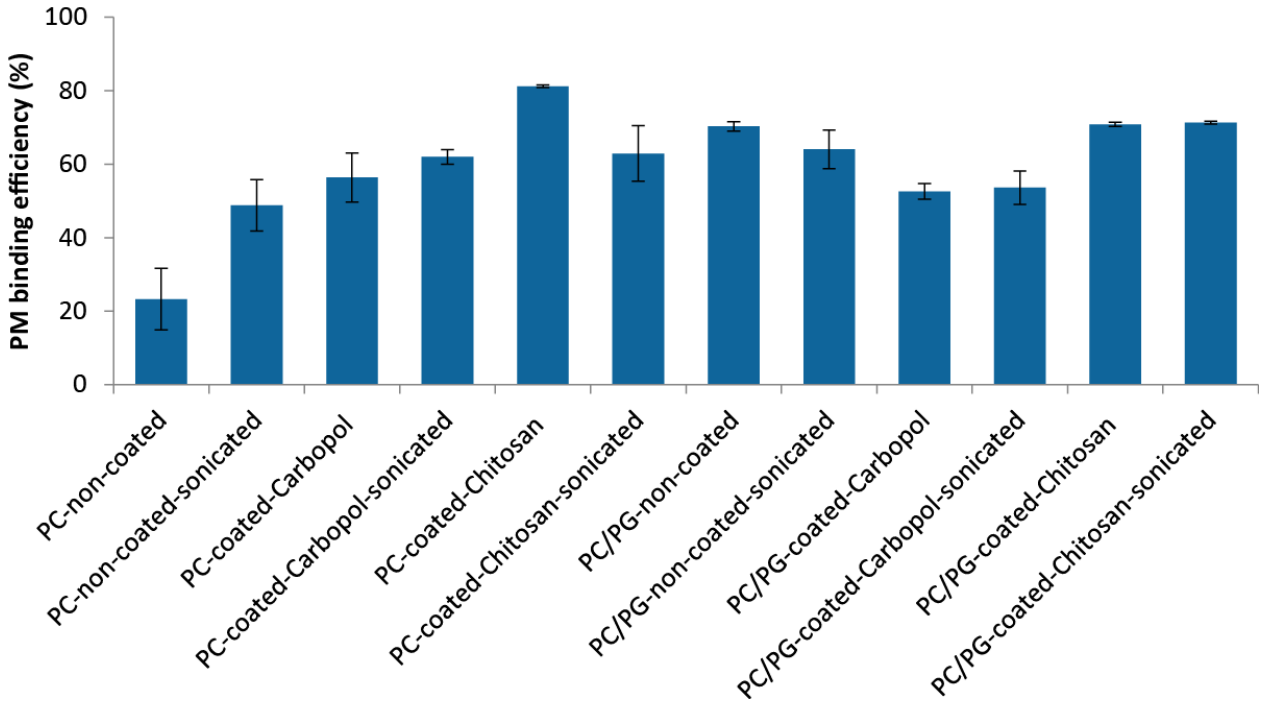

Figure 4.1: Pig mucin-binding efficiency of different formulation. Values are given as mean ± S.D.(n=3).

4.1.3. *In vitro* permeability studies using $PVPA_o$

To optimize the composition of drug carriers and their characteristics, models used for permeability screening might help to identify the best candidates for further development. More complex *in vitro* models such as the Caco-2 model have been used to assess the drug permeability from liposomes and mucoadhesive formulations (Vanic et al., 2013, Kudsiova and Lawrence, 2008). However, as our study involved the early development of ACV-containing drug carriers intended for mucosal targeting at both vaginal and intestinal sites, our aim was to use a more cost-effective and simpler permeability screening model. The novel screening model, $PVPA_o$ has been previously used to assess the permeability of drugs in formulations (i.e., micelles and solid dispersion) and has proven to be a suitable screening model for passive drug permeability (Fischer et al., 2011, Kanzer et al., 2010). Thus, this model was used to evaluate the permeability of AC in different formulations.

ACV was tested both in solution and in eight liposome formulations, and their P_{app} and ER were measured in these experiments. Figure 4.2 clearly shows that the permeability of ACV significantly increased for all the liposomal formulations except for the PC/PG-non-coated-sonicated liposomes, where the P_{app} values did not show any significant difference compared to ACV in solution. When comparing the neutral E-PC liposomes with the negatively charged E-PC/E-PG liposomes, the neutral liposomal formulations exhibited higher ACV permeability. The lower ACV permeability from the negatively charged liposomes could be explained by the fact that a change in the partition coefficient between the liposome formulation and the $PVPA_o$ barrier might have occurred, favoring the formulation/donor compartment. With respect to the coating of the liposomes, it appeared that both the E-PC/E-PG liposomes and sonicated E-PC liposome formulations exhibited enhanced ACV permeability. The enhanced permeability of the sonicated E-PC liposomes upon coating were thus in accordance with the increased mucoadhesion. This trend in increased permeability was observed for the non-sonicated E-PC liposomes, which can be explained by the fact that these liposomes are larger in size (above 1 μm) than the sonicated formulations and thus resulted in smaller contact surface between the barrier and the formulation. As the coating decreased the negative surface charge of the liposomes, we assumed that this allowed for closer contact between the liposome formulation and the $PVPA_o$ barrier and thus enhanced the permeability. The highest permeability of all the tested formulations was seen for

the E-PC-coated-sonicated liposomes, which also showed the highest mucin binding of all the formulations tested for permeability.

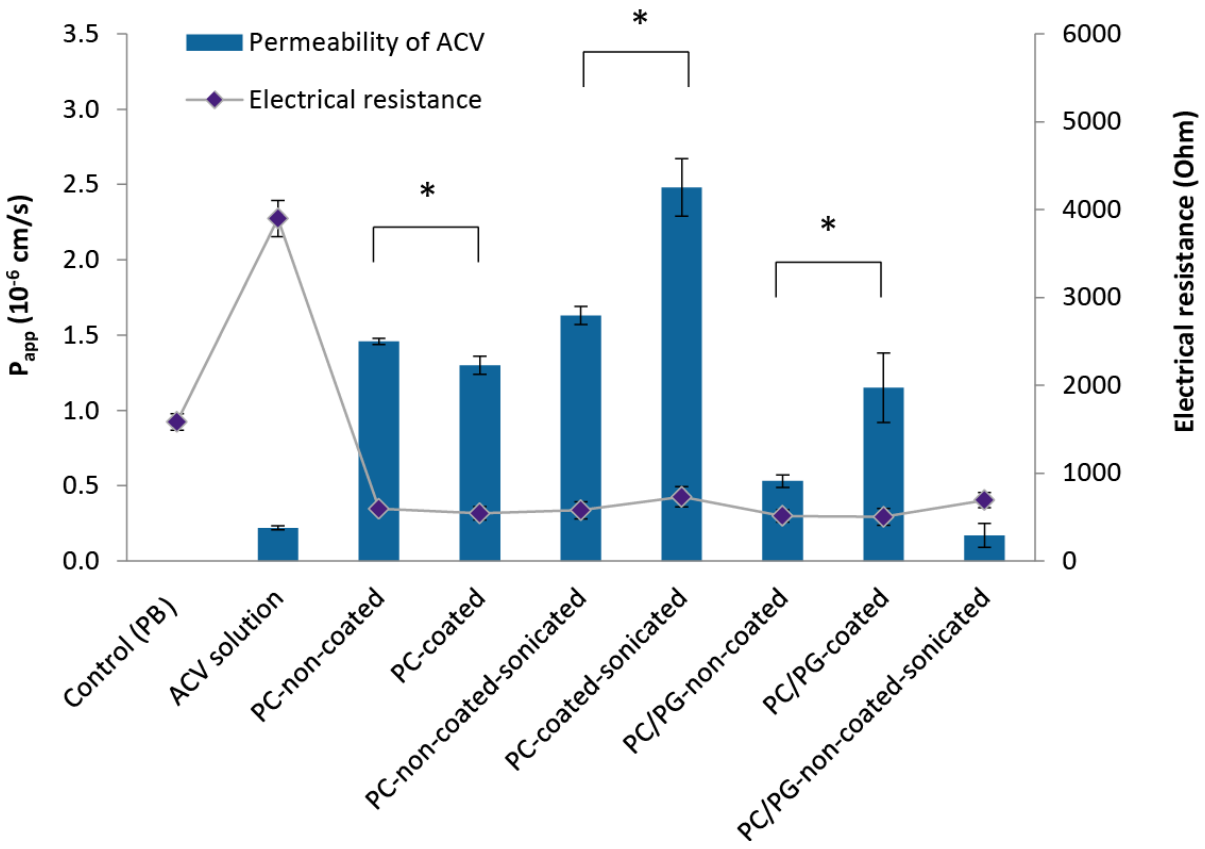

*Figure 4.2: P_{app} of ACV from the different liposome formulations together with ER across the permeation barrier. Values are given as mean± S.D.(n=3). *p<0.05.*

It has previously been reported that some drugs appear to influence the ER across the barrier during permeability experiments (Flaten et al., 2007). Thus, following each of the permeability experiments, the ER across the barrier was measured to confirm the barrier integrity. The measured ER values were off-scale in all of the liposomal formulations tested. An attempt was therefore made to remove the liposomal formulations from the donor compartment at the end of the permeability experiments, replace them with buffer, and re-measure the ER. This resulted in lower ER values compared with the control containing buffer. The decreased ER values could have been attributed to the turbulence in the donor compartment that occurred when the liposome formulations were replaced with buffer. The ER of the ACV in solution also resulted in increased

values relative to the control. High ER values have been reported to be associated with interactions between components in the formulation or the buffer present in the acceptor compartment, whereas low ER values are usually attributable to disruptions in the packing of the barrier. However, as both ACV in solution and in formulations initially resulted in high or off-scale values, it can be speculated that both the drug and the viscosity of the formulations interacted with the barrier and resulted in these observations (Fischer et al., 2011). We have also previously reported the same observation of increased ER values for other drugs (Flaten et al., 2007). Overall, the $PVPA_o$ appeared to distinguish between the permeability of ACV in solution and different mucoadhesive liposome formulations, and $PVPA_o$ was found to be a suitable *in vitro* model for the permeability screening of drugs in formulations.

4.2. Development of a biomimetic PVPA to estimate intestinal drug permeability (Paper II)

The goal of an *in vitro* permeability model is the ability to predict permeability values that correlate well with *in vivo* permeability data. To accomplish this, it is important that the model mimics the *in vivo* situation as closely as possible. Factors that have received much attention in this regard include the pH profile of the GI tract and the lipid composition of the artificial barriers.

4.2.1. Development of the preparation procedure for the biomimetic barrier

To develop a model that mimicked the intestinal lipid composition *in vivo*, the $PVPA_o$ barriers were altered using liposomes containing the following lipids: 26.5% PC, 26.5% PE, 7% PS, 7% PI, and 33% Chol. It has been shown that membranes consisting of only PC underestimate the permeability of basic and acidic drugs and that a better *in vivo-in vitro* correlation is obtained by using a lipid composition similar to the intestinal brush border membrane (Sugano et al., 2001a, Sugano et al., 2001b). Utilization of a biomimetic lipid composition in the PVPA barrier would provide us valuable information whether this new composition could improve the predictability of oral absorption.

The more complex lipid composition required additional changes in the original preparation process. In order to obtain liposomes of two different sizes the liposomal dispersion was extruded by hand using syringe holders as applied in the original extrusion procedure (Flaten et al.,

2006b). Unfortunately, this extrusion method was not suitable as the liposomes were not able to cross the filter. We assumed that inclusion of Chol in liposomes influenced the rigidity of the vesicles, thereby causing difficulties when extruding with syringe holders. The next attempt was therefore to employ a nitrogen driven extruder (Lauda DR. R. Wobster GmbH, Königshofen, Germany). This method resulted in great loss of the liposomes and was not found to be a successful method for extrusion neither. Lipofast (Avestin Europe GmbH, Mannheim, Germany) was further used to prevent less loss of liposomes during extrusion. Liposomes that were extruded through filters with pore sizes of 600 nm and 1200 nm resulted in vesicle size of 367 nm and 901 nm, respectively, thus resulting in a satisfactory size distribution.

Furthermore, use of the original preparation procedure to deposit the liposomes onto the filter support resulted in highly leaky barriers. This was monitored by means of both the ER across the barrier and the permeability. A key observation during the preparation performed with the original procedure was that following centrifugation, the small liposomes were not able to enter the filter pores as easily as the E-80 liposomes employed in $PVPA_o$. The centrifugation time and speed were therefore the first parameters investigated to obtain a tighter barrier. However, increasing the centrifugation speed from 2000 rpm (610*g*) to 2500 rpm (950*g)* for the small liposomes (6% w/v) still resulted in some liposomes laying on top of the filter support (650 nm pore size) and not entering the filter pores. An attempt to increase the centrifugation time was also made without any improvement (data not shown).

The next attempt was to dilute the liposome dispersion from 6% (w/v) to 3% (w/v) to determine whether this approach might fill the filter pores. Increasing the centrifugation speed from 2000 rpm to 2500 rpm for the small liposomes and using 3% (w/v) liposome dispersion, a barrier with a mean calcein permeability of 21.38×10^{-6} cm/s was obtained (procedure 1, Figure 4.3). In comparison the $PVPA_o$ barrier with E-80 had a permeability value for calcein of 0.08×10^{-6} cm/s. The preparation procedure 1 was therefore not satisfactory. Furthermore, a second centrifugation step involving the addition of large liposomes (3%, w/v) was performed twice, with the same total amount of lipids as in the original preparation procedure. Although this approach resulted in decreased calcein permeability, (4.35×10^{-6} cm/s, procedure 2, Figure 4.3) the result was still found to be unsatisfactory. The centrifugation speed was further increased from 2000 (610*g*) to 2500 rpm (950*g*) to settle the large liposomes on top of the filter (procedure 3, Figure 4.3). Not

even this approach led to any major changes in the calcein permeability. Further increases in the centrifugation speed resulted in the destruction of filters, and due to this the approach was not found to be successful either. As freeze-thaw (ft.) techniques and a number of freezing cycles have been reported to promote the fusion of liposomes (Flaten et al., 2006b), different thawing temperatures and heating durations during freeze-thaw cycles were investigated with the intention of obtaining a tighter barrier (procedures 3-9, Figure 4.3). Neither of these strategies resulted in any significant effects. Increasing the number of ft. cycles from one to two resulted in no significant changes in barrier tightness (procedure 5 vs. 6 and procedure 8 vs. 9, Figure 4.3). These results clearly show that further adjustment to the preparation procedure was necessary to meet the requirements for a tight barrier.

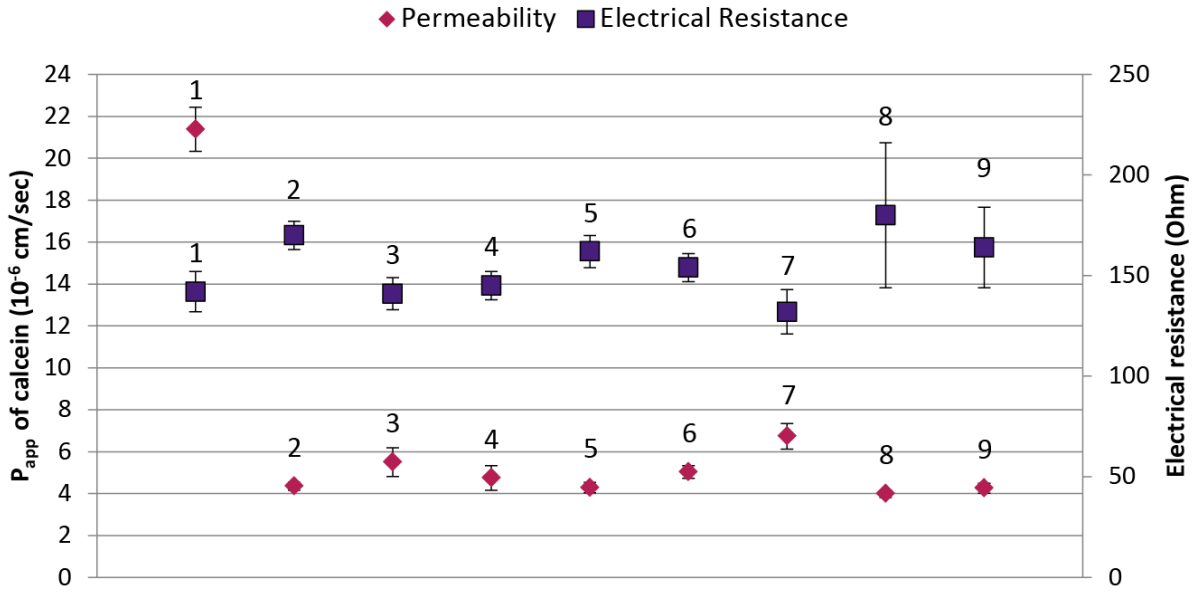

Figure 4.3: P_{app} of calcein and ER across barriers prepared with different parameters. Values are provided as mean ± S.D. (n=3).The different procedures represent changes in the following parameters:1) 2000 rpm with large liposomes, thawing at 65°C for 60 min, 2) 2000 rpm with large liposomes x 2, thawing at 65°C for 60 min, 3) 2500 rpm with large liposomes x 2, thawing at 65°C for 90 min, 4) 2500 rpm with large liposomes x 2, thawing at 65°C for 30 min, 5) 2500 rpm with large liposomes x 2, thawing at 50°C for 105 min, 6) 2500 rpm with large liposomes x 2, thawing at 50°C for 105 min, 2 ft. cycles, 7) 2500 rpm with large liposomes x 2, thawing at 40°C for 120 min, 8) 2500 rpm with large liposomes x 2, thawing at 30°C for 270 min, 9) 2500 rpm with large liposomes x 2, thawing at 30°C for 270 min, 2 ft. cycles.

We assumed that less efficient packing of liposomes both into the pores and on top of the barrier could result in a leaky barrier. Therefore, the next attempt was to use the original method with small E-80 to fill the pores. This was followed by the addition of larger liposomes consisting of the biomimetic lipids. The centrifugation speed was set to 2500 rpm for 30 minutes to settle the larger liposomes on top of the filter. Upon centrifugation, the excess buffer was removed by inverting the inserts before the ft. cycle was performed. The ft. conditions were performed according to the original preparation procedure (Flaten et al., 2006b). The resulting barrier exhibited a mean calcein permeability of 2.31 x 10^{-6} cm/s (procedure 1, Figure 4.4), which was an improvement; but still lacked satisfactory ER values within the limit of 1000 ohm/inserts (Flaten et al., 2006b).

The preparation of a new, skin-mimicking PVPA model composed of a lipid composition matching that of the skin barrier, has previously been successfully altered from the original procedure by omitting the last centrifugation step and instead removing the buffer by evaporation. The next approach was therefore to omit the inverted centrifugation step before the ft. cycle and remove the buffer by evaporation at 50°C for 60 minutes. This approach resulted in significantly decreased calcein permeability, with a mean of 0.063 x 10^{-6} cm/s and an ER value within the limits discussed above (procedure 2, Figure 4.4).

Although the last step met the requirements for a tight barrier, a further attempt was made to simplify the procedure. The last centrifugation step prior the evaporation step, which is intended to settle the big liposomes on top of the filter was therefore omitted to investigate whether this approach could influence barrier tightness. This approach resulted in a mean calcein permeability of 0.073 x 10^{-6} cm/s (procedure 3, Figure 4.4), and was not significantly different in terms of permeability compared with procedure 2. The last centrifugation step was therefore skipped to simplify the procedure and reduce the preparation time.

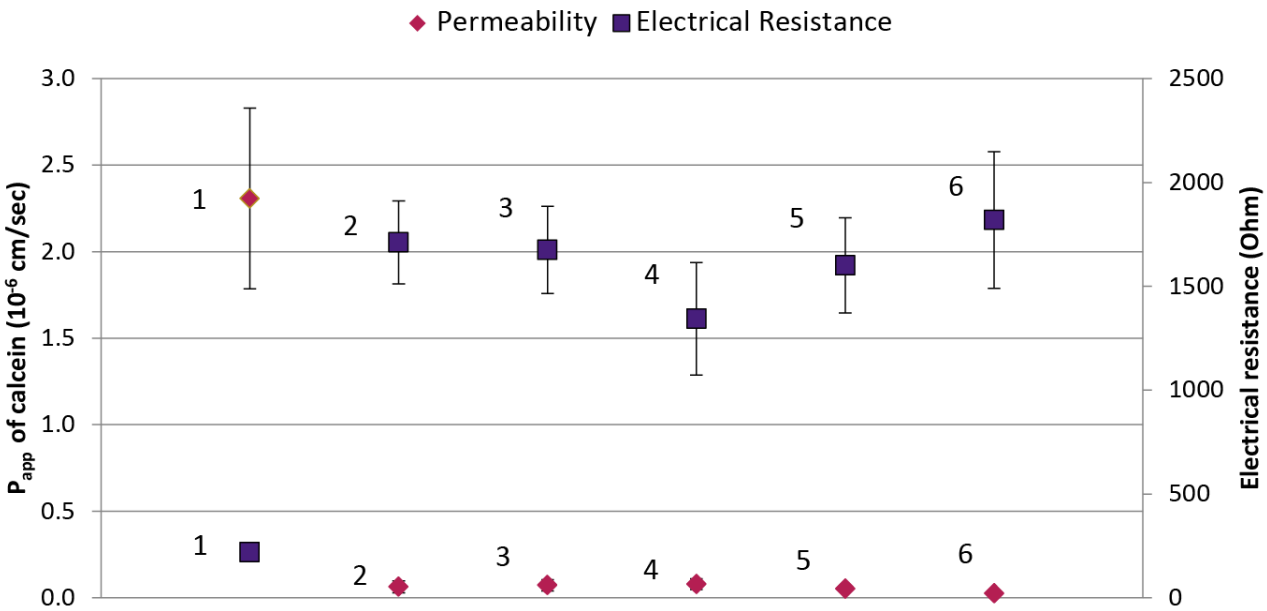

Figure 4.4: P_{app} of calcein and ER across barriers prepared with different parameters. Values are provided as mean ± S.D. (n=3).The different procedures represent changes in the following parameters:1)2500 rpm with large liposomes (3%) x2, invert centrifugation, thawing at 65 °C for 30 min; 2) 2500 rpm with large liposomes (3%) x2, heating at 50°C for 60 min, thawing at 60°C for 60 min; 3) addition of large liposomes (3%), heating at 50°C for 30 min x2, thawing at 60°C for 60 min; 4) addition of large liposomes (6%), heating at 50°C for 30 min x2, thawing at 60°C for 60 min; 5) addition of large liposomes (3%), heating at 50°C for 30 min x2, thawing at 50°C for 105 min; 6) addition of large liposomes (6%), heating at 50°C for 30 min x2, thawing at 30°C for 270 min.

Another approach was to investigate the influence of the liposomal concentration on the tightness of the barrier. The influence of 6% liposome dispersion, as used in the original preparation procedure, and the 3% liposome dispersion were evaluated for their effects on tightness to avoid extensive use of lipids and to reduce costs. The resulting barriers (procedure 3 vs. 4, Figure 4.4) exhibited no significant differences in calcein permeation and ER across the barrier. The 3% liposome concentration was therefore retained in the final preparation procedure.

Finally, the use of lower thawing temperatures over a prolonged period of time was investigated to elucidate their impact on the barrier tightness (procedures 5 and 6, Figure 4.4). Procedure 5 resulted in a calcein permeability of 0.053 x 10^{-6} cm/s, while procedure 6 resulted in slightly tighter barriers with a mean calcein permeability of 0.026 x 10^{-6} cm/s. Although procedures 5 and

6 both exhibited high barrier reproducibility, the simpler and faster preparation procedure using of 50°C for 105 min (procedure 5) was chosen for the final preparation process.

4.2.2. The final preparation procedure for the $PVPA_{biomimetic}$ barrier

The final preparation procedure for the $PVPA_{biomimetic}$ barrier consists of the following steps:

- Preparation of two liposome dispersions:
  - liposome dispersion (1) consisting of the lipid E-80 (6%, w/v)
  - liposome dispersion (2) consisting of an intestinal mimicking lipid composition (biomimetic liposomes) PC/PE/PS/PI/Chol (3%, w/v).
- Extrusion of E-80 liposomes through a 400 nm pore filter.
- Extrusion of biomimetic liposomes through a 1200 nm pore filter.
- Deposition of small E-80 liposomes into the pores of a mixed cellulose ester filter (650 nm pore size) by centrifugation at 610*g*. Procedure performed twice with centrifugation for 5 and 10 min, respectively.
- Deposition of large liposomes on top of the mixed cellulose ester filter by evaporation.
- Freeze-thawing cycle to promote liposome fusion to obtain tight a barrier.

The mixed cellulose ester filter inserts that were used as supports for the barriers are not commercially available. These were prepared in-house with an IBR Heat-Press HP80-3500 sealing machine (Figure 4.5) designed to fuse the filters onto 24 inserts simultaneously.

In detail, the barrier for permeation experiments was produced by adding an aliquot of small E-80 liposomes (100 µl) to each insert followed by centrifugation at 610*g* for 5 min. The procedure was repeated with the addition of small liposomes (100 µl), and the centrifugation was extended to 10 min. The inserts were transferred to dry well plates and incubated at 50°C for 45 min. This step was performed to allow evaporation of the ethanol-buffer mixture and to promote the fusion of liposomes. Following heating, large liposomes consisting of PC/PE/PS/PI/Chol (50 µl) were added to settle on top of the filter support. The inserts were then incubated at 50°C for 30 min to allow the solvent to evaporate. This procedure was performed twice. Subsequently, the inserts were frozen at -70°C for a minimum of 60 min, followed by thawing at 50°C for 105 min prior to permeation experiments.

Figure 4.5: Pictures of the sealing machine IBR Heat-Press HP80-3500. A and B gives a closer look at the sealing part of the instrument.

4.3. Permeability screening performance of the PVPA (PAPER II)

4.3.1. Comparison of the permeation experiments with an *in silico* model

The permeability of a selection of 25 marketed drugs were assessed using the original $PVPA_o$ model and further compared with an *in silico* model to evaluate the extent to which $PVPA_o$ could distinguish and rank the selected drugs as expected from our previously introduced *in vivo* classification system (Flaten et al., 2006b). The classification system divides the compounds into poor, moderate and excellent absorption categories according to their permeability values obtained from the PVPA experiments. Compounds with P_{app} values $<0.1 \times 10^{-6}$ cm/s are poorly absorbed (<30% fraction absorbed *in vivo*), compounds with P_{app} values between 0.1 and 0.9×10^{-6} cm/s are moderately absorbed (30-70% fraction absorbed *in vivo*), and compounds with P_{app} values of $>0.9 \times 10^{-6}$ have excellent absorption (> 70% fraction absorbed *in vivo*). Based on this classification system, 22 out of 25 drugs (approximately 90%) were correctly classified using the $PVPA_o$ model (Table 4.2). The three drugs for which the P_{app} values did not lead to correct classification were salicylic acid, sulphasalazine and tranexamic acid. Salicylic acid was classified as a moderately permeable drug when using $PVPA_o$ while according to the *in vivo* data it should be classified as excellently absorbed (see Table 4.2). Sulphasalazine was also classified

as a moderately permeable drug using our model, however, according to the *in vivo* data it should be classified as poorly absorbed (see Table 4.2). Tranexamic acid was classified as an excellent permeable drug in the $PVPA_o$ while it is reported to be moderately absorbed according to the *in vivo* data (see Table 4.2). Tranexamic acid has earlier shown to differ in permeability properties compared to other drugs with the same fraction absorbed *in vivo* (Sugano et al., 2002).

However, when $PVPA_o$ was compared with the *in silico* model, only 19 of the 25 drugs were correctly classified based on the QikProp calculated absorbed fractions. When comparing the calculated absorbed fractions with the absorbed fractions *in vivo*, it appeared that the calculated data were elevated relative to the *in vivo* data for poorly absorbed drugs, as shown in Table 4.2. The opposite was observed for drugs with high absorption *in vivo*. In summary, the $PVPA_o$ appeared to be able to distinguish and predict the intestinal absorption with a higher degree of accuracy than the *in silico* model. The next step was thus to investigate whether the use of more biologically relevant pH conditions and a biomimetic lipid barrier compositions could further improve the model.

Table 4.2: Experimental P_{app}-values from the $PVPA_o$ at pH 6.2 and 7.4, molecular properties and calculated and experimental literature values on percent absorbed in humans (F%)

Compound	$PVPA_o$		$F\%_{calc}$	pKa[b]	F%[c]
	$P_{app} \pm SD\ 10^{-6}$ cm/s				
	pH 6.2	pH 7.4[a]			
Acetylsalicylic acid	1.95 ± 0.65	1.49 ± 0.23	72	3.5	100
Alprenolol	1.57 ± 0.09	4.92 ± 0.34	97	9.2	93
Atenolol	0.18 ± 0.01	0.22 ± 0.05	55	9.5	50
Caffeine	6.24 ± 0.17	6.04 ± 0.57	81	-	100
Chlorothiazide	0.04 ± 0.01	0.03 ± 0.003	52	6.7	13
Chloramphenicol	1.57 ± 0.25	1.81 ± 0.07	64	5.5	90
Cimetidine	0.40 ± 0.05	0.89 ± 0.05	72	6.9	64
Diclofenac	3.05 ± 0.14	2.71 ± 0.16[d]	100	4.0	100
Enalapril	0.35 ± 0.03	0.14 ± 0.01	52	2.9/5.2	65
Flurbiprofen	8.72 ± 0.34	6.24 ± 0.30[d]	96	4.03	95
Flufenamic acid	1.61 ± 0.14	1.68 ± 0.59	89	4.09	NA[e]
Hydrochlorothiazide	0.44 ± 0.06	0.51 ± 0.06	53	8.9/10.3	67
Ibuprofen	13.34 ± 0.97	8.42 ± 0.86	92	4.45	95
Metoprolol	1.26 ± 0.33	3.23 ± 0.78	89	9.6	95
Metronidazol	4.34 ± 0.12	3.92 ± 0.25[d]	92	-	100
Nadolol	0.54 ± 0.10	0.70 ± 0.11	67	9.7	35
Naproxen	10.17 ± 0.40	8.73 ± 0.61	90	4.15	99
Propranolol	1.18 ± 0.17	3.41 ± 0.41[d]	97	9.52	90
Ranitidine	0.11 ± 0.02	0.24 ± 0.01	70	8.3/2.1	50
Salicylic acid	0.45 ± 0.04	0.59 ± 0.03	76	3.0	100
Sulphasalazine	0.48 ± 0.16	0.60 ± 0.18	50	2.8/8.3/ 11.0	13
Sulpiride	0.35 ± 0.07	0.22 ± 0.01	61	9.0	35
Terbutaline	0.80 ± 0.09	0.40 ± 0.05	62	8.7/10.0/ 11.0	62
Timolol	1.65 ± 0.08	1.70 ± 0.12	84	9.5	90
Tranexamic acid	5.34 ± 0.46	12.36 ± 3.64	43	4.5/10.7	55
Calcein	-	0.061 ± 0.005	-	1.8/9.2	-

[a] The P_{app} values are from and Flaten et al. (2006b), Flaten et al. (2008) and Engesland et al. (2013) unless stated otherwise.
[b] The pKa values are from Flaten et al. (2006b), Avdeef (2003), Sugano et al. (2002), Li and Cooper (2012).
[c] Fa values are from Kansy et al. (1998), Yazdanian et al. (1998), Sugano et al. (2001a), Sugano et al. (2002), Österberg et al. (2001).
[d] Measured for this study.
[e] Not available.

4.3.2. Influence of a more biorelevant pH condition on drug permeability

The pH of the GIT increases from the acidic environment of the stomach to the more basic environment of the large intestine. There are in addition pH differences under fasted and fed conditions. This provides a challenge when considering an estimation of permeability. To achieve a better understanding of drug permeability *in vivo*, the models must maintain their integrity across the pH range normally found in the intestine. Thus, there is great interest in investigating how changes in pH influence the drug permeability.

The $PVPA_o$ barrier has previously shown to be stable at a pH range from 2.0 to 8.0 according to the ER and permeability values of the hydrophilic marker fluorescein (Flaten et al., 2006a). As the $PVPA_o$ was found to be a suitable model for obtaining information regarding segmental absorption in the GIT, the permeability of the 25 selected drugs were evaluated at the biologically relevant pH levels of 6.2 and 7.4 to determine the influence of pH on drug permeability.

When comparing the permeability values obtained at the two pH conditions, the degree of correct classification according to the *in vivo* absorption were not different (Table 4.2). Some drugs showed the same permeation regardless of pH, whereas most of the basic and acidic drugs showed P_{app} values consistent with the pH partition hypothesis; at pH 6.2 acidic drugs showed increased permeability while basic drugs showed decreased permeability relative to their performance at pH 7.4. However, because the classification system was set based on data obtained at pH 7.4, one could suggest narrowing the range of the moderate absorption category to P_{app} values between 0.1 and 0.7 x 10^{-6} cm/s. However, this would thus lead to the incorrect classification of cimetidine at pH 7.4 into the class of excellently absorbed drugs.

4.3.3. Evaluation of the $PVPA_{biomimetic}$ model

As the majority of membrane lipids are comprised of a head group region with one or more charged units (commonly zwitterionic and anionic lipids), it is expected that basic drugs will have a higher affinity for a negatively charged barrier (Ingebrigtsen and Brandl, 2002, Sugano et al., 2001a, Sugano et al., 2001b). The charged head groups at the membrane surface have been reported to attract counterions from the buffer solution to the interface, creating a so-called electrical double layer and further establishing an electrical potential profile at the barrier surface. The surface potential appears to affect the partition of positively charged compounds, thus

increasing the surface concentration and leading to a higher probability of permeation. The opposite has been reported for negatively charged compounds, for which the permeation is considered to decrease. The permeation of neutral compounds, however, is less likely to be affected by the surface potential (Malkia et al., 2004).

As an attempt to alter the membrane lipids in the $PVPA_{biomimetic}$ resulted in a negative net charge and a more biomimetic barrier, the next approach was to evaluate the permeability performance of this barrier. Consequently, a selection of 19 drugs covering a broad range of fractions absorbed in humans (Fa 13-100%) was chosen. The permeability experiments with the selected acidic, basic and neutral drugs were carried out at both pH 6.2 and 7.4 to facilitate a comparison with the $PVPA_o$. Salicylic acid and sulphasalazine, which were incorrectly classified in the $PVPA_o$ were also included in this selection to challenge the newly developed PVPA barrier. However, before the permeability assessment at pH 6.2 could be performed, the stability of the $PVPA_{biomimetic}$ barrier was studied to reveal the robustness of this model at the desired pH. Based on the permeability of calcein and ER values, the $PVPA_{biomimetic}$ model proved to be stable at pH 6.2 (Table 4.3). Thus, further permeability experiments could be performed.

Permeability values through the $PVPA_{biomimetic}$ model and the fractions absorbed in humans are shown in Table 4.3. At pH 7.4, positively charged basic drugs showed increased permeability through the biomimetic barrier compared to the $PVPA_o$ barrier, which primarily consisted of PC. These results were thus expected, as discussed above. Conversely, the negatively charged acidic drugs exhibited either increased or decreased permeability, thus providing no clear trend relative to the $PVPA_o$. However, Sugano et al., reported a similar trend for the permeability of a selection of acidic drugs in a PAMPA consisting of a biorelevant lipid composition (Sugano et al., 2001a). When the permeability values of the neutral compounds were evaluated, a trend towards decreased permeability was observed through the $PVPA_{biomimetic}$ barrier compared to the $PVPA_o$ barrier. However, due to high P_{app} values observed in both models, these results did not affect the drug classification.

Regarding drug classification, salicylic acid and sulphasalazine remained incorrectly classified in the $PVPA_{biomimetic}$ model relative to *in vivo* classification. Additionally, the basic drug, nadolol exhibited increased permeability through the negatively charged barrier and did not correspond to the correct *in vivo* classification. Nadolol exhibited excellent absorption, although its correct

classification is moderate absorption. The increased permeability was thus expected as other selected basic drugs showed increased permeability. The acidic drug sulphasalazine has previously been shown to interfere with the barrier during permeation experiments, resulting in a barrier that is less tight based on ER values (Flaten et al., 2007). This might explain the increased permeability observed in both models compared to what was expected from the *in vivo* data. Furthermore, salicylic acid demonstrated a P_{app} value that indicated moderate absorption *in vivo*, while the correct class should be excellent absorption. This underestimation is primarily attributable to the route of transport for salicylic acid, as it is known to be actively transported in humans. A similar underestimation of permeability has also been reported for salicylic acid using the PAMPA model compared to Caco-2 model supporting the theory of active transport of salicylic acid (Koljonen et al., 2008).

When the permeation of the drugs under different pH conditions was compared, the acidic drugs showed a trend towards increased permeability and the basic drugs showed a trend towards decreased permeability at pH 6.2 compared with the permeability values obtained at pH 7.4. Several other studies using the Caco-2 cell model have also reported that the permeation of basic drugs increases when the pH of the apical side is increased from 5.0 to 8.0. These previous results are in agreement with the results obtained in our model (Neuhoff et al., 2003, Palm et al., 1998). Thus, our results are in accordance with the pH partition hypothesis, which states that the partitioning of the charged state of the drug into phospholipid bilayers is largely restricted when compared to the partitioning of the uncharged form. However, as also discussed above, several studies have shown that the ionized form of a molecule is able to partition into phospholipid bilayers and, to a certain degree, and permeate across cell monolayers (Avdeef et al., 1998, Palm et al., 1998, Thomae et al., 2005). Furthermore, both pH conditions (pH 6.2 and pH 7.4) contributed the same degree of correct classification of drugs with the exception of timolol into the *in vivo* absorption categories. Timolol exhibited a decreased P_{app} value at pH 6.2 compared to pH 7.4. The P_{app} value of 0.8×10^{-6} cm/s at pH 6.2, classified timolol as a moderately absorbed drug, although the correct class should be excellent absorption. Timolol thus fell below the limit of 0.9×10^{-6} cm/s to be classified correctly.

Table 4.3: Experimental P_{app} values from the $PVPA_{biomimetic}$ at pH 6.2 and 7.4, molecular properties and fraction absorbed (Fa%) in humans.

Compound	$PVPA_{biomimetic}$		Charge at pH 6.2	Charge at pH 7.4	F%[a]
	$P_{app} \pm SD\ 10^{-6}$ cm/s				
	pH 6.2	pH 7.4			
Alprenolol	1.07±0.04	1.59±0.07	+	+	93
Atenolol	0.49±0.06	0.76±0.11	+	+	50
Caffeine	4.04±0.08	4.32±0.28	0	0	100
Chlorothiazide	0.07±0.01	0.05±0.01	0	-	13
Diclofenac	4.25±1.00	2.23±0.39	-	-	100
Enalapril	0.42±0.04	0.43±0.05	-	-	65
Flurbiprofen	5.77±0.57	3.26±0.57	-	-	95
Ibuprofen	12.11±1.98	9.01±1.45	-	-	95
Metoprolol	1.23±0.18	1.61±0.45	+	+	95
Metronidazol	3.31±0.36	3.15±0.27	0	0	100
Nadolol	1.47±0.09	1.55±0.13	+	+	35
Naproxen	5.63±0.33	3.79±0.30	-	-	95
Propranolol	0.89±0.08	1.76±0.11	+	+	90
Ranitidine	0.25±0.02	0.40±0.07	+	+	50
Salicylic acid	0.69±0.02	0.54±0.01	-	-	100
Sulphasalazine	0.46±0.02	0.20±0.02	-	-	13
Sulpiride	0.37±0.03	0.89±0.08	+	+	35
Terbutaline	0.50±0.13	0.64±0.11	+	+	62
Timolol	0.79±0.03	1.57±0.02	+	+	90
Calcein	0.08±0.003	0.05±0.002	-	-	-

[a] See Table 4.2 for references.

4.4. Solubilizers and cosolvents (Paper II)

Insufficient solubility is one important factor associated with limited oral bioavailability. Due to the increasing number of new drug candidates with poor water solubility, different solubilizers are used in pharmaceutical drug formulations for oral delivery. As a consequence, the number of permeability screenings performed in the presence of solubility enhancing agents has also increased (Buckley et al., 2012, Ingels and Augustijns, 2003, Kanzer et al., 2010). Poorly water-soluble drugs may result in analytical difficulties during permeability screening due to low donor/acceptor concentrations and/or the possibility of drug precipitation. To overcome these difficulties, solubility enhancing agents can be utilized in the donor compartment to increase the drug concentration. Thus, it is also important that the screening model maintains its integrity in the presence of the employed surfactants during experiments.

4.4.1. The influence of various surfactants and cosolvents on barrier integrity

The stability of the $PVPA_{biomimetic}$ barriers in the presence of surfactants and cosolvents was studied to determine the feasibility of using the model to estimate the permeability of poorly soluble drugs of interest. To investigate barrier integrity, a double approach was chosen in which both the ER across the barriers and the calcein permeability were evaluated. The surfactants were all used at concentrations greater than their CMC. The nonionic surfactant, Triton X-100, which is known to interact with lipid bilayers and solubilize phospholipids, was included as a reference, and buffer was included as a control.

The integrity of the $PVPA_o$ model has previously shown to be affected by various surfactants and cosolvents at different concentrations (Fischer et al., 2011, Flaten et al., 2008). Consequently, one of the objectives in this thesis was to compare the barrier integrity of the $PVPA_{biomimetic}$ with that of the $PVPA_o$ in the presence of various surfactants and cosolvents. It is known from the literature that surfactants have the ability to interact with membrane lipids and, in particular, with the lipid lecithin (Schubert et al., 1986, Thoren et al., 2007). As the lipid bilayer was altered to consist of a more biomimetic lipid composition, we assumed that the new PVPA model would be a more robust model towards different surfactants as well as cosolvents.

The results for the calcein permeability and the ER, measured in presence of the different surfactants and cosolvents, are presented in Table 4.4. In the case of the cosolvents ethanol,

dimethyl sulfoxide (DMSO), and polyethylene glycol (PEG), no significant changes were observed in terms of the ER and calcein permeability when compared with the control (buffer). The barrier therefore appeared to be compatible with all tested concentrations of the cosolvents. Similar results were previously shown for the $PVPA_o$ (Flaten et al., 2008). Furthermore, the $PVPA_{biomimetic}$ model appeared to be significantly more robust towards the surfactants compared with the $PVPA_o$. Poloxamer 188 was found to be compatible with the barrier at a concentration of 60 mg/ml. This result was in accordance with earlier findings for the $PVPA_o$, indicating that these barriers are compatible with the presence of Poloxamer 188 up to a concentration of 50 mg/ml (Fischer et al., 2011). The presence of Span 20 did not result in significant changes at a concentration of 20 mg/ml. This was an improvement in barrier integrity compared with the $PVPA_o$ in which only concentrations of up to 5 mg/ml were found to maintain barrier integrity. The presence of Tween 80, Brij 35, and Cremophor EL resulted in slightly significant changes in the calcein permeability. However, in the presence of the surfactants, the increase in calcein permeability was tremendously higher in the $PVPA_o$ model than the $PVPA_{biomimetic}$ model. The increase in the calcein permeability of the $PVPA_{biomimetic}$ barrier was small, and the ER values remained within the limits (1000-3000 ohm/insert) for the barriers to be considered to have maintained their integrity. It has also been previously stated that the $PVPA_o$ barriers are considered to be stable up to a calcein permeability of 0.15×10^{-6} cm/s (Fischer et al., 2012). All the permeability data acquired in the presence of the cosolvents and surfactants were below this limit. Even Triton X-100 (reference) demonstrated a calcein permeability value below this limit. These results indicated a tremendous increase in the barrier integrity of the $PVPA_{biomimetic}$ model compared to the $PVPA_o$ model, where even the lowest concentrations of surfactants resulted in a 10-20-fold increase in calcein permeability and ER values below 500 ohm (Fischer et al., 2011, Flaten et al., 2008). The reason for this increased stability is most likely the presence of the lipids Chol, PS and PE, which have previously been reported to stabilize the membrane against aggressive surfactants (Schubert and Schmidt, 1988). The highly increased barrier stability in the presence of surfactants is, however, an important finding, that demonstrates the enhanced capability of the model to estimate the permeability of poorly soluble compounds of interest.

Table 4.4: P_{app} values for calcein ± S.D. together with the [ER across the PVPA barriers (ohm)] in the presence of the surfactants and cosolvents.

Additive in donor compartment	0 mg/ml	0.5 mg/ml	1 mg/ml	5 mg/ml	10 mg/ml	20 mg/ml	40 mg/ml	60 mg/ml
Control (buffer)	0.053 ±0.013 [1532]	-	-	-	-	-	-	-
Triton X 100	-	0.131* ±0.024 [1257]	-	-	-	-	-	-
Tween 80	-	0.102* ±0.021 [1377]	0.095* ±0.015 [1390]	-	-	-	-	-
Brij 35	-	0.109* ±0.026 [1429]	0.117* ±0.025 [1258]	-	-	-	-	-
Span 20	-	-	-	-	-	0.062 ±0.032 [1547]	-	-
Cremophor EL	-	-	-	0.106* ±0.026 [1323]	-	0.110* ±0.021 [1480]	0.119* ±0.028 [1322]	-
Poloxamer 188	-	-	-	-	-	0.062 ±0.012 [1509]	0.041 ±0.012 [1608]	0.051 ±0.019 [2033]
PEG 400	-	-	-	-	0.053 ±0.014 [1453]	-	0.062 ±0.023 [1328]	-
Ethanol	-	-	-	-	0.075 ±0.022 [1452]	-	0.054 ±0.012 [1705]	0.074 ±0.024 (1727]
DMSO	-	-	-	0.053 ±0.012 [1585]	0.094 ±0.022 [1448]	-	0.078 ±0.019 [1544]	-

* The permeability values that were significantly ($p \leq 0.05$) different from control.

4.5. The influence of FaSSIF and FeSSIF on drug permeation (Paper III)

Over the last decade, the employment of simulated intestinal fluids have gained increased attention for use in *in vitro* solubility and permeability studies to better mimic the physiological conditions in the test setup (Bergström et al., 2014). Furthermore, simulated intestinal fluids have been used successfully in different permeability studies such as the Caco-2 model (Fossati et al., 2008, Ingels et al., 2004, Markopoulos et al., 2014). FaSSIF has also previously been shown to be compatible with the original $PVPA_o$ barriers (Fischer et al., 2012). However, the more challenging FeSSIF has never been tested in any of the PVPA models.

To investigate the performance of a drug in presence of the biorelevant media FaSSIF and FeSSIF, the $PVPA_{biomimetic}$ was applied as a tool for screening the drug permeability profile in the presence of the biorelevant media. This was the first time that the PVPA models were used to assess permeability in the presence of FeSSIF.

4.5.1. Barrier integrity in the presence of FaSSIF and FeSSIF

To evaluate the compatibility of the $PVPA_{biomimetic}$ barrier in the presence of the biorelevant media (FaSSIF and FeSSIF), other integrity approaches in addition to calcein permeability and ER measurements were applied. The bile salt sodium taurocholate, which is present at different concentrations in both FaSSIF and FeSSIF, is known to interact with several membrane lipids at low concentrations (Schubert et al., 1986, Schubert and Schmidt, 1988). Therefore, the release of phospholipids from the barriers as well as the loss of sodium taurocholate from the donor compartment were determined to obtain valuable additional information regarding barrier integrity.

The permeability values of calcein in presence of PB were compared to those in the presence of FeSSIF (Figure 4.6), and no significant differences between these results were observed. In contrast, the permeability of calcein in the presence of FeB resulted in significantly decreased permeation ($p < 0.05$) compared to FeSSIF. We assumed that the high bile salt concentration in FeSSIF (10 mM) might have contributed to these changes by potentially inducing some minor disruption of the barrier. However, the increased calcein permeability was significantly lower in the presence of FeSSIF compared to Triton X-100 (reference), which was, as mentioned earlier, found to be compatible with the model (Paper II). Both FaB and FaSSIF exhibited significant

increases in calcein permeability compared to PB. However, no significant differences in calcein permeability were observed between FaB and FaSSIF. The compatibility of the $PVPA_o$ model with the presence of FaB and FaSSIF has previously been examined by Fischer and co-workers (Fischer et al., 2012). According to their observations, the ER and permeability values of calcein were slightly higher and lower, respectively, in the presence of FaSSIF than compared to FaB, but the difference was not significant. When the $PVPA_{biomimetic}$ model was compared with $PVPA_o$, the permeability results for calcein in FaB and FaSSIF appeared to be comparable. Furthermore, the ER values remained within the limits set for the barriers to indicate the maintenance of integrity (1000-3000 ohm/insert). Overall, the integrity of the $PVPA_{biomimetic}$ appeared to be maintained in the presence of FaSSIF and FeSSIF according to both the calcein permeability results and the ER across the barrier.

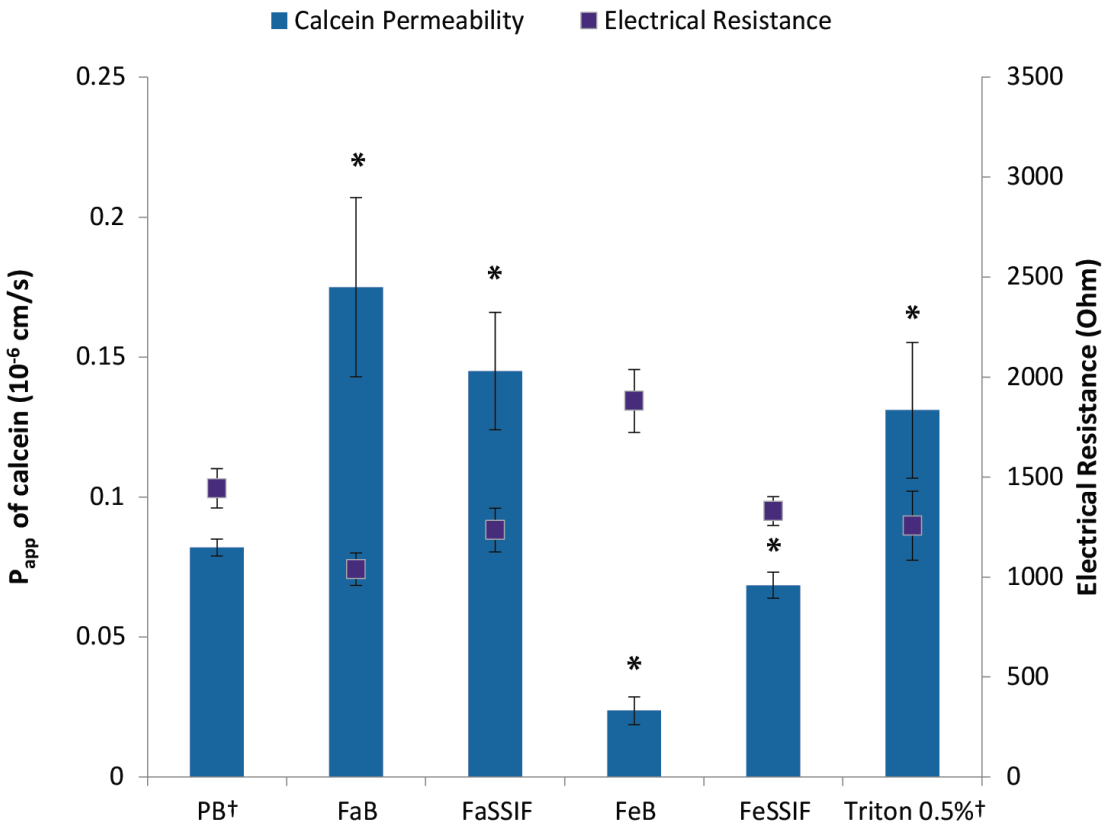

*Figure 4.6: P_{app} of calcein (bars) and ER measured across the barriers (markers) in presence of PB pH 6.2, FaB, FaSSIF, FeB, FeSSIF and 0.5% Triton X-100. Bars and markers represent the mean ± S.D. (n=3).†From Paper II. *significant difference ($p \leq 0.05$) compared to PB pH 6.2 (control).*

To further confirm the barrier integrity and to evaluate the possible disintegration of the barrier, the amount of phospholipids released from the donor chamber during the experiments were measured. The amount of phospholipids released from the barrier is indicative of the disintegration of the lipids in the PVPA barrier. When the release of phospholipids in the presence of FaSSIF and FeSSIF were compared, a slight but non-significant increase in the release was observed relative to PB, FaB and FeB (Figure 4.7). The amounts of phospholipids released from the barrier during incubation with FaSSIF and FeSSIF were 22 μg and 24 μg, respectively. These data indicate that the aggressive surfactant, sodium taurocholate, which is present in a higher concentration in FeSSIF, did not exert a superior influence on barrier disintegration. Moreover, when the reference 0.5% Triton X-100 was compared with FaSSIF and FeSSIF, Triton X-100 exhibited a release of approximately 40 μg of phospholipids, which is a significant ($p < 0.05$) two-fold increase. A similar approach has previously been reported in a study employing FaSSIF in the $PVPA_o$ (Fischer et al., 2012). During incubation with Triton X-100, the $PVPA_o$ model exhibited highly elevated levels of phospholipids (approximately 900 μg). The results for the $PVPA_{biomimetic}$ model indicated a 20-fold decrease in the amount of phospholipids released compared with the $PVPA_o$, demonstrating a tremendous increase in the integrity and stability of the $PVPA_{biomimetic}$ barrier. As discussed earlier, the lipids Chol, PS and PE within the barrier appear to have contributed to the stabilization of the membrane against the aggressive detergent-like action of sodium taurocholate. These findings thus confirmed previous integrity studies that evaluated membrane stability using calcein permeability and ER values.

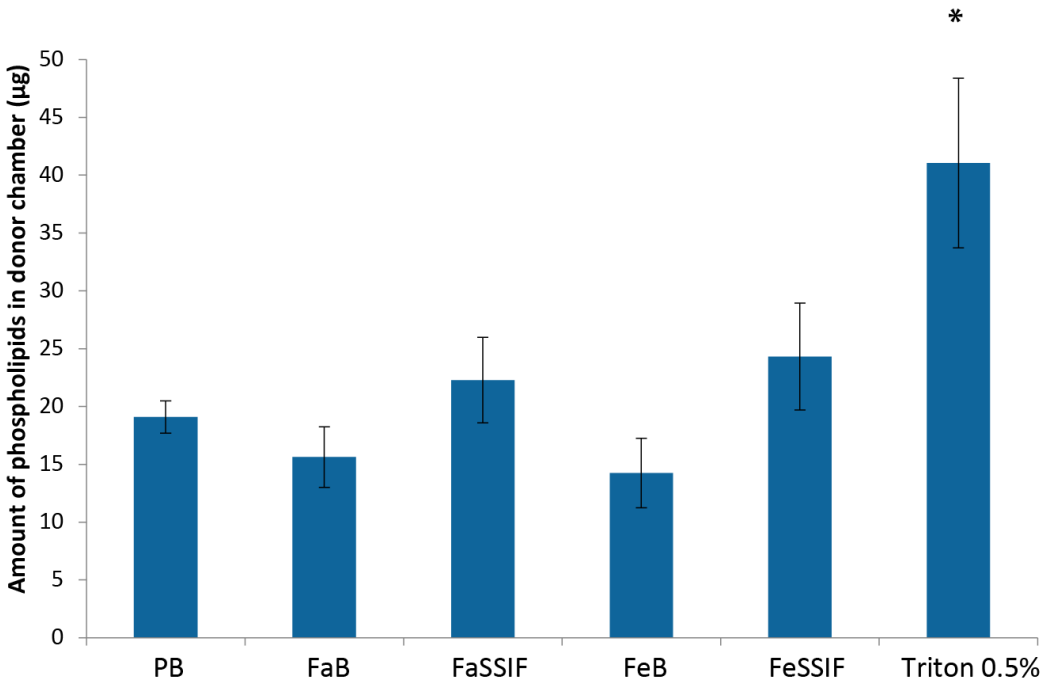

*Figure 4.7: Amount of phospholipids released from the PVPA$_{biomimetic}$ barrier to the donor chamber upon 5 hours incubation with PB pH 6.2, FaB, FaSSIF, FeB, FeSSIF and 0.5% Triton X-100 (reference). Bars represent the mean ± S.D. (n=3). *significant difference ($p \leq 0.05$) compared to PB pH 6.2 (control).*

To evaluate any further feasible interaction between sodium taurocholate and the PVPA$_{biomimetic}$ barrier, bile salt content in the donor phase was measured during a 5-h incubation period with FaSSIF and FeSSIF. The loss of sodium taurocholate in the donor compartment would confirm an interaction between the bile salt and the lipid bilayer. As shown in Figure 4.8, the loss of sodium taurocholate both in FaSSIF and FeSSIF reached a plateau of approximately 30% after 4 h of incubation. This finding demonstrates that approximately 30% of the sodium taurocholate was likely bound to or incorporated into the barrier. However, the loss of sodium taurocholate in FaSSIF resulted in no significant differences when compared to FeSSIF. Interestingly, it appears that the binding of sodium taurocholate to the membrane was hampered with increasing concentrations sodium taurocholate, which was probably attributable the interaction of sodium taurocholate with each other (Schubert and Schmidt, 1988, Schubert et al., 1986). When comparing these results with earlier studies of PVPA$_o$ in the presence of FaSSIF, an approximately 30% loss of sodium taurocholate after 4 h of incubation was also observed

(Fischer et al., 2012). This result correlates well with our observations. Given that $PVPA_o$ primarily consists of the lipid PC, a release of approximately 30% confirms that PC in particular interacted with sodium taurocholate. Overall, these results are in agreement with the release of phospholipids (Figure 4.7), for which no significant differences in release were observed from the barrier in the presence of FaSSIF and FeSSIF. Thus, the integrity studies collectively show that FaSSIF and FeSSIF are applicable biorelevant media for use in the $PVPA_{biomimetic}$ model. The compatibility of the $PVPA_{biomimetic}$ model with biorelevant media moved this model an important step forward and made it possible to investigate the impact of the biorelevant media on passive drug permeation.

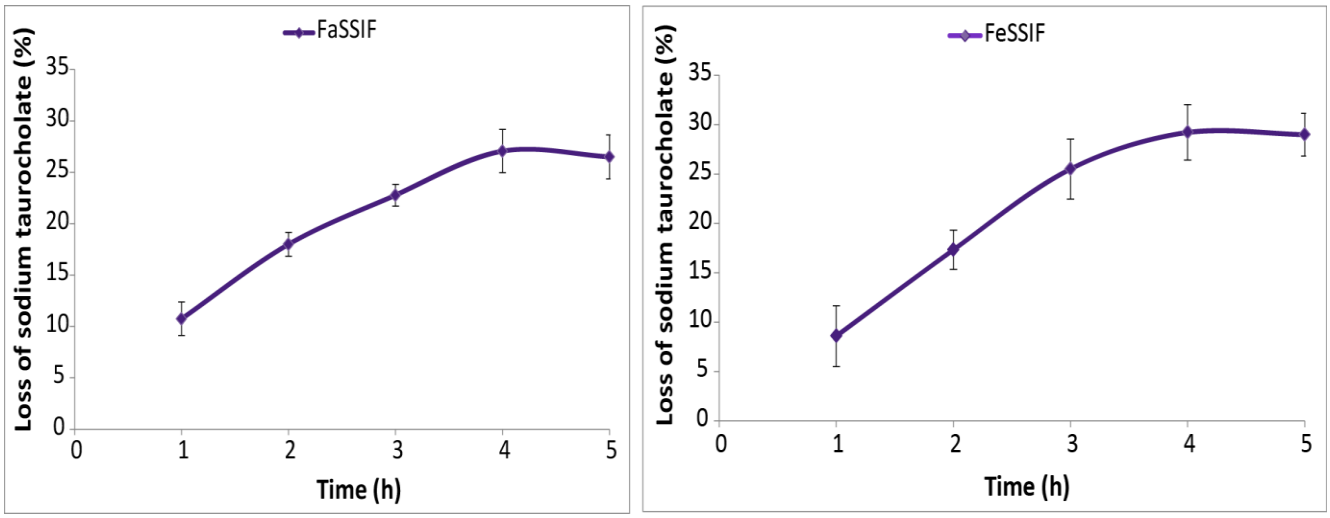

Figure 4.8: Loss of sodium taurocholate from the donor during 5 h of incubation with FaSSIF and FeSSIF. Values are provided as mean ± S.D.(n=3).

4.5.2. Effects of biorelevant media on passive drug permeation

Four compounds that offer different physicochemical properties (ketoprofen, nadolol, griseofulvin, and cimetidine) have been previously studied using the $PVPA_o$ model in the presence of FaSSIF. To our knowledge, this was the first time that FeSSIF was employed as a biorelevant medium in the PVPA model. A diverse set of model drugs (griseofulvin, indomethacin, ACV and nadolol) were assessed for their permeability abilities dissolved in PB, FaB, FaSSIF, FeB and FeSSIF.

Bile salt concentration and the amount of lecithin in the intestinal fluid are known to be important factors affecting drug solubility and permeability (Dahan and Miller, 2012). Moreover, it is known that different drugs interact uniquely with physiological surfactants depending on the physicochemical properties of both entities. The physiological surfactants present in the GIT act to incorporate lipophilic compounds into mixed micelles comprised of bile salts and phospholipids (Augustijns et al., 2014). The bile salt concentration of FaSSIF is below the CMC. Thus, FaSSIF is expected to exert less influence on the apparent permeability of drugs compared to FeSSIF, in which the bile salt concentration is above the CMC.

In the case of the highly lipophilic drug griseofulvin (class II drug by BCS classification), a trend in declining permeability was observed as the concentration of sodium taurocholate and lecihin increased in the media (Figure 4.9). It is most likely that the nonionizable drug, griseofulvin is associated within the micelles to a higher degree, consequently decreasing the free fraction of the drug. Markopoulos and co-workers have reported a similar trend in declining permeability of different BCS II drugs using the Caco-2 model (Markopoulos et al., 2014). The decline in permeability values was stronger when the lipophilicity of the compounds was increased. Our results are also in agreement with earlier studies using griseofulvin in FaSSIF and the $PVPA_o$ model (Fischer et al., 2012).

The highly lipophilic drug indomethacin (class II) was observed to have a significantly increased apparent permeability in FeSSIF compared with FaSSIF. This observation was in contrast to what was expected given the micellar incorporation of highly lipophilic drugs. However, the increased permeability of class II drugs in the presence of biorelevant media has also previously been reported. Kataoka and co-workers performed experiments using the Caco-2 model and observed increased permeation values of the class II drugs danazol and albendazole in the presence of FeSSIF compared to FaSSIF (Kataoka et al., 2006). A possible explanation for this observation might be the increased drug recovery from 30% to 39% in the presence of FaSSIF and FeSSIF (discussed in 4.5.3). It is assumed that FeSSIF, to a certain extent, prevented drug accumulation in the PVPA barrier and/or adsorption to the experimental materials resulting in the increased recovery and permeability of indomethacin.

The third model drug, ACV exhibited no significant changes in terms of apparent permeability in either FaSSIF or FeSSIF. It has been reported that drugs belonging to BCS III are less likely to

interact with colloidal systems and become incorporated within the micelles (Markopoulos et al., 2014). The results for ACV (class III) were thus in accordance with the literature. Furthermore, given that ACV is an amphoteric drug (pKa 9.23 and 2.34), it was assumed that this drug remained neutral since the pH of the different media were more than 3 pH units away from the pKa values of ACV.

Regarding the low permeability BCS class III drug, nadolol, the apparent permeability significantly decreased in FeB and FeSSIF compared to PB, FaB and FaSSIF. This result was the opposite of what was expected. However, an earlier study reported that the affinity of nadolol for micelles increases at higher concentration of cholate, which could possibly explain the decreased permeability observed in FeSSIF (de Castro et al., 2001). Further permeability studies performed in the presence of the surfactant Poloxamer 188, resulted in the same observation of decreased nadolol permeability. Fischer and co-workers reported that nadolol showed some association with the surfactant, and the decreased permeability was primarily attributed to that interaction (Fischer et al., 2011). Another explanation regarding the decreased permeability of nadolol in FeB and FeSSIF is a possible salt formation between maleic acid and the basic nadolol. Maleic acid is a component of both FaSSIF and FeSSIF and has pKa values of 1.9 for the first deprotonation and 6.1 for the second deprotonation. The pKa value of 6.1 results in a variation in the -1 and -2 charge states of maleic acid between the fed and fasted states, which might impact on salt formation, consequently leading to differences in the P_{app} values. However, nadolol has previously shown to be incorrectly classified regarding its *in vivo* absorption in the presence of PB (paper II). The permeability values in the presence of PB correspond to excellent *in vivo* absorption instead of the correct category of moderate *in vivo* absorption. However, in the presence of FeSSIF, nadolol falls into the correct classification system that we previously introduced. This might indicate that the use of biorelevant media could lead to better estimation of the *in vivo* permeability of drugs.

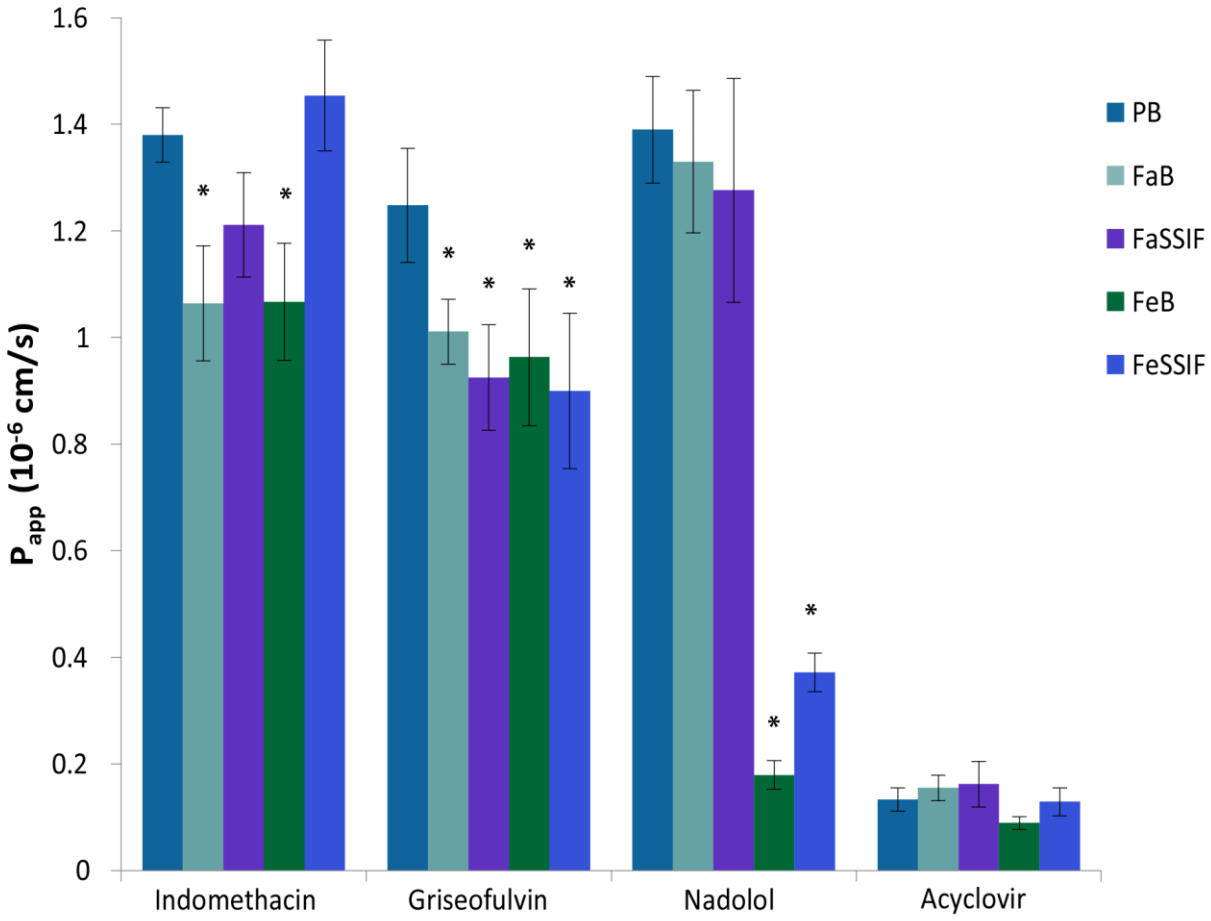

Figure 4.9: Apparent permeability (Papp) of model drugs in PB pH 6.2, FaB, FaSSIF, FeB and FeSSIF. Bars represent the mean ± S.D.(n=3).

4.5.3. Effects of biorelevant media on the recovery rates of the model drugs

Recovery is presented here as the quantity of drug found both in the donor and the acceptor compartments upon completion of the permeability experiments and is expressed as the percentage of the initial concentration in the donor compartment. As shown in Table 4.5, the recovery rates of nadolol and ACV were mostly above 80%. In contrast, low recovery rates (below 35%) were observed in the case of the highly lipophilic drugs, indomethacin and griseofulvin. Low recovery is typical for highly hydrophilic drugs and can be indicative of drug accumulation in the permeation barrier, drug adsorption onto the experimental materials, drug precipitation, or metabolic transformation of the drug (the latter occurs in cellular-based models

only) (Buckley et al., 2012). The occurrence of these effects may lead to a reduced drug concentration in the donor compartment (driving force), resulting in decreased drug permeability (Deferme et al., 2008). Several permeability studies have previously reported the impact of simulated intestinal fluids on the recovery of drugs (Fischer et al., 2012, Fossati et al., 2008, Ingels et al., 2004). According to their observations, lipophilic drugs exhibited increased recovery in the presence of simulated intestinal fluids compared with buffers. This might be due to the contribution of the media in preventing some of the aforementioned occurrences. In the case of indomethacin and griseofulvin, similar observations were observed in our studies regarding their increased recovery rates in the presence of FaSSIF and FeSSIF compared to the buffers. Collectively, the use of biorelevant media represents an improvement in the permeability assessments of poorly soluble drugs in the $PVPA_{biomimetic}$ model.

Table 4.5: Recovery of the model compounds in the different media. Values are provided as mean ± S.D.(n=3).

Medium	Recovery (%)			
	Indomethacin	Griseofulvin	Nadolol	Acyclovir
PB	24 ± 3	31 ± 3	85 ± 3	97 ± 2
FaB	25 ± 3	33 ± 2	97 ± 2	95 ± 3
FaSSIF	30 ± 1	45 ± 2	87 ± 1	86 ± 3
FeB	34 ± 2	25 ± 4	94 ± 2	92 ± 1
FeSSIF	39 ± 2	38 ±1	80 ± 1	70 ± 2

4.6. Storage stability of the $PVPA_{biomimetic}$

To increase the throughput of the model the stability of the $PVPA_{biomimetic}$ barrier was evaluated during storage at -70°C. Permeability of the hydrophilic marker calcein and ER across the barrier were determined, with barriers stored up to 6 months. The results shown in Figure 4.10 indicated negligible changes in calcein permeability during the storage from 12 h to up to 2 months. During storage from 2 to 6 months, a trend in decreased permeability was observed. These results

indicated tighter barrier with increased storage duration. However, the changes in the permeability values compared to the control were not significant. A tremendous improvement in stability was thus observed when comparing $PVPA_{biomimetic}$ with $PVPA_o$, which are stable up to 2 weeks (Flaten et al., 2006a). As inclusion of Chol was observed to increase the barrier robustness towards different tensides and cosolvents (paper II), we assume that this lipid might have an essential impact regarding the barrier storage stability. The Chol containing PVPA model mimicking *stratum corneum* has also shown improvement in storage stability compared to $PVPA_o$ (Engesland et al., 2013). These results of the barriers' increased robustness as means of storage stability gave the opportunity to produce larger batches of barriers.

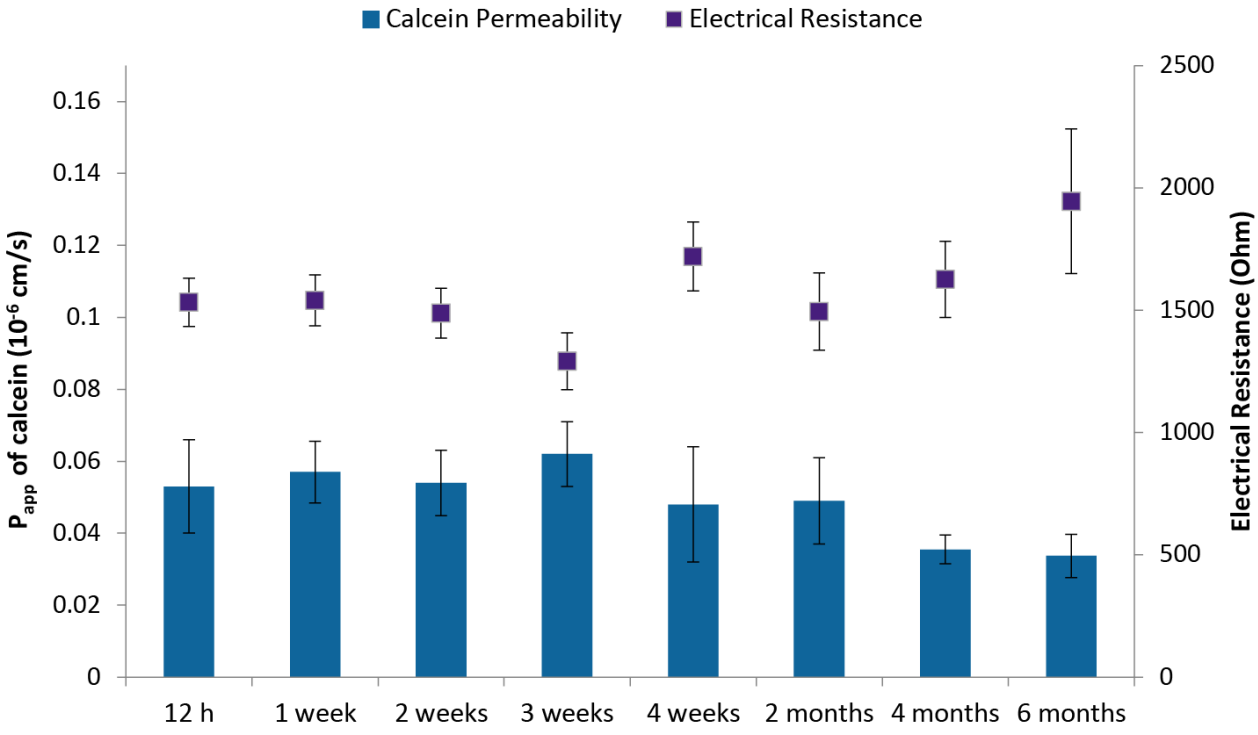

Figure 4.10: Storage stability testing of the $PVPA_{biomimetic}$ barrier according to calcein permeability (bars) and ER across the barrier (markers). Bars and markers represent the mean ± S.D.(n=3).

5. CONCLUSIONS

Based on the results presented in this thesis the following conclusions are made:

The $PVPA_o$ that was applied for the first time for *in vitro* permeability testing from mucoadhesive liposome formulations was shown to work well for screening of ACV in liposomal and mucoadhesive liposomal formulations developed for various routes of administration. Incorporation of ACV in liposomes was found to significantly enhance its *in vitro* permeability and coating with Carbopol further increased the permeability from small-sonicated E-PC liposomes, as well as E-PC/E-PG liposomes.

The $PVPA_o$ was successfully developed into a more robust, biomimetic PVPA ($PVPA_{biomimetic}$) by employing an intestinal-mimicking lipid composition. Based on a diverse set of drugs the $PVPA_{biomimetic}$ appeared to distinguish between substances with varying degree of intestinal absorption. Positively charged basic drugs exhibited increased permeability through the negatively charged biomimetic barrier, as expected. Furthermore, the degree of correct classification according to fractions absorbed *in vivo* was comparable for the $PVPA_o$ and the $PVPA_{biomimetic}$.

The $PVPA_{biomimetic}$ appeared to be tremendously more robust toward the presence of surfactants compared with $PVPA_o$. The $PVPA_{biomimetic}$ also showed improved storage stability for a period of 6 months compared to 2 weeks for the $PVPA_o$.

The biorelevant media (FaSSIF and FeSSIF) were found to be compatible with $PVPA_{biomimetic}$, thus enabling a closer intestinal mimicking environment in permeability assessment. Further, the permeability of the model drugs were differently affected in the presence of the biorelevant media that was in agreement with previous reports.

6. FUTURE PERSPECTIVES

The results obtained in this thesis serve as an important basis for further investigations to extend our knowledge about the PVPA models as screening tool in drug development.

The $PVPA_o$ has shown to be suitable for testing permeability of drugs from formulations. So far we have only been testing drugs in solutions in the $PVPA_{biomimetic}$ and further studies with both additional drugs and drugs in formulations would be of high importance. Studies on permeability of drugs in the presence of solubilizers found compatible with the model as well as an attempt to determine the interplay between the solubility, micelle association and permeability would also be interesting.

When developing a new model it is of high importance to compare the results with the current accepted standard. Further permeability studies of selected drugs both in presence of solubilizers and in formulations in the Caco-2 model should be performed and compared with results determined in the $PVPA_{biomimetic}$.

The $PVPA_{biomimetic}$ has shown to be stable for up to 6 months at -70°C, which is an improvement compared to only 2 weeks for the $PVPA_o$. However, further studies on storage stability over prolonged period of time and under different conditions should be performed. Additional markers and drugs to further elucidate the barrier stability should also be included.

Further, the administration of drug to the mucus membranes is of growing interest; however no one has so far succeeded in developing an artificial permeation models for these sites. The $PVPA_o$ has already shown to be able to distinguish between drug permeability from different mucoadhesive liposome formulations. Further studies to elucidate the PVPA's potential to serve as a model for mucus membranes like the vaginal epithelia involving examination of the penetration of drugs through tissue samples from the absorption barriers of interest would therefore also be interesting.

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Paper I

Paper II

Paper III