Review

Drug permeability profiling using cell-free permeation tools: Overview and applications

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A B S T R A C T

Cell-free permeation systems are gaining interest in drug discovery and development as tools to obtain a reliable prediction of passive intestinal absorption without the disadvantages associated with cell- or tissue-based permeability profiling. Depending on the composition of the barrier, cell-free permeation systems are classified into two classes including (i) biomimetic barriers which are constructed from (phospho)lipids and (ii) non-biomimetic barriers containing dialysis membranes. This review provides an overview of the currently available cell-free permeation systems including Parallel Artificial Membrane Permeability Assay (PAMPA), Phospholipid Vesicle-based Permeation Assay (PVPA), Permeapad®, and artificial membrane based systems (*e.g.* the artificial membrane insert system (AMI-system)) in terms of their barrier composition as well as their predictive capacity in relation to well-characterized intestinal permeation systems. Given the potential loss of integrity of cell-based permeation barriers in the presence of food components or pharmaceutical excipients, the superior robustness of cell-free barriers makes them suitable for the combined dissolution/permeation evaluation of formulations. While cell-free permeation systems are mostly applied for exploring intestinal absorption, they can also be used to evaluate non-oral drug delivery by adjusting the composition of the membrane.

1. Introduction

Despite the tremendous increase in approved (bio)pharmaceutical products intended for intravenous or subcutaneous administration, the oral route of administration remains of major interest since it is beneficial from an economical, convenience and safety point of view ("2016 FDA drug approvals - nrd.2017.14.pdf," n.d.; Ecker et al., 2014). Nevertheless, before reaching the systemic circulation and, subsequently, its site of action, the orally administered drug must cross the intestinal mucosa, a major barrier for oral drug delivery.

Numerous methods exist to estimate the extent of absorption across the human gastrointestinal wall (Bohets et al., 2001; Buckley et al., 2012). Using a fairly simple computational approach, different research groups proposed to estimate drug permeation based on physicochemical drug properties including molecular weight (MW), lipophilicity (log D), acid dissociation constant (pKa), polar surface area (PSA), and hydrogen bonding potential (Camenisch et al., 1998; Neuhoff et al., 2005; Palm et al., 1997; Veber et al., 2002). These molecular

descriptors, with the exception of pKa and PSA, are well covered in Lipinski's rule of 5 and provide a rational basis for understanding oral drug absorption processes in early stage drug development (Lipinski et al., 2001; Veber et al., 2002).

In contrast to this over-simplification of estimating intestinal drug permeation, tissue-based permeation models offer the advantage to closely mimic the *in vivo* situation from an anatomical, biochemical and structural point of view. For instance, the *in situ* rat intestinal perfusion technique with mesenteric blood sampling is often used for specific research scenarios aiming at (i) unravelling intestinal drug absorption mechanisms induced by drug transporters and cytochrome P450 enzymes allowing the investigation of transporter-metabolism interactions and (ii) exploring differences in regional drug absorption (Stappaerts et al., 2015; Ungell et al., 1998). Alternatively, the Ussing chambers model, in which rat or human intestinal tissue is mounted between 2 half chambers, also offers the opportunity to investigate differences in regional drug absorption, carrier-mediated transport and the impact of intestinal metabolism on drug transport (Mols et al.,

2005; Rogers et al., 1987). Despite their high relevance for the *in vivo* situation, these tissue-based absorption systems are associated with several drawbacks including low- to moderate-throughput, limited tissue availability, ethical burdens and challenging experimental procedures. More specific drawbacks, which further impede the use of tissue-based permeation models, are the unknown effect of anesthesia on drug absorption using the *in-situ* perfusion method and the possible underestimation of drug transport due to accumulation in the irremovable circular muscle layers in the Ussing chambers model.

Cell-based systems including the human colorectal adenocarcinoma (Caco-2) cell line and the Madin Darby canine kidney (MDCK) cell line are considered as valuable alternatives to assess intestinal drug permeation. In particular, the well-established Caco-2 cell line is widely used since this system generates reproducible and biorelevant permeability results on a high-throughput basis (Balimane et al., 2000). The presumed superior predictive power of cell-based models over molecular descriptors depends on careful selection of a reference compound set (Linnankoski et al., 2008). Furthermore, this permeation model allows for the investigation of carrier-mediated transport due to the expression of intestinal uptake and efflux transporters (Matsson et al., 2015; Ölander et al., 2016). Despite their high popularity, cell-based permeation systems suffer from several shortcomings including a relative incompatibility with food components and certain pharmaceutical excipients, the absence of CYP3A4 and the lack of a mucus layer (Sun et al., 2002). Furthermore, large inter- and intra-laboratory variability in transporter expression may impair comparability of the measured permeability values (Hayeshi et al., 2008; Lee et al., 2017).

Despite the reasonable predictive power of tissue- and cell-based permeation systems for the estimation of intestinal drug permeation, these models suffer from time-consuming and expensive preparation steps. As a result, a growing interest in the development of cell-free permeation systems has evolved wherein lengthy and expensive preparation steps are drastically reduced.

The present review provides an overview of the currently available cell-free permeation systems including the parallel artificial membrane permeation assay (PAMPA), the phospholipid vesicle based permeation assay (PVPA), Permeapad® and the artificial membrane insert system (AMI-system). The main focus of this review is the description of these permeation systems in the context of oral drug delivery; however, a section on their applicability in non-oral drug delivery evaluation is also included.

2. Mechanisms of intestinal drug absorption

While drug absorption in the stomach is of minor importance, the small intestine is the main site of absorption of orally administered drugs due to its unique anatomical properties. Particularly, the presence of (micro)villi drastically increases the surface area of the intestinal mucosa resulting in the enormous absorptive surface area of the small intestine (Helander and Fändriks, 2014; Niess and Reinecker, 2006). Drugs can cross the intestinal epithelium layer in several ways, as illustrated in Fig. 1. The type of intestinal transport is strongly connected to several physicochemical properties of the drug as described by Lipinski's rule of five which indicates whether a drug is likely to be absorbed after oral administration (Lipinski, 2000; Lipinski et al., 2001). For instance, depending on the lipophilicity of the drug, passive diffusion through the enterocytes (transcellular diffusion, Fig. 1(A)) is the preferred route for lipophilic compounds, while small hydrophilic compounds are mainly absorbed *via* passive diffusion between the enterocytes (paracellular diffusion, Fig. 1(B)) (Artursson et al., 1993; Camenisch et al., 1996). However, the contribution of passive paracellular diffusion to the overall drug transport is limited since the area available for this type of transport only accounts for 0.01% of the total surface area of the intestinal membrane (Zhu et al., 2017). In contrast to these passive routes of transport, some drugs reach the systemic circulation by means of active uptake (Fig. 1(C)), which requires energy

(Tsuji and Tamai, 1996). As a result of this energy-dependence, active transport enables drug transport against a concentration gradient. Additionally, efflux transporters (Fig. 1(D)) limit intestinal drug absorption by actively transporting drugs back to the luminal environment (Chan et al., 2004; Kapitza et al., 2007). Finally, transcytosis, *i.e.* compounds migrating from the luminal to the serosal side of the intestinal epithelium layer by incorporation in vesicles from the cell membrane, may contribute to the uptake of certain drugs (Fig. 1(E)) (Florence and Hussain, 2001).

In the past decades, the majority of new chemical entities (NCEs) in the pipeline of pharmaceutical companies have increased in lipophilicity and size; as a result, many of these NCEs are preferably absorbed by passive transcellular diffusion (Fig. 1(A)). Presently, 80%–95% of the commercially available drugs are mainly absorbed transcellularly (Mandagere et al., 2002), justifying the development of time-and cost-effective cell-free permeation systems. It should be noted, however, that these cell-free permeation tools could be exclusively applied for predicting passive transcellular drug transport; paracellular and active drug transport cannot be captured.

3. Currently available cell-free permeation systems and their predictive capacity

Depending on the composition of the barrier, cell-free permeation systems are typically classified into two classes including (i) biomimetic barriers which are constructed from (phospho)lipids and (ii) non-biomimetic barriers containing dialysis membranes. Below, an overview of the currently available cell-free permeation systems is provided in terms of their barrier composition. In addition, their predictive capacity is discussed in relation to well-characterized permeation systems.

3.1. Parallel artificial membrane permeation assay (PAMPA)

3.1.1. Original PAMPA

In 1998, PAMPA was introduced for the first time when the Roche team presented the use of artificial membranes in a 96 well microtiter plate format, as illustrated in Fig. 2 (Kansy et al., 1998). PAMPA barriers generally consist of a filter (*e.g.* polyvinylidene fluoride (PVDF)) soaked with (phospho)lipids dissolved in an organic solvent. Since the PAMPA barrier does not contain a physical boundary separating the donor media from the lipophilic barrier constituents, potential dissolution/emulsification of barrier constituents into the media may occur. Initially, an n-dodecane solution of egg lecithin (1–20%) (a mixture of lipids containing phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and cholesterol) was used to mimic the phospholipid composition of the mammalian membrane. Using this composition, Kansy and co-workers were able to correlate measured PAMPA fluxes at different pH values (6.5 and 7.4) with the fraction absorbed in humans (Kansy et al., 1998). Although the relationship obtained between the measured permeability values and the fractions absorbed in humans was similar to what was described for Caco-2 permeation studies, PAMPA suffers from the inability to predict paracellular and active transport, and potential membrane retention of lipophilic compounds. However, the contribution of paracellular transport to overall drug transport can be addressed using additional *in silico* models, which simulate the characteristics of the human epithelium; this approach may avoid underestimation of the fraction absorbed of small, hydrophilic molecules like atenolol, metformin, cimetidine or terbutaline (Adson et al., 1994; Sugano et al., 2002).

3.1.2. Variants of PAMPA

In the following years, several variations of the setup were published, which reflect tissues in the human body containing different lipid compositions (Proulx, 1996). The variants of the original PAMPA assay, which are listed in Table 1, differ by the nature of the filter support, the composition of the membrane constituents, the pH in
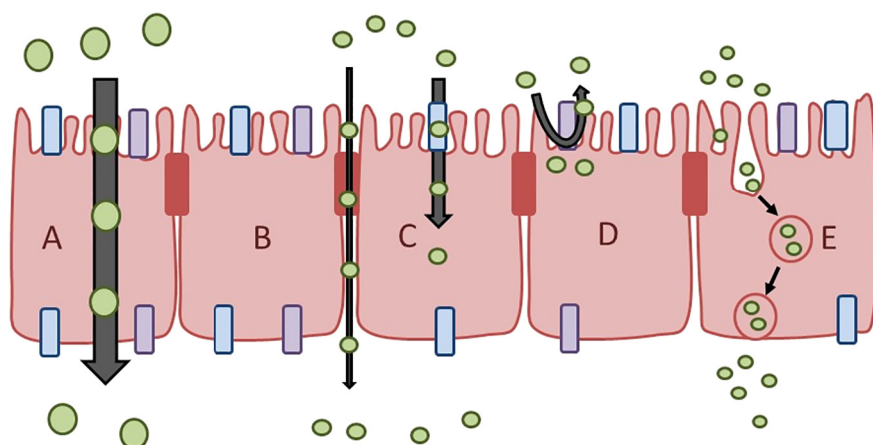
Fig. 1. Schematic overview of different types of intestinal drug transport including passive transcellular diffusion (A), passive paracellular diffusion (B), active influx transport (C), active efflux transport (D) and transcytosis (E). Blue and purple boxes represent uptake and efflux transporters, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

donor/acceptor, and the presence of a "sink" in the acceptor compartment (Faller, 2008). Since the (phospho)lipids and the organic solvent can be adjusted within certain limits according to the specific application, it is plausible that the compatibility of PAMPA barriers with co-solvents, excipients and biomimetic media may vary according to the composition of the barrier which is further discussed in Section 4.1. The incorporation of phospholipids modifies the physicochemical properties of the alkane solution and, as a result, affects the membrane permeability as well as the membrane retention properties. For example, in the PAMPA models described by Sugano et al. and Zhu et al., a relatively low concentration of (phospho)lipids is used (Sugano et al., 2001; Zhu et al., 2002). While the first approach aimed to consider paracellular transport (Sugano et al., 2001), the second approach used a hydrophilic PVDF filter (instead of a hydrophobic PVDF filter) as supporting barrier to reduce the transport time significantly (Zhu et al., 2002). In contrast, the (phospho)lipid concentration is increased in the Double-Sink™ PAMPA (PAMPA-DS) model consisting of a PVDF filter impregnated with 20% lecithin in n-dodecane. As the name indicates, the PAMPA-DS model employs both a pH-gradient and a 'sink' created by the addition of a surfactant or serum proteins to the acceptor medium which is beneficial with regard to the assessment of poorly water soluble drugs (Avdeef, 2005). The use of a surfactant containing acceptor medium suggests that PAMPA-DS possesses an intrinsic resistance against surfactants.

3.2. Phospholipid vesicle-based permeation assay (PVPA)

3.2.1. Original PVPA

The PVPA, which is depicted in Fig. 3, was introduced in 2006 by the University of Tromsø as a new artificial liposome based *in vitro* model representative for the intestinal epithelium intended for the estimation of passive drug permeability (Flaten et al., 2006b). The concept was that the building blocks (*i.e.* liposomes) of this permeation tool closely mimic the phospholipid bilayer of the intestinal cell membrane from a structural point of view. When depositing a tight barrier of

liposomes on a filter support, the obtained barrier could be considered as a valuable substitute for the intestinal epithelium (Flaten et al., 2006b). The original PVPA consists of egg phospholipids, mainly phosphatidylcholine, a component found in the intestinal epithelia as well as in many other biological absorption barriers (Naderkhani et al., 2014a).

It has been reported that the apparent permeability coefficients (P_{app}) obtained from the PVPA correlate well with literature data on the human fraction absorbed. More specifically, when dividing drugs into three categories including (i) poor absorption (< 30% absorbed *in vivo*), (ii) moderate absorption (30–70% absorbed *in vivo*), and (iii) excellent oral absorption (> 70% *in vivo* absorption), the *in vivo* absorption ability was correctly predicted for > 80% of the about 30 drugs tested. Further, compounds that were not correctly classified were found in the neighbouring class and none of the compounds were totally mispredicted (Naderkhani et al., 2015). Overall, based on the permeability values from the tested drugs, the PVPA seems to model the *in vivo* absorption better than molecular descriptors and equally well as the Caco-2 model and the PAMPA-DS model (Flaten et al., 2006b).

3.2.2. Variants of PVPA

The original PVPA has the potential to serve as a general model mimicking several biological absorption barriers (Naderkhani et al., 2014a). Nevertheless, by changing the lipid composition of the PVPA, specific barriers might be better mimicked. For instance, the biomimetic PVPA with a lipid composition more closely approaching that of the intestinal barrier was introduced to improve passive intestinal permeability assessment (Naderkhani et al., 2014b). This biomimetic PVPA barrier consists of 26.5% phosphatidylcholine, 26.5% phosphatidylethanolamine, 7% phosphatidylserine, 7% phosphatidylinositol and 33% cholesterol which provides the barrier with a negative surface charge thereby better mimicking the lipid composition found in intestinal epithelia (Naderkhani et al., 2014b). For positively charged compounds, an increased permeability was observed with the negatively charged biomimetic barriers. Overall, the degree of correct
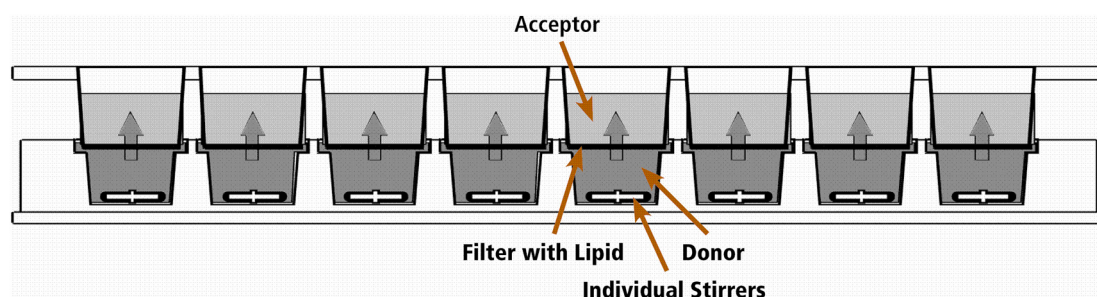

Fig. 2. Schematic illustration of PAMPA which is typically applied in a 96-well plate. Reprinted from website of Pion Inc. with permission (Pion Inc., 2018).

Table 1
Characteristics of PAMPA assays.

Assay short name	Barrier target	Support filter	Solvent	Membrane composition	pH (donor/acceptor)	Sink	Inc. time (h)	Lit. ref
Egg-PAMPA	GI-tract	Hydrophobic PVDF (125 μm)	n-Dodecane	10% egg lecithin	Iso 6.5 and 7.4	No	15	(Kansy et al., 1998)
HDM-PAMPA	GI-tract	Polycarbonate 10 μm, non-tissue culture treated	n-Hexadecane	n-Hexadecane	Iso 4.0 or gradient 6.8–8.0	Bile salt in donor	4	(Wohnsland and Faller, 2001)
BM-PAMPA	GI-tract	Hydrophobic PVDF (125 μm)	1.7-Octadiene	3% of a phospholipid mixture	Iso 6.5	No	15	(Sugano et al., 2001)
	GI-tract	Hydrophilic PVDF (125 μm)	n-Dodecane	1% egg lecithin	Iso 5.5 and 7.4	No	2	(Zhu et al., 2002)
DOPC-PAMPA	GI-tract	Hydrophobic PVDF (125 μm)	n-Dodecane	2% DOPC	Iso 7.4	No	15	(Avdeef et al., 2001)
PAMPA-DS	GI-tract	Hydrophobic PVDF (125 μm)	n-Dodecane	20% (phospholipid mixture)	Gradient 6–7.4	Anionic surfactant in acceptor	4	(Bermejo et al., 2004)
BBB-PAMPA	BBB	Hydrophobic PVDF (125 μm)	n-Dodecane	2% polar brain lipids	Iso 7.4	No	18	(Di et al., 2003)
PAMPA-skin	Skin	Hydrophobic PVDF (125 μm)	70% silicon oil, 30% iso-propyl myristate	70% silicon oil, 30% iso-propyl myristate	Variable to target neutral species	No	7	(Ottaviani et al., 2006)
Precoated PAMPA	GI-tract	PVDF (0.45 μm)	Hexane	Lipid/oil/lipid trilayer	Iso 7.4	No	4–5	(Chen et al., 2008)

Abbreviations: HDM-PAMPA, Hexadecane membrane-PAMPA; BM-PAMPA, Biomimetic-PAMPA; DOPC-PAMPA, Dioleyoylphosphatidylcholine-PAMPA; BBB-PAMPA, blood-brain barrier-PAMPA; GI-tract, gastro-intestinal tract; PVDF, polyvinylidene fluoride.
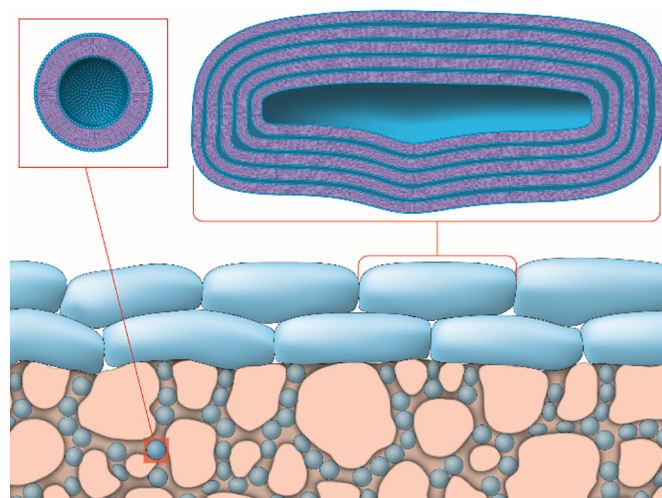

Fig. 3. Schematic illustration of the phospholipid vesicle-based permeation assay which could be used in Transwell inserts (0.33 cm^2) and Millicell well plates (0.6 cm^2). The filter (cellulose ester) support is shown in pink while the liposomes of two different size distributions and lamellarity are shown in blue; the liposomes can be found both within the pores as well as on top of the filter support. Reprinted from PhD thesis of Flaten with permission (Flaten, 2007). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

classification according to the *in vivo* absorption was comparable with the original PVPA (Naderkhani et al., 2014b). Since the biomimetic PVPA model also showed improved storage stability by maintaining its integrity in the frozen state for a period of 6 months compared to 2 weeks for the original PVPA, the biomimetic variant of the PVPA model might be considered as a step forward in permeability screening.

In addition to this biomimetic PVPA, the mucus-PVPA has recently been introduced as another variant of the PVPA (Falavigna et al., 2018). The mucus layer, covering all mucosal surfaces in the human body, is the first barrier encountered by drugs before absorption through epithelial tissues, implying that the mucus layer could affect drug absorption. It has been suggested that two major mechanisms might influence the ability of compounds to diffuse through the mucus layer: i) the interaction filtering where the overall hydrophilicity of the mucin gel mostly hampers the diffusivity of lipophilic compounds, whereas hydrophilic compounds tend to penetrate more easily through this hydrophilic mucus layer, and ii) the size filtering properties of the mucin mesh preventing diffusion according to the size of the diffusing compound or formulation (Boegh and Nielsen, 2015). The impact of a mucus layer on intestinal drug absorption has mainly been investigated using cell-based models. For instance, co-cultures of mucus producing HT-29 cells and Caco-2 cells have been developed (Lechanteur et al., 2017) or an additional artificial mucus layer has been applied on top of Caco-2 cells (Wuyts et al., 2015a). Since the integrity of the mucus barrier cannot be assured using these cell-based systems, there is a need to implement a fixed mucus layer to directly investigate the impact of mucus on (oral) drug delivery. To meet these shortcomings, the novel mucus-PVPA was developed which contains the same liposome composition as the original PVPA barriers supplemented with an additional layer of mucus on top of the PVPA-barrier (Falavigna et al., 2018). The mucus was prepared using unpurified mucin from porcine stomach type III in phosphate buffer pH 7.4. The reason for choosing this type of mucin was that the molecular weight, structure and mesh size of pig mucins resemble what is found in human mucins. This modified model has been proven to be stable in the presence of mucus with mucin concentrations from 10 to 40 mg/mL and demonstrated the ability to discriminate between compounds with different chemical structures and properties. More specifically, a decrease in drug permeability was found in the presence of mucus on top of the PVPA barriers. To

overcome this barrier, drug delivery systems have been designed with mucoadhesive (chitosan-coated) and/or mucus penetrating (PEGylated) properties (Naderkhani et al., 2014a; Netsomboon and Bernkop-Schnürch, 2016). The mucus-PVPA was able to distinguish between the different liposomal formulations, making it a reliable tool in the development and optimization of formulations intended for transmucosal delivery (Falavigna et al., 2018).

3.3. Permeapad®

Permeapad®, which has been developed at the University of Southern Denmark in 2015, is also a fully artificial phospholipid-based biomimetic membrane with a layered structure (di Cagno et al., 2015). In contrast to PAMPA and PVPA, Permeapad® is composed of phospholipids (soy bean phosphatidylcholine S-100) deposited between two support sheets (di Cagno et al., 2015). According to the general model of phospholipid hydration, the lipid crystals are assumed to swell in contact with water generating within minutes a tightly packed layer of spheroids consisting of stacks of bilayers with intercalating water layers, which mimic the cell membrane. As the phospholipids fill the space between the support layers, the vesicles will remain in close proximity to their neighbor vesicles and thereby mimic tissue morphology. Furthermore, the support layers protect the lipid layer from erosion and leakage of lipids into the aqueous environment. For a set of compounds, Di Cagno and co-workers observed a good linear correlation between permeability coefficients obtained with Permeapad® on the one hand and the Caco-2 cell assay or PAMPA from literature on the other hand (di Cagno et al., 2015).

Being available in several ready-to-use formats, as illustrated in Fig. 4, Permeapad® can potentially serve for several permeability applications: while high-throughput permeability screening of NCEs (Permeapad® Plate; Fig. 4(D)) is generally performed using a 96-well plate, disks of Permeapad® mounted in side-by-side diffusion cells or Franz diffusion cells (Fig. 4(B)) are mainly applied for rank ordering drug formulations. For example, Volkova and co-workers used Permeapad® to determine the permeability of a set of novel 1,2,4-thiadiazole derivatives to evaluate the predictive capacity of *in silico* models (Volkova et al., 2017). Furthermore, Permeapad® is also available in the form of inserts for 6-well plates (Fig. 4(C)).

3.4. Artificial membrane based systems

Dialysis experiments, using a cellulose membrane with a molecular cutoff weight of 12–14 kDa, are regularly performed to estimate the bioaccessible fraction since these membranes are impermeable for micellar and other colloidal structures. These artificial membranes can also be used for permeation experiments, which will be exemplified in this section by the AMI-system. In 2017, the AMI-system has been introduced by the KU Leuven as a new cell-free permeation tool which, in contrast to the previously described cell-free permeation systems, only contains an artificial membrane without any (phospho)lipids to investigate the passive intestinal permeability of poorly water soluble drugs (Berben et al., 2018a). To this end, a regenerated cellulose membrane (molecular weight cutoff 2 kDa) is mounted between two plastic rings, as depicted in Fig. 5. Using this AMI-system, the permeability of 14 different poorly water-soluble drugs dissolved in both fasted state simulated and human intestinal fluids (FaSSIF/FaHIF), was assessed. For this set of compounds, covering a broad range of physicochemical properties, a good correlation (Pearson correlation coefficient r of 0.95) was observed with the commonly used Caco-2 cell model when FaHIF was used as donor solvent system. Additionally, the apparent permeability coefficients obtained when using FaSSIF and FaHIF correlated nicely using the AMI-system, indicating the usefulness of FaSSIF as surrogate medium for FaHIF (Berben et al., 2018a). Overall, due to the limited preparation steps of this recently introduced permeation tool, the AMI-system may be considered as a high-throughput and cost-effective tool for passive permeability profiling of poorly water-soluble drugs.

As previously mentioned (cfr. 3.2.2.), the intestinal mucus layer, which is often ignored in permeability profiling, is of considerable interest since this layer may drastically affect the intestinal absorption of lipophilic drugs and may function as a drug depot for (mucoadhesive) nanoparticles (Behrens et al., 2001; Netsomboon and Bernkop-Schnürch, 2016). To investigate the impact of a mucus layer, Stappaerts et al. recently described a slightly modified version of the AMI-system where a mucus layer was added between a dialysis membrane (pore size 2 kDa) and a filter membrane (pore size 1 μm), as illustrated in Fig. 6 (Stappaerts et al., 2017). The thickness of the mucus layer (± 120 μm) and the mucin concentration (50 mg/mL) closely approach reported *in vivo* values (Ensign et al., 2012). Since the relatively large pore size of the filter membrane on top of the mucus layer successfully retains the mucus and allows free permeation of drugs, this model enables to study the interaction of drugs with mucus and its implications on intestinal drug permeation. For instance, it was demonstrated that the transport of the lipophilic compound heptylparaben was strongly impeded by the implementation of a mucus layer in the AMI-system. However, this diffusion limiting effect of the mucus layer was neutralized by (i) the addition of relatively low concentrations of hydroxypropyl-β-
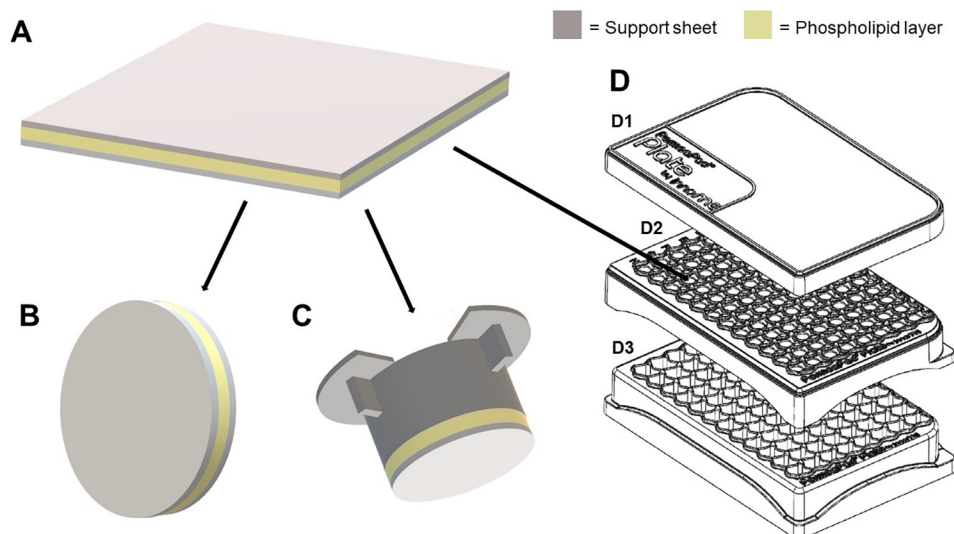

Fig. 4. Schematic representation of the available formats of the Permeapad® barrier. A. Bulk sheet of Permeapad®, B. Disk of Permeapad® compatible with side-by-side diffusion cells and Franz diffusion cells, C. Insert with Permeapad® for 6-well plates (surface area of 3.8 cm²), D. Permeapad® plate, a 96-well plate with a surface area of 0.13 cm² for high throughput permeation screening with: D1 lid, D2 middle plate with Permeapad® as barrier and D3 bottom well-plate.
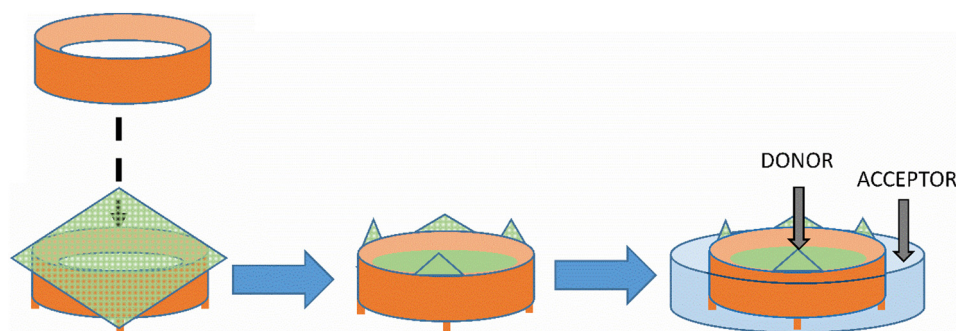
= Support sheet   = Phospholipid layer

Fig. 5. Schematic representation of the AMI-system; green surface (4.91 cm^2) represents the regenerated cellulose membrane as barrier (Berben et al., 2018a). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cyclodextrin (HP-β-CD) to the phosphate buffer in the donor compartment or (ii) by the use of biorelevant media (FaSSIF) as donor solvent system. These results indicate that the implementation of a mucus layer in the AMI-system enables to mechanistically evaluate the impact of mucus on drug permeation.

4. The usefulness of cell-free permeation tools in oral drug delivery studies

Since a sizable fraction of NCEs have a low solubility in intestinal fluids that may limit their oral absorption, solubility enhancers are regularly employed in formulation development; in addition, food may affect as well the absorption of these low solubility NCEs (Loftsson and Brewster, 2010). In view of the fact that (i) information on the impact of these pharmaceutical excipients and/or food components on drug permeability is scarce and (ii) they may compromise the barrier integrity of several cell-based permeation tools (*e.g.* Caco-2 cells), there is an increased interest to implement cell-free permeation systems in formulation testing. A number of mechanisms may contribute to the incompatibility of Caco-2 cells towards different intestinal conditions. (i) In contrast to the *in vivo* situation, Caco-2 cells are not covered by a mucus layer which protects the enterocytes in the human intestinal tract from toxic effects of *endo-* and exogenous substances. (ii) Caco-2 cells are exposed to the donor medium during the entire transport experiment; *in vivo*, transit along the intestinal tract results in a reduced exposure time to the intestinal epithelium. In specific cases, an effect of excipients on monolayer integrity is targeted. For example, it is known that permeation enhancers, which aim to increase the intestinal absorption of proteins and peptides, rely on the temporarily disruption of the intestinal lining by opening the tight junctions to allow oral absorption of the peptide or protein *via* the paracellular route (McCartney et al., 2016). Since cell-free permeation systems are limited to the prediction of passive transcellular diffusion, other permeation systems are required to investigate the effect of permeation enhancers.

In this section, an overview of the robustness of these cell-free permeation models towards the harsh conditions from the intestinal environment is provided and how these systems might be used for evaluating the performance of oral drug delivery systems within the field of drug discovery and development.

4.1. Robustness

Independent of the nature of the barrier (cellular or non-cellular), experimental evidence of barrier integrity is essential to establish a reliable permeation screening protocol. Traditionally, non-invasive techniques are preferred to check the barrier integrity continuously during a permeation experiment. Such techniques are mainly based on the measurement of the transepithelial electrical resistance (TEER). Although originally designed for confluently growing cells cultured on porous membranes, the same approach is frequently used for cell-free barriers (Fischer et al., 2011, 2012). Another, more direct assay in terms of functional integrity is to measure the permeability of hydrophilic, low permeability model compounds, such as calcein, carboxyfluorescein, Lucifer Yellow, Trypan Blue, mannitol or dextran. For practical reasons, fluorescence-labeled substrates are preferred; depending on the interference between marker and drug, they may be included in the same run as the drug of interest. Finally for biomimetic barriers, a leaching of barrier-constituents such as phosphatidylcholine into the donor compartment may serve as an indicator for partial breakdown of the barrier (Flaten et al., 2008). Hence, within this section, the functional stability of the lipid-based cell-free permeation assays towards different pH values, pharmaceutical excipients and biomimetic media is extensively described and summarized in Table 2. Given the fact that the AMI-system only contains a regenerated cellulose membrane, this permeation tool is not discussed since it is supposed that the AMI-system easily resists bile salts, enzymes, and (digesting) products; indeed, permeation experiments applying FaSSIF and FaHIF as donor medium did not result in any stability issues (Berben et al., 2018a).

4.1.1. pH

Riethorst et al. observed that, in a medium-scale clinical trial with 20 healthy volunteers, the pH of the duodenal fluids collected in the fasted state was highly variable, ranging from 3.4 to 8.3 (Riethorst
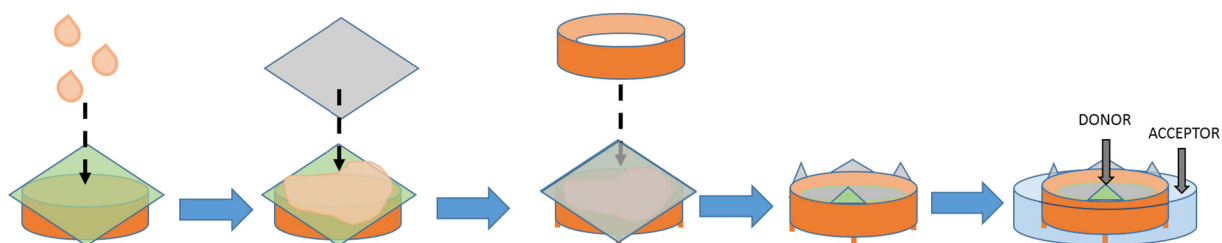

Fig. 6. Schematic representation of the AMI-system consisting of a mucus layer (brown droplets) on a dialysis membrane (green surface) which is retained by an additional membrane filter (grey surface) allowing to investigate the influence of a mucus layer on the intestinal drug permeation, reprinted with permission (Stappaerts et al., 2017). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Robustness of PAMPA, PVPA and Permeapad® in the presence of co-solvents, surfactants, other excipients and biomimetic media.

Permeation assay	Integrity test method	Time (h)	Additives (co-solvents, surfactants, biomimetic media)	Concentration	Compatibility	Ref.
PAMPA (2% DOPC in n-dodecane)	Permeation of Lucifer yellow	16	Ethanol	0.1–5%	✔	(Liu et al., 2003)
			Tween 80	0.1–5%	✔	
			Brij 35	0.1–5%	✔	
			Cremophor EL	0.5%	Not compatible	
PAMPA biomimetic	Permeation of trypan blue	2	Ethanol	Up to 30%	✔	(Sugano et al., 2001)
			DMSO	Up to 30%	✔	
			PEG 400	Up to 30%	✔	
Double-sink™ PAMPA	Permeation of Lucifer Yellow	20	FaSSIF-v2[e]	–	✔	(Markopoulos et al., 2013)
			FeSSIF-v2[e]	–	✔	
Pre-coated PAMPA	Permeation of 8 high and low permeability compounds	4–5	Methanol	20%	✔	(Chen et al., 2008)
PVPA_original	Permeation of calcein, electrical resistance, phospholipid release	5	Ethanol	20 mg/mL	✔	(Flaten et al., 2008)
			DMSO	20 mg/mL	✔	
			PEG 400	40 mg/mL	✔	
			Tween 80	0.5–1 mg/mL	Not compatible	
			Brij 35	0.5–1 mg/mL	Not compatible	
			Cremophor EL	1 mg/mL	Not compatible	
			Span 20	5 mg/mL	✔	
		4	FaSSIF[e]	–	✔	(Fischer et al., 2012)
	Permeation of calcein, electrical resistance	4.5	Vinylpyrrolidone/vinylacetate	–	✔	(Kanzer et al., 2010)
		5	Gelucire 44/14[a]	< 1 mg/mL	✔	(Fischer et al., 2011)
			Cremophor RH[b]	< 1 mg/mL	Not compatible	
			Solutol HS 15[c]	< 1 mg/mL	Not compatible	
			Triton-X	0.5 mg/mL	Not compatible	
			Poloxamer 188	10 mg/mL	✔	
PVPA_biomimetic	Permeation of calcein, electrical resistance	5	Ethanol	40 mg/mL	✔	(Naderkhani et al., 2014b)
			DMSO	40 mg/mL	✔	
			PEG 400	60 mg/mL	✔	
			Tween 80	0.5–1 mg/mL	(✔)[d]	
			Brij 35	0.5–1 mg/mL	(✔)[d]	
			Cremophor EL	5–40 mg/mL	(✔)[d]	
			Span 20	20 mg/mL	✔	
			Poloxamer 188	60 mg/mL	✔	
	Permeation of calcein, electrical resistance, phospholipid release	12	FaSSIF-v2[e]	–	✔	(Naderkhani et al., 2015)
			FeSSIF-v2[e]	–	✔	
Permeapad®	Permeation of calcein	5	Ethanol	40%	✔	(Bibi et al., 2015)
			DMSO	10%	✔	
			Tween 60	4%	✔	
			Tween 80	5%	✔	
			Cremophor EL	5%	✔	
			Sodium dodecyl sulfate	5%	✔	
			Triton-X	1%	✔	
			FaSSIF[e]	–	✔	
			FeSSIF[e]	–	✔	
			FaSSIF-v2[e]	–	✔	
			FeSSIF-v2[e]	–	✔	
AMI-system	Permeation of 14 low solubility compounds		FaSSIF[e]	–	✔	(Berben et al., 2018a)
			FaHIF	–	✔	

[a] Polyoxyl 32 glycerides.

[b] Polyoxyl 40 hydrogenated castor oil.

[c] Polyoxyl 15 hydroxystearate.

[d] Calcein permeability increased significantly, electrical resistance remained within the limits set which indicate maintenance of barrier integrity.

[e] Commercially available from *biorelevant.com*.

et al., 2016). Another research group reported that the pH at the surface of the epithelial cells is significantly lower (pH 5.5) than the pH of the bulk solution due to the presence of the mucus layer on top of the cells (Shiau et al., 1985). In order to reliably evaluate the permeation potential of drugs and/or the effect of pH on drug permeation, it is essential that the cell-free permeation systems are robust over this wide pH range. While cell monolayer assays tolerate only a narrow pH range (monolayer integrity tends to be compromised at pH values lower than 6.5), PAMPA and PVPA barriers have been proven to be stable in a pH range from 2 to 8. These systems are thus suitable as permeability model to evaluate segmental intestinal absorption as well as absorption from sites with a broad pH range (Bermejo et al., 2004; Flaten et al., 2006a; Wohnsland and Faller, 2001; Zhu et al., 2002). Using the PVPA, acidic and basic drugs also showed pH-dependent permeability according to the pH partition theory (Flaten et al., 2006a; Naderkhani

et al., 2014b). For example, permeation of acids is regularly underestimated when measuring the permeation *in vitro* at pH 7.4 only. Likewise, the Permeapad® barrier demonstrated to withstand the physiologically relevant pH range since the permeation of the non-ionisable compound hydrocortisone was unaffected at different pH values between 1 and 9 (di Cagno et al., 2015) and this barrier showed pH-dependent permeability for dissociating APIs (see also Section 5.3.).

4.1.2. Pharmaceutical excipients

Permeability is considered as a key parameter to evaluate the developability of NCEs. Hence, permeability screening is an integral part of drug discovery and/or early stages of drug development. During drug discovery, spiked dimethyl sulfoxide (DMSO) stock solutions are typically applied in high throughput screening, including permeation screening of NCEs. In early stages of drug development, high doses are

often required to assess the therapeutic safety margin, and the formulations used are relatively simple (*e.g. co*-solvent systems based on polyethylene glycol (PEG) 400). Since most of the cell-free permeation assays discussed so far contain lipid components, co-solvents represent a challenge for permeation barrier integrity due to potential extraction of the barrier's lipidic constituents (Flaten et al., 2008). Robustness of the permeability barriers against the co-solvents used should thus be established to obtain valid permeation data in the presence of co-solvents. During later development phases, the selection of the best formulation strategy for a drug candidate is in focus. Especially poorly soluble drugs require advanced formulations (*i.e.* absorption enabling formulations), often containing solubilizing excipients, to render them bioavailable. In order to rank the performance of the formulations, permeation tools are increasingly employed in recent years as they may allow to rank various pharmaceutical formulation concepts according to their ability to enhance bioavailability (Buckley et al., 2013). Generally, the ranking of different formulations according to their *in vitro* (permeation) performance is desirable to minimize animal usage. However, it should be noted that solubilizing excipients such as surfactants or lipids represent a challenge for permeation barrier integrity due to potential dissolution and/or emulsification of the barrier's lipidic constituents. Additionally, cyclodextrins represent a challenge for artificial barriers containing cholesterol, which can be employed as stabilizing barrier component, as β-cyclodextrins are able to chelate cholesterol (Zidovetzki and Levitan, 2007). Robustness of the employed permeability barriers against co-solvents and solubilizing formulation additives should thus be thoroughly established.

The robustness of PAMPA towards pharmaceutical excipients could be highly different across the multiple variants of PAMPA due to differences in composition of the barrier. By determining the permeability of Lucifer Yellow in the presence and absence of additives, Liu et al. reported that a PAMPA barrier consisting of a hydrophobic filter impregnated with 2% dioleoylphosphatidylcholine in n-dodecane maintained its integrity in the presence of up to 5% ethanol and 5% of the non-ionic surfactants polysorbate 80 (Tween 80) and polyoxyl 23 laurylether (Brij 35), respectively. On the other hand, PAMPA barrier integrity was compromised in the presence of 0.5% ethoxylated castor oil (Cremophor EL) (Liu et al., 2003). Sugano et al. investigated the effect of co-solvents on a biomimetic PAMPA model to determine the optimal conditions for the prediction of oral drug absorption (Sugano et al., 2001). Here, biomimetic refers to the (phospho)lipid solution containing 0.8% phosphatidylcholine, 0.8% phosphatidylethanolamine, 0.2% phosphatidylserine, 0.2% phosphatidylinositol and 1% cholesterol (total lipid content 3%) in 1.7-octadiene used for filter impregnation, which should reflect the (phospho)lipid composition found in the intestinal brush border (Proulx, 1991). By measuring the appearance of the non-permeable marker Trypan Blue in the acceptor compartment, the maintenance of integrity of the biomimetic PAMPA barrier was demonstrated in the presence of up to 30% ethanol, DMSO or PEG 400 (Sugano et al., 2001). It is well established that cholesterol has a stabilizing effect on phospholipid bilayers and vesicles as cholesterol is known to increase the rigidity of the bilayer (Brandl, 2001). Thus, it may be hypothesized that the comparatively high cholesterol content (1/3 of lipids) may aid PAMPA barrier stability in the presence of co-solvents at this relatively high concentration.

To the best of our knowledge, no specific stability data in the presence of co-solvents or excipients are available in literature for PAMPA-DS employing a surfactant to create sink conditions. However, the effect of excipients on the permeability of eight sparingly soluble compounds was studied using PAMPA-DS. The excipients studied included 2-hydroxypropyl-β-cyclodextrin, sodium taurocholate and the co-solvents 1-methyl-2-pyrrolidone, propylene glycol, and PEG 400. Stability issues, based on drug permeation and membrane retention, were not addressed with the exception of sodium taurocholate which appeared to compromise PAMPA-DS barrier integrity at a concentration of 15 mM (Bendels et al., 2006). Using the PAMPA model, Avdeef et al. assessed

the impact of single excipients including solubilizing agents, co-solvents and organic compounds on the absorption potential of test compounds and concluded that the presence of an excipient mainly decreased permeability, but most often not by the same extent as solubility was increased (Avdeef et al., 2008).

With a markedly reduced organic solvent content as compared to the original PAMPA models, the barrier of the Corning® Gentest™ pre-coated PAMPA plate system consists of a PVDF filter and phospholipid-hexadecane-phospholipid tri-layers dissolved in the volatile solvent hexane (Chen et al., 2008). By determining the permeation of 8 compounds, including high and low permeability compounds, in the presence of 10% methanol, the barrier integrity of the pre-coated PAMPA model was maintained as only slight increases in permeability for highly permeable compounds and no increase in permeability for low permeability compounds were observed. In the presence of 20% methanol, a noticeable increase in permeability for low permeable compounds was observed implying that the integrity of the artificial barrier was impaired. Moreover, barrier integrity was completely lost in the presence of 30% methanol as permeability values were dramatically increased (Chen et al., 2008). According to technical information from the manufacturer, the pre-coated PAMPA model is also compatible with 5% DMSO, 10% acetonitrile and other organic solvents.

Furthermore, the pre-coated PAMPA plate system was used to conduct a head-to-head comparison of different solubility-enabling formulations of the poorly soluble anticancer drug etoposide (Beig et al., 2015). The etoposide formulations tested were solutions of hydroxypropyl-β-cyclodextrin, sodium dodecyl sulfate (SDS) and PEG 400 as well as a co-povidone based amorphous solid dispersion (ASD). Beig et al. showed that formulations reducing the free fraction of etoposide lead to a concomitant decrease in etoposide apparent permeability. In a later study investigating the solubility-permeability interplay of etoposide, Beig et al. used the same PAMPA model to investigate the permeability for etoposide formulated as D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) solution compared to a polymer-based etoposide ASD. Again, it was demonstrated that TPGS-based formulations resulted into a concomitant decrease in *in vitro* etoposide permeability (Beig et al., 2017b). The same PAMPA model was used to evaluate the performance of rifaximin ASDs whereby higher concentrations were translated into a higher flux due to lack of the solubility-permeability interplay (Beig et al., 2017a). It should be mentioned that, in none of these studies, barrier compatibility with the tested excipients was explicitly tested. However, the effects of surfactants on the integrity of artificial barriers should be carefully considered to obtain unbiased results as observed effects on permeability in the presence of these compounds may result not only from (micellar) complexation (solubility-permeability interplay), but also from potential changes of the membrane properties. According to technical information by the manufacturer, surfactants may potentially disturb integrity of the pre-coated hexane-based PAMPA membrane. This is in agreement with results from Könczöl et al. and Müller et al. who argued that hexane-based PAMPA barriers may be more susceptible for barrier instability than n-dodecane-based PAMPA barriers in harsh environments (Könczöl et al., 2013; Müller et al., 2015), which could possibly be explained by the more volatile properties of hexane.

In the case of the original PVPA, it was found that the integrity of this barrier did not seem to be influenced by 40% of PEG 400, 20% of ethanol or 20% DMSO (Flaten et al., 2007, 2008). Also Poloxamer 188 did not affect barrier integrity in all the tested concentrations (up to 50%) (Fischer et al., 2011). Conversely, Brij 35, Tween 80 and Cremophor EL were found to be incompatible with the model at all concentrations as the barriers became leaky (Flaten et al., 2008). Furthermore, the original PVPA model has been used to study different solubility-enabling formulations. Kanzer et al. tested three prototypes of melt extrudates containing the poorly water-soluble drugs HIV-PI 1 and HIV-PI 2 (Kanzer et al., 2010). All formulations contained a vinylpyrrolidone/vinylacetate copolymer matrix supplemented with

different surfactants (sorbitan monolaurate, Cremophor EL and propylene glycol laurate). The integrity of the barriers were investigated in the presence of the vinylpyrrolidone/vinylacetate copolymer matrix alone, placebo versions of the three formulations as well as the drug-containing formulations (Kanzer et al., 2010). Except for the Cremophor EL containing solid dispersion, no influence on the integrity of the PVPA barriers was observed according to calcein permeability and electrical resistance measurements for up to 3 h. In other studies, different drug containing liposome formulations composed of phosphatidylcholine or a combination of phosphatidylcholine and phosphatidylglycerol have been investigated using the PVPA (Falavigna et al., 2018; Naderkhani et al., 2014a). These formulations were further PEGylated or coated with Carbopol or chitosan. When using electrical resistance measurements, none of the tested formulations appeared to impair the integrity of the barrier. In comparison with the original PVPA, the biomimetic PVPA demonstrated to be more robust (Naderkhani et al., 2014b), since its barrier integrity was not disrupted by any of the tested surfactants and co-solvents, not even in the presence of 0.5 mg/mL Triton X (originally included as a positive control).

Given the fact that the biomimetic membrane Permeapad® consists of one or more layers of (phospho-)lipids sandwiched between two polymeric supports, the (phospho)lipid layer is separated from the surrounding media. Plausibly, this may aid to maintain the barrier integrity in harsh environments. As such, Bibi et al. have investigated the integrity of the Permeapad® barrier in the presence of various (co)solvents and surfactants by determining the permeability for calcein over the Permeapad® barrier using both a Franz and side-by-side diffusion cell set-up for a period of 5 h (Bibi et al., 2015). Results demonstrated that Permeapad® was compatible with at least 10% DMSO and 40% ethanol. In addition, integrity of Permeapad® was investigated for several surfactants including Cremophor EL (0.1–5%), polysorbate 60 (Tween 60, 2 and 4%), Tween 80 (0.05–5%), SDS (0.1–5%) and Triton-X (0.5 and 1%). Using Franz diffusion cells, the Permeapad® barrier appeared to be compatible with all tested surfactants except for Cremophor EL (slight increase in calcein permeability), which also compromised the integrity of the original PVPA (Flaten et al., 2008) and PAMPA barrier (Liu et al., 2003). To investigate whether the increased calcein permeability in the presence of Cremophor EL was caused by barrier instability or other effects, explorative barrier functionality/stability studies were carried out using another set up (side-by-side diffusion cells instead of Franz diffusion cells). To this end, a calcein permeation experiment in the presence of Cremophor EL was performed for 5 h, followed by washing the Permeapad® barrier with PBS and a new calcein permeability experiment in the absence of the surfactant showed unchanged permeation properties (Bibi et al., 2015).

4.1.3. Biomimetic media

When considering oral drug administration, the influence of gastrointestinal colloidal structures on the absorption of especially poorly soluble drug compounds is well recognized. In recent years, biomimetic media, *i.e.* media mimicking the composition of human intestinal fluid in different prandial states containing bile salts, phospholipids and/or fatty acids, have become increasingly popular in dissolution testing (Galia et al., 1998; Mann et al., 2017; Wagner et al., 2012) and subsequently also in permeation testing (Ingels et al., 2004; Wuyts et al., 2015b, 2015a). Bile salts, fatty acids and other compounds present in the gastrointestinal environment are known to be detrimental for Caco-2 cell monolayers and may potentially compromise the integrity of permeation barriers containing lipid constituents as these surfactants may lead to emulsification. Also (pancreatic) enzymes able to cleave (phospho)lipids into digestion products (fatty acids, mono-/di-glycerides, lyso-phospholipids) are considered as an additional threat to the permeability barriers. Obviously, prior to prediction of oral drug absorption, compatibility of the permeability barrier with intestinal structures including bile salts, (phospho)lipids, (pancreatic) enzymes, and digestion products should be carefully investigated.

With regard to compatibility of PAMPA with (simulated) intestinal fluids, Markopoulos et al. validated PAMPA-DS barrier integrity by determining the permeability of Lucifer Yellow in the presence and absence of simulated intestinal fluids (Markopoulos et al., 2013). The authors observed that the PAMPA-DS barrier retained its integrity for at least 20 h when fasted or fed state simulated intestinal fluid version 2 (FaSSIF-v2 or FeSSIF-v2, respectively) were used as donor medium. Furthermore, PAMPA-DS barrier integrity was maintained for up to 8 h in the presence of aspirates from the upper small intestine collected from healthy volunteers at various time points after the administration of a heterogenous danazol and olive oil containing meal. However, PAMPA-DS barrier was stable for only 40 min in the presence of the micellar phase of aspirates (supernatant of ultracentrifuged aspirate). While the bile salt content in this supernatant was in the same order of magnitude as in the aspirates, the content of lipidic constituents was reduced considerably. It was hypothesized that emulsification of PAMPA barrier constituents by bile salts was prevented by encapsulation of bile salts in coarse lipid particles (Markopoulos et al., 2013). Analogous findings were obtained in a Caco-2 experiment utilizing FeSSIF as donor medium (Patel et al., 2006). Here it was found that, by reducing the bile to lecithin ratio, compatibility of the Caco-2 permeation assay with a modified FeSSIF medium could be achieved.

Also the original PVPA was demonstrated to be compatible with FaSSIF while the integrity towards the more challenging FeSSIF has never been tested (Fischer et al., 2012). However, the biomimetic PVPA barrier maintained its integrity in the presence of both FaSSIF-v2 and FeSSIF-v2 implying that the intestinal environment could be more closely mimicked during permeability estimations compared to the original PVPA (Naderkhani et al., 2015). The compatibility was demonstrated using permeability for the hydrophilic marker calcein as well as electrical resistance across the barriers.

The Permeapad® barrier demonstrated to be compatible for 5 h with FaSSIF, FaSSIF-v2 and FeSSIF-v2; however, calcein permeability was significantly increased in the presence of FeSSIF(-v1), which could possibly be attributed to an increased osmolality of FeSSIF in comparison to the other simulated intestinal media. Nevertheless, after removing FeSSIF, permeability of calcein (now dissolved in PBS), was no longer significantly increased and thus compatibility established (Bibi et al., 2015). Moreover, Permeapad® is the only artificial barrier system that has been used under lipolytic conditions for *in vivo* relevant formulation evaluation so far. More specifically, the performance of self-nanoemulsifying drug delivery systems (SNEDSS) of cinnarizine was investigated using the Permeapad® barrier (Bibi et al., 2017). Validating studies using calcein showed that the Permeapad® barrier maintained its integrity in the presence of lipolysis medium containing a pancreatic extract with a lipase activity of 600 USP/mL over a period of 4 h. Additionally, the Permeapad® barrier was found compatible with self-emulsifying drug delivery systems consisting of oleic acid, sesame oil, ethanol, Cremophor EL and Brij 97. Barrier integrity was even maintained in the presence of both lipolysis medium and SNEDDSs allowing to reliably investigate lipolysis and permeation in parallel (Bibi et al., 2017).

Overall, it can be concluded that the holding time for the different biomimetic barriers in the presence of excipients and/or colloidal structures may vary widely, implying that the time window to perform the permeation experiments should be selected properly.

4.2. Importance of stirring and sink conditions

The unstirred water layer (UWL) is generally considered as an additional permeation barrier in *in vitro* assays, mainly hampering the permeation of lipophilic compounds. While the UWL is relatively small (30–100 μm) in the human intestinal tract due to relatively high motility, values of > 2000 μm are frequently reported for *in vitro* assays (Lennernäs, 1998). For example, the calculated thickness of the UWL adjacent to Caco-2 cell monolayers amounted to 2000 μm while values

up to 3800 μm have been estimated in PAMPA assays (Hidalgo et al., 1991; Nielsen and Avdeef, 2004). However, several research groups have demonstrated that by increasing the stirring speed of the permeation set-up, the thickness of the UWL could be reduced to the *in vivo* range (Avdeef et al., 2004; Hidalgo et al., 1991). Hydrodynamics also appeared to be important when studying permeation using the AMI-system: the apparent permeability coefficient for 11 out of 14 compounds was clearly higher when applying stirring (300 rpm *versus* no agitation); no further enhancement was observed upon increase of agitation to 700 rpm (Berben et al., 2018a). Conversely, PVPA differs from other permeability models in the sense that mechanical agitation did not result into increased permeability, not even for highly lipophilic drugs such as testosterone. This might be explained by the structure of the PVPA barriers which consist of liposome filled filter pores together with a layer of liposomes on top of the filter (Flaten et al., 2006a); inside the liposomes, there are aqueous compartments that will not be affected by the stirring of the system. As a result, even though a wider range of permeability values could be observed for drugs with human fraction absorbed above 90%, permeability data obtained without the use of agitation already correlate well with the fraction absorbed in humans after oral administration, also for the highly lipophilic compounds (Flaten et al., 2006b; Naderkhani et al., 2015).

Along with an adequate agitation during (non)-cell-based transport experiments, sink conditions are often maintained by including a surfactant-based solution in the acceptor compartment to avoid back flux of permeated drug. For instance, TPGS is often added as micelle-forming agent to entrap the permeated drug. In addition to the use of surfactants, sink conditions can also be created by applying serum proteins (*e.g.* bovine serum albumin (BSA)) or by frequently moving the permeation barriers to fresh acceptor medium. Moreover, surfactants and BSA in the receiver compartment are also beneficial to minimize drug adsorption to the permeation device avoiding loss of drug and a biased assessment of drug permeation.

All currently employed geometries have a relatively small permeation area in relation to the volume of the donor phase. The physiological area-to-volume ratio is estimated to be between $1.9\,cm^{-1}$ and $2.3\,cm^{-1}$ (Mudie et al., 2012). In contrast, the area-to-volume ratio of most permeation setups described in literature is $< 0.5\,cm^{-1}$. Accordingly, only a very minor fraction of the drug permeates within physiological relevant time frames, *i.e.* steady-state permeability values are reported by such approaches. Such steady-state permeability values have found widespread use within predictive pharmacokinetic modelling although they do not capture the potential mutual influence between dissolution and permeation, as it is expected to occur *in vivo*, especially with poorly soluble drugs and enabling formulations. The use of permeation screens within combined dissolution-/permeation-approaches and adequate geometries employed for such type of studies are discussed in more detail in the next chapter.

4.3. Integration of cell-free permeation systems in dissolution testing

Pharmaceutical excipients and/or concomitant food intake may lead to an increase of intraluminal concentrations of poorly water soluble drugs. Over the past two decades, a range of cellular dissolution/permeation systems have been designed to simultaneously evaluate the impact of pharmaceutical excipients and/or food components on dissolution and permeation behavior of drugs (*e.g.* dissolution/permeation system using a Caco-2 cell monolayer) (Kataoka et al., 2003). Due to the inherent poor robustness of cell monolayers against formulation excipients and/or food (digestion) products, cell-based systems are limited in their applicability for formulation evaluation. Since the robustness of cell-free permeation systems against harsh intestinal conditions is thoroughly validated and described in literature (cfr. 4.1), cell-free permeation systems are very useful alternatives to integrate a permeation compartment into formulation testing (Buckley et al., 2012).

As such, Lovering and Black already pointed out in their pioneering work that polydimethylsiloxane dialysis membranes are useful to determine the permeable fraction during a dissolution experiment and may thus help to predict intestinal absorption for a series of drugs (Lovering and Black, 1973). An early use of a cell-free permeation screening includes PVPA for surfactant-based formulations (Fischer et al., 2011). Furthermore, a modified version of the original PVPA has been successfully used in a combined dissolution/permeation system for testing of solid dosage forms. Permeation data from both fast and slow releasing tablet formulations showed an excellent correlation with a similar system using Caco-2 cells as intestinal permeation barrier (Gantzsch et al., 2014).

Sironi et al. used a pair of side-by-side diffusion cells separated by a hydrophilic cellulose hydrate membrane to predict the gastrointestinal and absorptive behavior of two commercial fenofibrate formulations by measuring dissolution and permeation simultaneously for raw fenofibrate powder, a nano- and a microparticle formulation (Sironi et al., 2017b). When using phosphate-buffered saline (PBS), the fenofibrate permeation from the different formulations increased according to the dissolved concentrations measured in the donor compartment: fenofibrate powder < microparticles < nanoparticles. The same rank order was observed when comparing the flux values using biomimetic media (FaSSIF and FeSSIF) as donor solvent system. However, permeation rate was unaffected when comparing different donor media (PBS, FaSSIF or FeSSIF) implying that micellar solubilization by FaSSIF- or FeSSIF-micelles did not have a significant impact on the permeation rate.

Recently, Bibi et al. evaluated the feasibility of combining lipid digestion (lipolysis) and *in vitro* permeation using Permeapad® as permeation barrier for a lipid based formulation of cinnarizine (Bibi et al., 2017). Although the focus of this work was on the functional stability of the barrier during lipid digestion (cfr. 4.1.3), a significant change in drug permeation from SNEDDS was demonstrated when comparing *in vitro* permeation from the formulation as such with that obtained during digestion. These findings indicate that meaningful *in vitro* biopharmaceutical evaluation of lipid-based formulations requires a dynamic simulation of lipolysis and permeation.

In order to monitor donor drug concentrations during dissolution/permeation experiments, another technique has been suggested by Fong et al. and Koplin et al. (Fong et al., 2017; Koplin et al., 2017). As illustrated in Fig. 7A, the suggested microdialysis-dissolution/permeation (M-D/P) system is intended for the biopharmaceutical assessment of oral drug formulations. The M-D/P system consists of a side-by-side diffusion chamber, a commercial microdialysis probe submersed into the dissolution chamber for continuous sampling, and the biomimetic Permeapad® barrier separating the dissolution compartment from the acceptor compartment. The concentration of molecularly dissolved drug in the dissolution compartment can be monitored over time using microdialysis, while the concentration of the permeated drug is followed by conventional sampling. In a proof-of-concept study, the applicability of the M-D/P system for slowly dissolving crystals of hydrocortisone solvate (HCS) in buffer and FaSSIF was evaluated. Compared to the traditional direct sampling from the donor compartment using filters of 0.1–0.45 μm pore size, microdialysis-sampling exhibited distinct advantages including (i) minimal disturbance of the interactive dissolution/permeation-process, (ii) discrimination between "molecularly" dissolved drug (which is considered as the free drug concentration) and micelle-associated dissolved drug (in the case of FaSSIF), and (iii) close to real-time monitoring with probe-equilibration times of 10 min or less. The authors concluded that the M-D/P system allows a comprehensive evaluation of the dynamic interplay of dissolution and permeation, which is promising for oral formulation performance ranking and mechanistic studies.

In recent decades, dissolution/permeation models based on non-biomimetic barriers have been designed, like the TNO model of dynamic gastric and intestinal transit and absorption, which is being used for food and drug studies (Blanquet et al., 2004). Recently, the
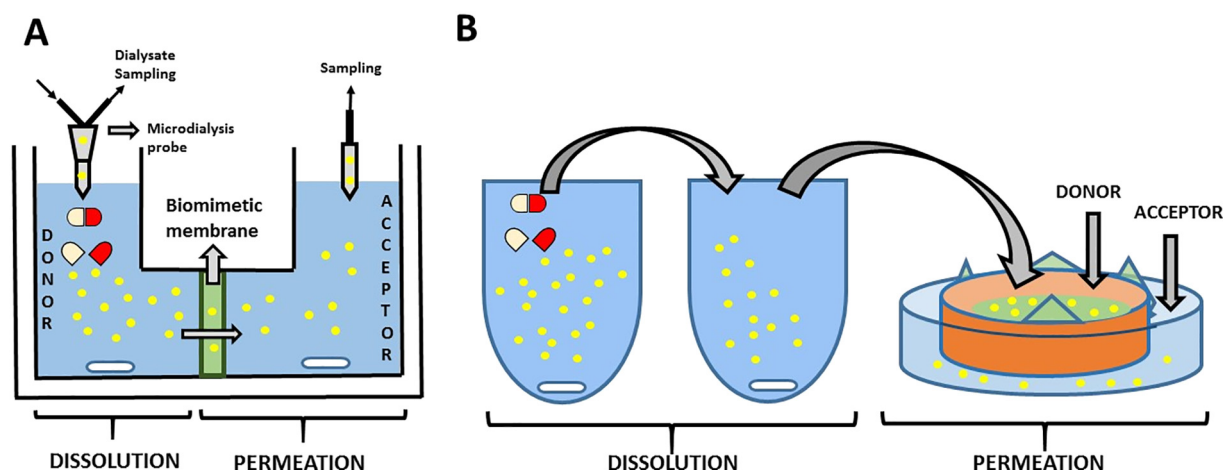
Fig. 7. Schematic illustration of (A) a microdialysis-dissolution-permeation system and (B) a two-stage dissolution test combined with a permeation compartment to evaluate dissolution and permeation simultaneously.

capability of the dialysis membrane-based AMI-system as predictive tool to explore the ranking performance of different absorption-enabling formulations was evaluated (Berben et al., 2018b). Prior to permeation assessment using the AMI-system, a two stage dissolution test was performed, as depicted in Fig. 7B, for three selected formulations for which *in vivo* intraluminal and systemic data were available. The acquired permeation data clearly demonstrated that the AMI-system enables to correctly predict the effects of (i) formulation pH on posaconazole dissolution and absorption (Hens et al., 2016), (ii) dilution on cyclodextrin-based itraconazole absorption (Berben et al., 2017), and (iii) food intake on fenofibrate absorption (Hens et al., 2015). Based on these data, simple dissolution testing combined with this newly developed permeation system appears suitable to be implemented as a time- and cost-effective tool in the early-stage evaluation of absorption-enabling formulations.

When combining dissolution testing with permeation studies, Buckley et al. suggested to investigate the kinetics of the interrelated dissolution and permeation processes (Buckley et al., 2013). In such scenarios, the donor concentration is subjected to continuous changes and the donor and acceptor profiles are dynamically inter-connected. For instance, slow dissolution of the drug or depletion of the donor compartment will result in non-steady state conditions. In view of the importance of balancing these processes in a predictive model, there is currently limited experimental evidence on the optimal geometry of such a combined dissolution/permeation setup in order to quantitatively predict the *in vivo* behavior of (enabling) formulations.

A recent study aimed at exploring the capabilities and geometrical constraints of a conventional side-by-side (Ussing chamber) dissolution/permeation setup, which was equipped with a biomimetic barrier (Permeapad®). In order to demonstrate the interplay between dissolution and permeation, a simplified model was constructed, where the dissolution rate was restricted on purpose and depletion in the donor chamber was monitored (Sironi et al., 2017a). Sironi et al. concluded that a substantial (> 10-fold) increase of the permeation area per donor volume ratio is needed to achieve a substantial decrease in donor concentration within a reasonable period of time. However, none of the setups described in literature have a donor (dissolution) chamber connected to a permeation barrier with a sufficiently high area available for absorption so that the permeation area to donor volume ratio is in the order of magnitude as described by Sironi and co-workers.

5. The usefulness of cell-free permeation tools in non-oral drug delivery studies

Since different tissues in the human body have different lipid compositions, and as the lipid composition in the cell-free permeation systems can easily be modified, several research groups have explored the ability of these permeation systems to investigate drug delivery *via* non-oral routes. Below, the implementation of the previously mentioned permeation tools in blood-brain barrier, transdermal, and buccal delivery is briefly discussed and schematically illustrated in Fig. 8.

5.1. Blood-brain barrier permeability

The blood-brain barrier is known as a highly selective barrier where the paracellular transport is negligible but uptake and/or efflux transporters affect permeation of drugs more dramatically as compared to the gastrointestinal tract. By adjusting the lipid composition of the original PAMPA intended to evaluate gastrointestinal drug permeation, a blood-brain barrier specific PAMPA (PAMPA-BBB) using porcine brain lipid extract (PBLE; 20 mg/mL) in n-dodecane was proposed (Di et al., 2003). PBLE consists of phosphatidylethanolamine (33.1%), phosphatidylserine (18.5%), phosphatidylcholine (12.6%), phosphatidic acid (0.8%) and phosphatidylinositol (4.1) supplemented with cerebrosides, sulfatides and pigments. In this study, only drugs that are substrates for active uptake and efflux transporters were misclassified
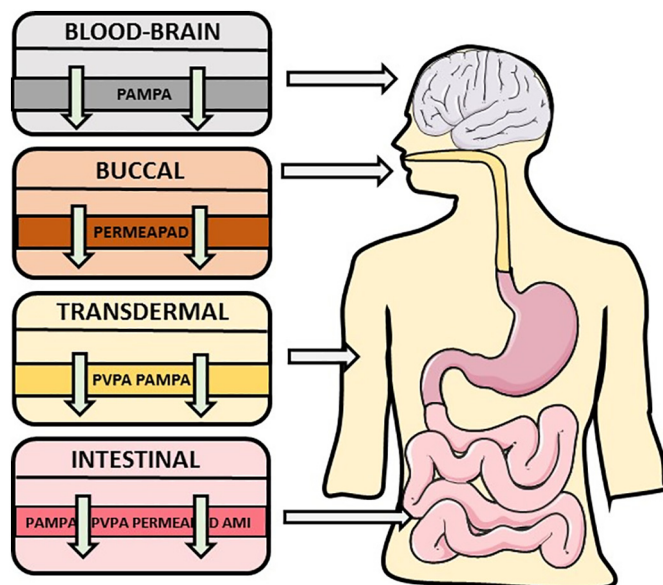

Fig. 8. Overview of the applications of the different cell-free permeation systems in (non)-oral drug delivery.

while, classification of passively transported drugs was correct for a set of 25 compounds (Di et al., 2003). Also Mensch and co-workers observed that PAMPA-BBB enabled to accurately predict blood-brain barrier permeability for 19 structurally diverse, commercially available drugs, except for caffeine and verapamil which are known as substrates of an uptake and efflux transporter, respectively. Nevertheless, in combination with a model covering active uptake and/or efflux such as the Caco-2 assay, this PAMPA assay is highly useful as a first method to discard molecules with a low ability to cross the blood-brain barrier (Mensch et al., 2010).

5.2. Transdermal delivery

Most of the artificial models used to mimic healthy, and to lesser extent compromised skin, are relying either on phospholipid mixture models or various types of diffusion cells. Several attempts have been made to develop (i) chromatographic methods, (ii) non-lipid based models like the silicone membranes (Ottaviani et al., 2006), and (iii) lipid based models like PVPA and PAMPA as simple alternative models for predicting dermal absorption (Flaten et al., 2015).

Sinko et al. reported the development of the skin-PAMPA consisting of synthetic certramides, together with cholesterol, stearic acid and silicon oil (Sinkó et al., 2009, 2012). The skin-PAMPA exhibited poor correlation with skin epidermis; however, a good correlation with full thickness skin was observed (Sinkó et al., 2012). The permeability data of a selection of drugs solubilized in different vehicles in the skin-PAMPA model was compared with results from other artificial models as well as porcine skin. The results demonstrated that the highest correlation was observed for the skin-PAMPA, which ranked four out of six vehicles correctly (Karadzovska and Riviere, 2013).

Originally, as thoroughly discussed above, PVPA was introduced as a screening model for intestinal permeability, but by changing the lipid composition of the liposomes used to produce the permeation barrier, the PVPA model could be applied to predict transdermal absorption (Engesland et al., 2013; Flaten et al., 2006b). The first PVPA model mimicking the stratum corneum barrier of the skin was introduced by Engesland et al.; thereafter, several modified versions have been released (Engesland et al., 2013, 2015, 2016; Ma et al., 2017; Palac et al., 2014; Zhang et al., 2017). In general, two main categories of the PVPA model for estimating skin penetration have been presented: the simple skin PVPA was prepared by liposomes of cholesterol and egg phospholipids (Engesland et al., 2013; Zhang et al., 2017), while the more complex skin PVPA contains all the main lipid classes that are present in the skin including ceramide, cholesterol, free fatty acid, cholesteryl sulfate, and egg phospholipids (Engesland et al., 2013; Ma et al., 2017). Evaluation of transdermal permeation using these barriers is usually performed in a 24 well plate format (Engesland et al., 2013, 2016; Palac et al., 2014) or in a Franz diffusion cell setup (Ma et al., 2017; Zhang et al., 2017). When comparing the permeability of a selection of drugs, a similar ranking was observed between the simple or more complex skin PVPA models on the one hand and animal skin penetration models or calculated *in silico* values on the other hand (Engesland et al., 2013). In addition, the complex skin PVPA was compared with the reconstructed human EpiSkin® model. The complex skin PVPA demonstrated the ability to distinguish between drug solutions and liposomal formulations, and was superior to the EpiSkin® in terms of ease of use, efficiency and cost-effectiveness (Engesland et al., 2015). In another study, Palac and co-workers used skin PVPA models to evaluate different liposome formulations containing diclofenac sodium, including conventional liposomes, deformable liposomes and propylene glycol liposomes (Palac et al., 2014). The permeation of the drug through the PVPA barriers was clearly affected by the lipid composition of the formulation and increased, as expected, in the presence of penetration enhancers (Palac et al., 2014). In addition, complex skin PVPA, containing all the classes of lipids found in the stratum corneum, allowed to explore the penetration enhancing effect of menthol and the underlying

mechanism (Ma et al., 2017).

Lastly, the barrier function of the skin PVPA models could be modified in a controlled manner allowing to investigate drug permeation through compromised skin with reduced barrier functionality (Engesland et al., 2013, 2016). Since these models have so far only been used to test drugs in aqueous solutions, their potential use in formulation optimization still needs to be evaluated.

5.3. Buccal delivery

Delivery of drugs through the buccal route is attractive because of ease of access and patient compliance. The oral, in particular sublingual, mucosa is well permeable because the epithelium is not keratinized and no tight junctions are present. Since the buccal barriers are well supplied with blood vessels whereby the drug molecules are directly released into the systemic circulation, this pathway benefits from circumventing harsh gastric and intestinal environments as well as first pass effect connected to oral administration. Evaluation of buccal drugs and formulations has classically been performed *in vitro* using cell-based models (in many cases TR146 cell type from human cancer) or *ex vivo* using animal tissues (*e.g.* from pig or rabbit, hamster pouch). Such studies are widespread because these tissues are relatively easily available. However, artificial membranes are attractive alternatives for evaluation of buccal permeation in high throughput formats as compared to cell-and tissue-based models as they are less laborious in preparation and are expected to give a more reproducible outcome since biological variation is excluded. Multiple studies describe the use of filter (*e.g.* cellulose acetate, cellulose nitrate) (Delvadia et al., 2012; Pongjanyakul and Suksri, 2009) and dialysis membranes (Dhiman et al., 2008) to evaluate buccal permeation by comparing with *ex vivo* permeation through porcine buccal mucosa or an oral transmucosal *in vivo* study. Khdair and co-workers reported that, using a variant of PAMPA comprising cellulose acetate-nitrate filters and cellulose acetate filters impregnated with L-phoSphatidylethanolamine in an octanol solution, linear relationships between the permeation of carvedilol through this artificial membrane and rabbit and porcine mucosa were observed (Khdair et al., 2013). Using Permeapad® as buccal permeation barrier with the same lipid composition as for the intestinal site, a recent study discussed the direct comparison for formulations of different pH values of metoprolol with previously published *in vitro*, *ex vivo* and *in vivo* studies. As such, permeability values obtained from Permeapad® showed excellent correlation (Pearson correlation values > 0.97) with *in vitro* TR146 cell culture and *ex vivo* porcine buccal mucosa in the Ussing Chamber setup; in addition, data were in line with the absolute bioavailability of metoprolol administered buccally to mini-pigs (Bibi et al., 2016). Overall, these studies indicate that cell-free permeation systems can be used to reliably predict buccal absorption in a faster and less expensive way as compared to cell- and tissue-based systems or *in vivo* studies.

6. Concluding remarks

During the past two decades, several artificial permeation models have been developed for the prediction of the passive permeability of various biological barriers. In addition to cutting costs of cell-and tissue-based permeation tools, cell-free permeation models also proved to be more robust against exogenous substances like pharmaceutical excipients and/or food (digestion) components. As a result, these systems allow formulation scientists to simultaneously investigate dissolution of the formulation of interest and drug permeation without compromising barrier integrity. While cell-free permeation systems were initially intended to predict intestinal drug permeation, their utility towards non-oral drug delivery including blood-brain barrier, transdermal and buccal delivery was also demonstrated in recent years by adjusting the composition of the permeation barrier.

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