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Instrumented In Vitro Approaches to Assess Epithelial Permeability of Drugs from Pharmaceutical Formulations

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Abstract The following chapter gives an overview of instrumented approaches to investigate the interactions of orally or pulmonary administered formulations with epithelial cell cultures in vitro. The first section is focused on the combined assessment of drug release/dissolution and subsequent absorption/permeation for solid oral dosage forms. Experimental approaches to mimic the complex physiologically surroundings in the gastrointestinal tract are presented as well as combinations of dissolution and permeation apparatus.

The second part describes different experimental approaches to simultaneously assess deposition and subsequent absorption of pharmaceutical aerosol formulations, typically by adapting some existing impactor/impinger devices to accommodate pulmonary epithelial cell culture systems. Differences between longtime and low-dose aerosol deposition in environmental toxicology and short time bolus inhalation of pharmaceutical aerosols are elucidated.

Keywords: Dissolution; Permeability; Dosage forms; Bioavailability; Blood–air barrier; Cascade impactor

Abbreviations

AIC	Air-interfaced culture
API	Active pharmaceutical ingredient
ASL	Alveolar surface liquid
AUC	Area under the curve
BCS	Biopharmaceutical classification system
BML	Bronchial mucus layer
CLSM	Confocal laser scanning microscope
CMC	Critical micellar concentration
FaSSIF	Fasted state-simulating intestinal fluid
FeSSIF	Fed state-simulating intestinal fluid
GI	Gastrointestinal
hAEpC	Human alveolar epithelial cells
HBSS	Hanks balanced salt solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IVIVC	in vivo/in vitro correlation

KBR	Krebs bicarbonate ringer buffer
KRB	Krebs ringer buffer
LCC	Liquid-covered condition
LID	Liquid-interfaced deposition
MES/HEPES	Morpholineethanesulfonic acid/HEPES
MSLI	Multistage liquid impinger
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromid
P_{app}	Apparent permeability coefficient
PEPT1	Di/Tri-peptide transporter 1
P-gp	P-glycoprotein
SEM	Scanning electron microscopy
SIA	Sequential injection analysis
TEER	Transepithelial electrical resistance
USP	United States Pharmacopoeia

19.1. Introduction

For most drugs, extravasal administration of the pure active pharmaceutical ingredient (API) to patients is not feasible mainly due to low dose/mass. Hence, drugs are formulated in order to facilitate dosing and to improve patient compliance. Furthermore, for various routes of drug delivery, the dosage form is a key factor determining in vivo bioavailability. However, only little is known about interaction formulated drugs and the several mucosal barriers in vivo. The following chapter comprises an overview of instrumented approaches which are assessing the interactions between formulated drugs and epithelial cells in vitro, with the aim of elucidating effects occurring in vivo.

The first section is focused on orally administered solid dosage forms, whereas within the second section, approaches for pulmonary administered aerosols are highlighted.

19.2. Intestinal Permeability of Drugs Delivered as Solid Dosage Forms

19.2.1. Rationale for Connecting Dissolution and Permeation Measurements

Release from the dosage form and subsequent permeation through intestinal epithelia are the two dominant processes in oral drug delivery, whereby each of both may be rate limiting. Unnumbered parameters affect these processes, such as subsequent wetting, swelling, and disintegration of the dosage form and dissolution of the active compound within the intestinal transit time, depending on the surrounding microclimate pH, osmolarity, and surface tension. While sufficient disintegration and dissolution are prerequisite for oral absorption, critical parameters can also be seen in the pH value of the gastrointestinal (GI) lumen and the cell covering, rather acidic mucus. Further factors include the apical concentration of drug, affinity to active cellular uptake or efflux systems, sink conditions in the trans-mucosal receptor fluid, concomitant administration of food, and disposability of active excipients, just to mention the most important ones. Summarized as L(iberation) and A(bsorption) of the LADME model,

both processes are influenced by and therefore relevant to the design of solid oral dosage forms.

Regarding dissolution testing, many successful efforts have been made to imitate the physiological surroundings in the GI lumen more and more closely. Variables such as temperature, osmolarity, pH, surface tension, and presence of bile salts were altered. As a result of the effort, two in vitro dissolution buffers coming most closely to in vivo conditions were reported as fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) by the group of Dressman [1]. Dissolution testing is routinely carried out using complete dosage forms, except for the assessment of intrinsic solubility at very early stages in the drug development process. In contrast, permeability testing is typically performed with nonformulated compounds, because it is considered as an intrinsic property of a given API. Hence permeability testing does usually address the role of formulation factors and excipients. A number of issues underpin this statement: (i) the dissimilarity between a normal dose of a solid dosage form and the permeation area of Caco-2 monolayers in a Transwell® system, potentially resulting in enormous osmotic pressure differences between apical and basolateral compartment; (ii) the sheer impossibility to insert a complete dosage form into a standard Transwell® device; (iii) nonappropriate dissolution features of the apical compartment (volume, pH, hydrodynamics, etc.). In conclusion, researchers tend to determine two related and connected issues—solubility and permeability—separately. Of course, when dissolution and permeation assays are to be connected, experimental and technical efforts will increase dramatically. Thus, the question raise for the benefits of the concomitant determination of dissolution and permeation that might justify such efforts. However, significant progress in the development of solid dosage forms by combined dissolution and permeation testing may be expected under the following aspects:

1 Improved in vivo/in vitro correlation for bioavailability screening of new APIs.
2 Improved screening of innovative formulation approaches.
3 Investigation of potential food effects on drug absorption.

19.2.1.1. Improved In Vivo/In Vitro Correlation for Bioavailability Screening of New APIs

As described by Artursson et al. [2], in vitro permeability of drugs through Caco-2 cell monolayers is correlating well with absorption in humans. However, pure permeation experiments disregard possible solubility issues of a compound that might potentially limit absorption from the gut. By combining dissolution and permeation assay, a deeper insight into the processes happening in vivo in the intestine may be given, especially, if one considers the clinical dose to be dissolved in the limited liquid volume of the intestinal fluid. Furthermore, dissolution testing is generally accepted as a validated tool for generating valid in vivo/in vitro correlations (IVIVC), but this works only for drugs where absorption is not limited by poor permeability, that is, classes I and II of the biopharmaceutical classification system (BCS).

However, for BCS class III (and IV), an in vitro/in vivo correlation of the dissolution result is often not possible, since dissolution is not rate limiting and other parameters cannot be detected in a dissolution setup. The latter therefore

appears possible only when both the dissolution process and the absorption process are considered.

19.2.1.2. Improved Screening of Innovative Formulation Approaches

Considering the LADME model, liberation and absorption of drugs pose crucial parameters for bioavailability after oral administration and are the only factors accessible for manipulation by designing adequate dosage forms. Evidently, detection of formulation parameters affecting dissolution processes is easier accessible, than effects of the formulation on absorption processes. Dissolution studies have been shown to be a good predictor for in vivo performance of solid dosage forms, for example, Refs. [1, 3, 4], under the assumption that the more compound gets into solution within an acceptable time in a more or less physiological surrounding, the higher the bioavailability and the better the in vivo performance. This central paradigm of predictive dissolution testing relies on the assumption that intestinal permeation is not rate limiting and that excipients administered along with the drug do not alter the permeability of intestinal epithelia. In contrast, during the past decades, more and more drugs have been recognized as absorbed by active cellular uptake mechanisms or hindered from absorption by active cellular efflux systems. Simultaneously, several excipients have been detected to interact with intestinal epithelia and have been discussed as intestinal absorption enhancers. The mini-review of Aungst [5] and the review of Swenson et al. [6] may serve as an overview on that particular topic. Terao et al. [7], for example, reported an increased AUC of furosemide in rats by decreasing intraluminal pH at distal portions of the GI by means of Eudragit L100-55, a proton-releasing polymer. For the influx transporter di/tri-peptide transporter 1 (PEPT1), a proton gradient coupled oligopeptide transporter, it has been reported that lower luminal pH values increase the driving force for the uptake, due to a secondary uptake coupled with a proton gradient. Accordingly, Nozawa et al. [8] found that by decreasing the pH in the lumen of rats' small intestines by means of proton-releasing polymers, the bioavailability of PEPT1 substrates, such as cefadroxil and cefixime, increases. Although the authors use remarkably high amounts of excipient (500 mg excipient per kg rat weight), a proof of principle that excipients can affect the active uptake of drugs in vivo was given. In addition to improving uptake, other excipients enhance oral absorption by means of interaction with the tight junctions of intestinal epithelia. Lindmark et al. [9] reported an increased oral absorption after addition of medium-chain fatty acids in the Caco-2 model. Medium-chain fatty acids affect membrane fluidity or interact with tight junction proteins such as occludin and increase in vitro permeability of the hydrophilic marker compound fluorescein-sodium. Sakai et al. [10] supported this theory by means of confocal laser scanning microscopy (CLSM) and by increasing permeability of hydrophilic, passively permeating compounds after coadministration of sodium caprate and sodium deoxycholate. Borchard et al. [11] reported that mucoadhesive polymers, such as chitosan-glutamate and carbomer, widen tight junctions following CLSM studies. However, only coadministration of carbomer together with chitosan-glutamate leads to significant increase in permeability. Ranaldi et al. [12] found that chitosan and other polycationic polymers decrease the transepithelial electrical resistance (TEER) reversibly and thus increase permeability of inulin across Caco-2 monolayers (for a review on that particular topic, see [13]).

On the other hand, several publications are suggesting that commonly used surface active excipients affect cellular efflux systems, namely, P-glycoprotein (P-gp) or other efflux pumps, for example, Refs. [5, 14–20]. Many authors report increased drug fluxes from apical to basolateral and decreased fluxes in the adverse direction in presence of polyethoxylated surface active excipients such as Tween 80, vitamin E TPGS, pluronic block polymers, and Cremophor EL. While the mechanisms of action are still under discussion, there is increasing evidence that the inhibition of apical efflux pumps by this class of excipients is determined by an interaction with the energy-providing ATPase rather than by effects on membrane fluidity or competitive interactions of substrate binding ([21]). The application of these excipients in oral solid dosage forms affects the absorption process from the GI lumen in two ways. First, they can enhance the dissolution rate by decreasing surface tension and improving wetting and hence increase the solubility by means of micellar entrapment. Second, they can increase permeability through intestinal epithelia by inhibiting P-gp-mediated efflux. However, these excipients can also decrease permeability, since micellar entrapped active compound may no longer be thermodynamically as active and lower fluxes may result. However, the findings reported in literature remain contradictory. Although statistically significant results in permeability assays have been found when adding these excipients in solution to the transport media, in vivo relevance remains questionable. Brouwers et al. [22] reported for amprenavir, being marketed as a dosage form (Agenerase®) with the P-gp inferring excipient vitamin E TPGS, a much more complicated behavior than generally anticipated. The authors showed that increasing concentrations of vitamin E TPGS lead to higher dissolution rates and solubility of amprenavir in vivo. The in vitro permeation experiment showed initially increasing and subsequently decreasing apparent permeability (P_{app}) values for increasing vitamin E TPGS concentrations, which can be due to micellar entrapment above the critical micellar concentration (CMC). However, at concentrations of vitamin E TPGS and amprenivir which are relevant in vivo, a P-gp-inhibiting action of vitamin E TPGS might be made irrelevant by the enormous luminal concentration of amprenavir as a result of the solubilizing effect of vitamin E TPGS. Ramsay-Olocco [23] published data on a clinical evaluation of a P-gp substrate formulated in soft capsules along with vitamin E TPGS. Although increase of flux in presence of vitamin E TGPS was observed in the absorptive direction in vitro, no significant difference was detectable in clinical studies, performed in mini pigs and humans. In contrast, Bogman et al. [24] reported for talinolol perfused intraduodenally along with vitamin E TPGS, a statistically increased AUC, compared to talinolol administered alone or with Poloxamer 188, a non-P-gp-interfering surfactant. To conclude, for formulation development, it is crucial to know the actual luminal concentration not only of the API but also of the active excipients. Beside the increase in bioavailability, researchers hope to use these excipients to lower the intersubject variability of bioavailability, normally associated with low-permeability and low-solubility compounds. Although being potent absorption enhancer in vitro, the in vivo relevance of these findings remains unclear. From a clinical point of view, the relative contribution of intestinal P-gp to overall drug absorption has been questioned, unless only a small amount of API is administrated or dissolution and permeation rate is rather low [25]. Additionally, it remains unclear, whether cell culture-derived in vitro data can be directly scaled up

to the human situation [25]. Obviously, the in vivo relevance of P-gp is not depending exclusively on the affinity of the API to cellular efflux systems, but also on the actual luminal concentration of the API and excipient.

In this context, a combination of dissolution and permeation assessment may be very valuable, especially when excipients interfere at different stages in a concentration-dependent manner. As shown above, a combined setup yielding physiological relevant concentrations may reveal these phenomena before testing the dosage form in animal and human studies. Moreover, a concept of combined dissolution/permeation might also be imagined to serve as a quality assurance tool for dosage forms containing active excipients.

19.2.1.3. Investigation of Potential Food Effects on Drug Absorption

Effects of concomitant intake of food with drugs on disintegration [26] and dissolution [14, 27–31] of oral solid dosage forms have been extensively described in the literature. For dissolution testing, the development of biorelevant dissolution media by Galia et al. [1], simulating intestinal fluid in fed (FeSSIF) and fasted state (FaSSIF), led to a generally accepted in vitro method to forecast food effects on dissolution. Nevertheless, effects of food components on absorption processes are getting more and more into the focus of research [14, 32]. Wu et al. [33] suspected that concomitant intake of high-fat meals with drugs is inhibiting intestinal influx and efflux drug transporters and thus may lead to decreased bioavailability of BCS class III compounds; however, due to increased solubility in the intestinal lumen after fatty meals, BCS class II compounds may show an increased postprandial bioavailability. Inhibition of intestinal efflux transporters may also be mediated by flavonols in grapefruit and orange juice, as stated in a review by Wagner et al. [14]. Ingels et al. [34] reported that the application of FaSSIF in Caco-2 permeability assessment does not affect the permeability of passively permeating compounds. However, actively secreted compounds show a significant lower permeability in basolateral to apical direction, whereas apical to basolateral transport is not affected and thus polarity in transports diminishes. Ingels et al. suggested that the bile salts present in FaSSIF may inhibit P-gp and other efflux transporters and concluded that the interferences between efflux pumps and food components which have been detected in vitro might be generally overestimated.

Consequently, the interplay between solubility and permeability with too many free variables is hindering the researcher or developer from clear statements. Interactions of food with oral absorption may happen at different stages, which again can be titled as dissolution and permeation, and a combined dissolution and permeation assessment might give a deeper insight into possible interaction and food effects.

To summarize, there are several rationales to justify the approach for permeation and dissolution testing. Nevertheless, it has to be pointed out that for the rather "uncomplicated" BCS class I and some BCS class II compounds, where it can be anticipated that increased luminal concentrations will lead to increased transepithelial fluxes, no significant additional information should be expected to the conventional dissolution testing. However, for "difficult" compounds, mainly belonging to BCS classes III and IV, important information about the drug in the dosage form can be obtained.

19.2.2. Connecting Dissolution and Permeation Measurement in One Instrumented Setup

19.2.2.1. Limitations/Specifications Concerning Dissolution/Permeation Media

The choice of dissolution/permeation media is limited, mostly due to the need to maintain viability and integrity of Caco-2 cell monolayers. Main requirements of the media to be in contact with Caco-2 cells are (i) isotonic conditions (Caco-2 cell monolayers are basolaterally more sensitive to osmotic pressure than apically); (ii) appropriate pH, which may be in a range from 5 to 8 [35], again this should be handled more restrictive basolaterally; (iii) presence of calcium and magnesium ions in order to maintain monolayer integrity by stabilizing adherens junctions; and (iv) physiological ratio between extracellular sodium and potassium, in order to warrant an appropriate potential difference across the epithelia. To circumvent these limitations, rendering dissolution media before getting into contact with Caco-2 cells may be appropriate. However, it has to be kept in mind that the original dissolution concentrations are diluted by doing so, and that the system is getting more and more complicated. Therefore, one has to find some compromise concerning the dissolution/permeation media between simplicity, in vivo relevance, and analytical requirements. For dissolution testing, numerous media as described in current pharmacopoeiae are generally accepted and mimic to some extent the physiological surroundings of disintegration and dissolution processes. For permeation assessment, however, it still remains unclear which transport media generates best results. Permeability assessment through Caco-2 monolayers is routinely carried out using Krebs Ringer buffer (KRB), Krebs bicarbonate Ringer buffer (KBR), or Hank's balanced salt solution (HBSS), though those might pose too restrictive conditions for low-soluble compounds, due to the lack of lipids and surface active ingredients and their excessive amount of counter-ions. A shortcoming for low-solubility compounds can generally be seen in the addition of either Dimethylsulfoxide or other cosolvents (a comprehend review of applied substance together with concentrations is comprised in [36]). For more comparable in vivo permeation conditions in Caco-2 assays, Ingels et al. [37] recommend to use FaSSIF on the apical side, while basolaterally an analytical-friendly buffer, such as HBSS, may be the best choice. Table 19.1 summarizes the composition of the previously mentioned buffer systems. Differences among KRB, KBR, and HBSS are not that distinct and the most important difference may be seen in the absence of carbonate in KRB. Degassing procedures prior to dissolution testing may affect the carbonate concentration and thus alter the pH. Therefore, KRB appears to be most suitable for combined dissolution/permeation measurement.

Ingels et al. tested FaSSIF and FeSSIF for compatibility with Caco-2 monolayer integrity, a prerequisite for valid permeation experiments [37]. Only FaSSIF turns out to maintain Caco-2 monolayer integrity throughout a 3-h experiment, as indicated by TEER measurements and flux studies using fluorescein-sodium. These data were confirmed by means of a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromid (MTT) cytotoxicity assay. FeSSIF is immediately toxic to the cell monolayer; Ingels et al [37] attribute this to the high osmolarity (≈600 mOsm), low pH (5.0), and the high content of Na-taurocholate (15 mM). In addition, the lack of glucose, and especially calcium

Table 19.1 Composition of various buffer systems possibly eligible for combined dissolution and permeation testing. Concentrations may vary from reference to reference. Composition of FaSSIF and FeSSIF is shown as reported by Galia et al. [3]; in the meantime, several publications denoted a change from potassium to sodium as the main cation [91] or others changes. Abbreviations are given in the text.

Compound [mM]	HBSS [36]	KRB [56]	KBR [38]	FaSSIF [3]	FeSSIF [3]	FaSSIF modified by [38]	FeSSIF modified by [38]
$CaCl_2$	1.26	1.41	1.20	–	–	1.67	1.67
Fumaric acid	–	–	5.40	–	–	–	–
KCl	5.36	3.00	4.69	103.27	203.86	5.37	5.37
$MgCl_2$	0.49	2.56	–	–	–	–	–
$MgSO_4$	0.41	–	1.20	–	–	0.81	0.81
NaCl	136.89	142.03	108.01	–	–	136.89	136.89
$NaHCO_3$	4.26	–	16.0	–	–	0.42	0.42
K_2HPO_4	–	–	0.47	–	–	–	–
KH_2PO_4	0.44	–	–	28.66	–	0.44	0.44
Na_2HPO_4	0.34	0.44	–	–	–	0.34	0.34
NaH_2PO_4	–	–	5.38	–	–	–	–
Na-pyruvate	–	–	4.90	–	–	–	–
D-Glu	25.00	4.00	11.50	–	–	5.55	5.55
L-Glutamine	–	–	5.67	–	–	2.00	2.00
MES/HEPES[a]	10.0/20.0	10.0/20.0	–	–	–	–	–
Acetic acid	–	–	–	–	144.17	–	–
Na-taurocholate	–	–	–	3.00	15.00	3.00	15.00
Lecithin	–	–	–	0.75	3.75	0.75	7.50
NaOH	ad 7.4/6.5	ad 7.4/6.5	ad 7.4	ad 6.5	ad 5.0	ad 6.5	ad 6.0
Osmolarity [mOsm/kg H_2O]	~ 300	~ 300	~ 300	~ 300	~ 630	~ 343	~ 336

[a] For resulting pH of 6.5: MES; for 7.4: HEPES; HBSS: Hanks balanced salt solution; KRB: Krebs ringer buffer; KBR: Krebs bicarbonate ringer buffer; FaSSIF: Fasted state-simulating intestinal fluid; FeSSIF: Fed state-simulating intestinal fluid.

and magnesium, may increase damage to the monolayers. Recently, Patel et al. [38] modified FaSSIF and FeSSIF in order to obtain Caco-2 compatible in vivo relevant transport media. Changes of the composition are outlines in Table 19.1. Briefly, magnesium and calcium salts are added, osmolarity of FeSSIF is reduced to 330 mOsmol/kg H_2O, sodium and potassium ions are present in a physiological ratio, lecithin concentration in FeSSIF is doubled, in order to mitigate toxicity of taurocholic acid and buffering phosphate, and carbonate salts are added. Caco-2 monolayers retain their integrity throughout 2 h indicated by mannitol flux and TEER. By means of scanning electron microscopy (SEM), no severe damage to the cells is detectable, except a minor microvilli loss and shortening. Apparent permeability of metoprolol is significantly reduced in the presence of FaSSIF/FeSSIF; the authors attribute that to micellar entrapment into the bile salt lecithin mixed micelles. Nevertheless, the influence of the modification of FaSSIF/FeSSIF on dissolution and solubilization properties of the media is not tested yet.

19.2.2.2. Historical Evolution of Combined Dissolution and Permeation Testing

First attempts to combine both dissolution and permeation in one device have been made in the mid 1970s. Typical devices from that era employed distribution between three phases, each of different polarity, that is, the distribution between the aqueous and lipoid phases and subsequently between the lipoid and the aqueous phases was measured. Both aqueous phases were kept apart by baffles. Some inevitable compromise resulted from the fact that the "membrane" (i.e., the lipoid phase) was much thicker than the in vivo membranes, and transport rates found in vitro appeared to be too strongly controlled by that. In 1969, Dibbern et al. [39, 40] circumvent this drawback by separating aqueous acceptor and donor phase by lipoid-treated polymeric membranes and thus were able to improve prediction of in vivo behavior of dosage forms containing passively permeating compounds (the apparatus became commercially available under the name "Resomat" of the company Desaga). However, the validity of the obtained data was limited due to the employment of artificial lipoid membranes and thus omitted conclusions concerning actively secreted or absorbed drugs and metabolism. In 1980, Koch et al. [41] reported a simplified experimental tool ("Resotest Apparatus"), again reviving the experimental determination of the partition between organic and aqueous phases. However, the apparatus was reduced to a rotating two-neck pear-shaped flask containing two aqueous phases conjointly covered with a layer of suitable organic solvent. Although being a crude simplification and exhibiting a nonphysiologic lipoid membrane, the model made it into the textbooks may be just because it was very easy to handle, due to its simplicity. However, none of these early dissolution-permeation devices were listed in the pharmacopoeiae. As computing power started to increase tremendously from the early 1980s, and computational modeling sciences emerged, those simplified models for dissolving and passively permeating substances became obsolete.

In the meantime, dissolution testing was more and more standardized and gained enormous impact on the fields of quality assurance and drug development in pharmaceutical industry. Several dissolution apparatus made their way into the pharmacopoeiae, such as the rotating paddle, rotating bas-

ket, reciprocation cylinder, and the flow through cell, just to mention the most important ones. For comprehensive discussion on dissolution equipment, the reader is referred to previously published books, a good example being Ref. [42].

At the beginning of the 1990s, the general acceptance of Caco-2 cell monolayers as a model of the small intestinal mucosa caused a significant push toward the field of permeability testing [2, 43]. For detailed information about the Caco-2 cell line, the reader is referred to Chap. 8. As a consequence, the pharmaceutical community became more aware that permeation through intestinal epithelia is oftentimes more sophisticated than mere diffusion through a lipophilic membrane.

In 1995, Amidon et al. [44] initiated the theoretical base for a biopharmaceutical drug classification, which was also addressed in several food and drug administration (FDA) guidelines [45, 46]. The BCS assigns drugs into classes I–IV according to their solubility and permeability properties, thus highlighting the importance of both fundamental parameters—dissolution and permeation. Nowadays, the BCS has gained tremendous impact on drug development process.

19.2.2.3. Published Approaches for Combined Dissolution and Permeation Assessment

Recently, an approach mimicking the GI tract and feasible for pharmaceutical dissolution studies has been published [47]. Coming from the area of nutritional research, this approach reflects some promising aspects, such as relevant luminal pH values, peristalsis, luminal bacterial colonization, and relevant fluid volumes. However, permeation is only reflected by diffusion through hollow fiber membranes and thus is not in the scope of this chapter. Nevertheless, it will be interesting to see whether it will be possible to expand such a perfect in vitro device toward a more realistic intestinal epithelium.

Several approaches combining Caco-2 permeation assessment with dissolution testing have been published in the past. In the following, these systems are described in chronological order of their publication.

Approach of Ginski and Polli [48]: In 1999, Ginski et al. [48] published a continuous dissolution/Caco-2 system. Dissolution takes place in a paddle apparatus (apparatus I, United States Pharmacopoeia, USP) and Caco-2 monolayers are mounted in a side-by side diffusion cell, whereas a peristaltic pump transfers media from the dissolution vessel to the permeation chambers. Dissolution and permeation media is HBSS (pH 6.8). The system (Figure 19.1a) can be seen as a closed model, since no drug was allowed to leave the experimental setup. The study had several objectives. The main objective was to test whether the experimental data fit to the previously described mathematical model determining the dissolution/absorption relationship [48, 49]. Another objective was to detect possible effects of the formulation (disintegrating agent, solubilizers, such as Tween 80 and cyclodextrins) on the dissolution/absorption relationship. Although being a rather simple experimental setup, the results were promising. Ginski et al. [48] were able to confirm the validity of their setup with in vivo data using piroxicam and ranitidine oral solid dosage forms with varying release kinetics. The addition of croscarmellose-Na as an in vitro permeation enhancing agent led to an increased permeated amount across Caco-2 cell monolayers, although in vivo no absorption

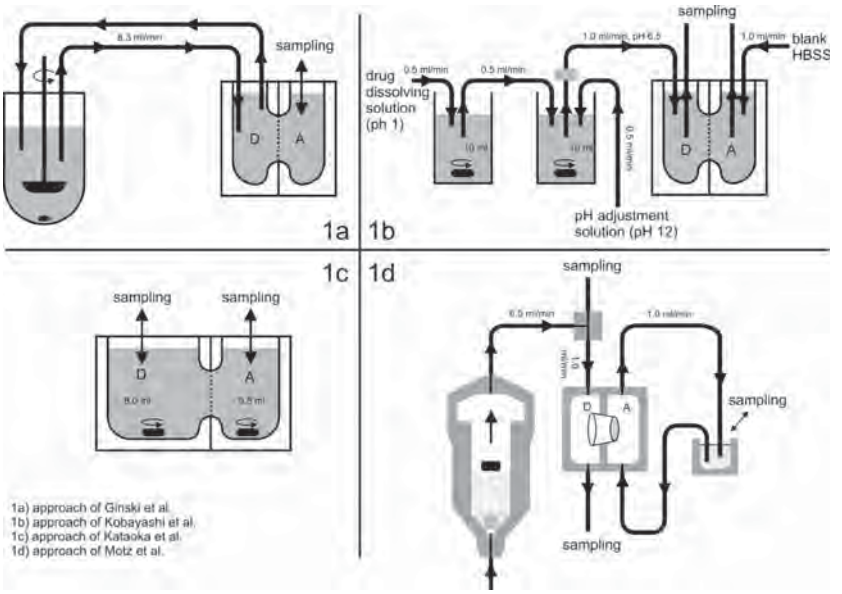

Figure 19.1 Schematic depiction of the published approaches for concomitant assessment of dissolution and permeation. Figure 19.1a adopted from Ref. [48], Figure 19.1b from Ref. [51], and Figure 19.1c from Ref. [54], respectively. Donor compartments are denoted as "D," acceptor compartments as "A." Doted lines between "D" and "A" represent Caco-2 monolayers. Flow rates are given where necessary.

enhancing effect of croscarmellose-Na was detected. This phenomenon was hence attributed to the calcium sequestering properties of croscarmellose-Na and was identified as a false positive in vitro finding, since complete sequestering of all extracellular calcium may not happen in vivo. For the variation of solubilizers the authors prove that only dissolution is altered, not permeation. Finally, the authors concluded that this continuous dissolution/Caco-2 may be a tool to forecasting formulation effects on in vivo dissolution and in vivo permeation.

Approach of Miyazaki et al. [50–53]: Shortly after Ginski et al. [48], Kobayashi and coworkers [50] published an open dissolution/permeation approach in 2001. A stirred glass vessel serves as a flow through dissolution device, from where the eluted solution is pumped into a second glass vessel for pH adjustment to a pH of 6.0. The solution is then transferred into a flow through diffusion chamber with a Caco-2 monolayer between the acceptor and donor compartments. A schematic drawing of the most recently published setup is shown in Figure 19.1b. The authors routinely carry out their experiment with an HBSS-like buffer, which is generated prior to contact of the buffer with the Caco-2 monolayers. The system is able to mimic effects of pH on the drug; however, the device is not capable of changing the pH acting on the drug in one experiment and thus one might argue that in vitro, the undissolved drug appears to stay in the gastric compartment rather than being transferred to the small intestine with its increasing pH values. This issue is almost circumvented in later publications by employing several gastric acid models [52]. Nevertheless, the authors are able to mimic precipitation of weak bases in the "pH adjustment vessel," such as for albendazol [53]. Furthermore, the

apparatus of Kobayashi et al. [50] and He et al. [51] shows a good linear relationship between the amount cumulatively permeated into the acceptor compartment and the fraction absorbed in humans, although clinical doses of the drugs are disregarded. Interestingly, the authors used this system also for assessing the metabolism of prodrugs to be cleaved in intestinal cells, for instance, ampicillin/pivampicillin. The experiments with either rat intestine or Caco-2 monolayers indicate that pivampicillin is undergoing cleavage during the permeation through the intestinal barriers and again the obtained values correlated well with in vivo data.

In comparison to the approach of Ginski et al. [48], the Miyazaki's method appears to be more elaborate and complex and is thus coming closer to the in vivo situation. The device can simulate various effects of pH on dissolution and is, as an open system, closer to in vivo conditions compared to a closed one. However, it exhibits the drawback of not freely "adjustable" pH values acting on the drug. Low flow rate in the dissolution vessel may limit applications of complete dosage forms and allows predominantly only the use of granules, pellets, or grinded tablets. Furthermore, the application of compendial dissolution devices appears to be a more robust approach.

Approach of Kataoka et al. [54]: In 2003, Kataoka et al. [54] published an in vitro system for simultaneous evaluation of dissolution and permeation of poorly water soluble drugs. In contrast to Ginski et al. [48], this system assesses dissolution and permeation in a downsized dissolution vessel (1% of the volume estimated in vivo, 8 ml) with a Caco-2 monolayer mounted between acceptor (5.5 ml) and donor compartments with 8 ml (Figure 19.1c). When reducing the volumes by a factor of ~100 compared to concentration of dissolution testing, the dose of the drug is also to be reduced by the same scale, which may raise some balancing and handling issues when adding 0.1 mg drug or even less to the dissolution compartment. In comparison to previously published papers, the authors highlight the importance of clinical doses of the compounds tested, as compounds are assigned into the four BCS classes according to their solubility of the highest dose in 250 ml [44]. The authors find a good correlation between the amount permeated after 120 min and the fraction absorbed in humans, whereas the 120-min time point was chosen with regard to the mean small intestinal transit time. The setup was also able to assign correctly all compounds into BCS classes I–III; however, the study was lacking any BCS class IV compound. Furthermore, experiments with apically added taurocholate (at 5 mM, thus, between FaSSIF and FeSSIF concentration) in the dissolution compartment showed increased permeated amount for griseofulvin, mainly due to higher dissolution rate and solubility. Moreover, the addition of 4.5% bovine serum albumine (BSA) in the acceptor compartment increased the permeation rate of griseofulvin, which may be attributed to increased sink conditions.

The approach of Kataoka et al. [54] shows some desirable features, such as downsized, in vivo relevant volumes, and low complexity of the device and thus low error proneness. However, the device lacks the possibility to add complete dosage forms and the use of compendial dissolution devices would be a more robust approach.

Approach of Motz et al. [55, 56]: In 2006, Motz and coworkers published a dissolution permeation device combining a compendial flow through dissolution

cell (apparatus 4, USP) with a flow through permeation cell. In order to connect both, a stream splitter providing appropriate low flow rates for the sheer stress sensitive Caco-2 monolayer was installed. The dissolution module was designed open, whereas the transmucosal receptor compartment was closed. Nevertheless, sink conditions were given for the published experiments. Several sampling ports were implemented, in order to monitor dissolution and permeation thoroughly: sampling port D (dissolution), shortly after apparatus 4; sampling port A (apical), after the stream splitter and after passage through the flow through permeation cell; and finally sampling port B (basolateral) in the acceptor compartment. As the apparatus is intended to assess permeability across Caco-2 monolayers after drugs being released from solid dosage forms, several propranolol HCl immediate release tablets with increasing dosage strength, and two different propranolol HCl extended release tablets, were manufactured and subsequently tested. Mean time approach for the different sampling ports D, A, and B yielded plausible and conclusive results for this setup. In addition, the stream splitter was found to work reliable. The permeated amount is linearly dependent on the dosage strength, expected for a passively permeating compound. For the extended release tablets, a delay in release of propranolol from the solid dosage form was observed in the permeation profile. Nevertheless, the cumulatively permeated amount was not statistically different for all the differently releasing tablets. Advantage of such a setup can be seen in the hydrodynamically separated modular setup providing the possibility to employ the dissolution module with compendial, sinusoidal flow profile generating pumps. A further advantage may be seen in the possibility to use complete solid dosage forms together with physiological flow rates and volumes in the dissolution module and thus facilitating the application of relevant concentrations. In addition, the three sampling ports allow thorough monitoring of both dissolution and permeation. After this preliminary testing of the apparatus, the time for quantification was found to be suboptimal. Therefore, the system got automated using sequential injection analysis (SIA) [55], the throughput is increased, and comparison of the results obtained with the automated setup reveal results similar to those from the nonautomated version. The automated setup allows online measurement at the three different sampling ports D, A, and B or partially online analysis in combination with high performance liquid chromatography (HPLC). The automation facilitates in the future more and faster experiments, as well as higher reproducibility of the measurement due to the less error sources.

The approach of Motz et al. [55, 56] can be seen as a combination of the approach of Ginski et al. [48] (compendial dissolution equipment) and the approach of Kobayashi et al. [50] (open dissolution module). The most dominant advantage may be seen in the application of complete dosage forms and the application flow rates resulting in physiologically relevant concentrations. Furthermore, the apparatus appears to be robust being equipped with compendial dissolution equipment. However, the apparatus is still lacking a pH simulation unit.

19.2.3. Critical Evaluation of the State of the Art and Further Needs

To connect two in vitro assays displaying fundamental processes occurring during oral absorption in vivo appears to be a promising approach. Such setups, as

they are already published, allow the researcher intense comprehension of the interplay of dissolution and permeation with surrounding media and excipients. Application of complete dosage forms bears the advantage to detect possible interactions of excipients with intestinal epithelia and might help to better understand and select promising formulation approaches in drug development. However, the mentioned apparatus still need improvements. Simulation of the pH profile in the human intestine has to be improved in order to be able to freely adjust the pH acting on the dosage form. In addition, devices have to be improved and tested, as well as media feasible for detecting possible food–drug interaction. Also, studies focusing on the detection of formulations effects as already started by Ginski et al. [48] have to be continued in order to finally disclose the value of the obtained data. For the future, assessment of permeation of solid oral dosage forms may lead to more realistic and more relevant devices, improving drugability of difficult drugs.

19.3. Permeability Assessment of Pulmonary Aerosol Formulations

The inhalation route is of general interest for the application of drugs, in order to treat systemic and local diseases [57–59]. Recent advances in the development of inhalation devices and particle technology allow to deliver small molecules as well as proteins and peptides with sufficient efficacy to the lung [60]. However, this approach is often limited by missing data regarding safety and efficacy of aerosol formulations. In particular, the lack of safety data is one reason that to date only few drugs and excipients are approved for pulmonary application by the regulatory authorities, and this situation decelerates the development of modern inhalable medicines. Innovative development tools must therefore acknowledge the complexity of the interplay between the lung, the inhalation maneuver [61], and pulmonary deposition, which is difficult to simulate in vitro.

Depending on particle characteristics and breathing pattern, aerosols are deposed in various regions of the lung. In the bronchial as well as in the alveolar region, the particles are settling on an epithelium, in both cases the main barrier for pulmonary drug absorption. Already back in the 1980s, attempts have been made to simulate the respiratory epithelia using isolated organs or organ slices [62]. However, these approaches have been limited by functional breakdown of the tissue [63], lacking reproducibility, and high costs involved [64]. Finally, progress in cell culture overcame these issues and led to standardized and validated models of the blood–air barrier. The continuously growing bronchial epithelial cell lines, Calu-3 [65] and 16HBE14o- [66], develop tight and polarized monolayers suitable for transport studies, when grown on filter inserts. For the alveolar region, up to now, no immortalized cell line with sufficient barrier properties is available. However, primary cell cultures isolated from different species [67], including human alveolar epithelial cells (hAEpC), are reported in literature [68–71].

Inspired by the successful implementation of the intestinal cell line, Caco-2, as a model to predict oral drug absorption, pulmonary-based models are used in a similar manner. The use of pulmonary cells which are covered by a comparatively thick fluid layer (liquid-interfaced deposition, LID) is probably

not adequate to model the blood–air barrier [72]. Structure and surface properties of the pulmonary surface liquid are locally different in the human lung. Because of difficulties of its preservation, only few facts are known about composition and especially thickness. The bronchial tree is comprised by a columnar epithelial cell lining. The airway epithelium consists in its majority of ciliated cells and interspaced goblet cells for the production of mucus. Mucus consists primarily of mucin, a highly glycosilated peptide. A bilayer structure with an aqueous sol phase of mucus adjacent to the epithelial cells that includes the beating cilia and an overlying more viscous gel phase of mucus covers all conducting airways. Particle embedding and subsequent particle clearance to the pharynx, wetting of the inhaled air, and temperature control are the three main functions of the bronchial mucus layer (BML). Its thickness has been reported to vary between 20 and 60 μm [73]. This means that particles in a size between 2 and 5 μm could be completely submersed in and would need to travel distances between 4 and 12 times of their diameter before reaching the actual epithelial cell membrane.

The situation is, however, different in the alveolar region of the lung where the respiratory gas exchange takes place. Its thin squamous epithelium is covered by the so-called alveolar surface liquid (ASL). Its outermost surface is covered by a mixture of phospholipids and proteins with a low surface tension, also often referred to as lung surfactant. For this surfactant layer only, Scarpelli et al. [74] reported a thickness between 7 and 70 nm in the human lung. For the thickness of an additional water layer in between the apical surface of alveolar epithelial cells and the surfactant film no conclusive data are available. Hence, the total thickness of the complete ASL layer is actually unknown, but is certainly thinner than 1 μm.

Particles in the typical size range of pharmaceutical aerosols (2–5 μm), when deposited in the bronchial region of the central lung, can be completely submersed in the BML where they either dissolve, are cleared by the mucociliary escalator, or eventually reach the apical membrane of the bronchial epithelial cells. On the other hand, when deposited in the alveolar region of the peripheral lung, particles of the same size will be wetted by the ASL, but the amount of liquid to dissolve them is much smaller than in the bronchial region. If particles deposited in the alveolar region dissolve in this limited volume of the ASL, the concentration gradient driving transepithelial absorption is much steeper than after bronchial deposition. In absence of the mucociliary clearance escalator in the distal region of the lung, nondissolved particles will interact with and perhaps penetrate the alveolar epithelial cells, if they are not rapidly cleared by alveolar macrophages.

Application of a given drug to an in vitro model should aim to mimic the physiological situation of drug delivery in vivo as closely as possible. Limitations, however, may be imposed either by biological demands of the cell culture systems or by technological challenges to realistically mimic the process of drug administration. This is particularly true in pulmonary drug delivery where the drug is administered as an aerosol and deposition and subsequent absorption occurs across epithelial cells which are covered by an aqueous film of perhaps less than 1 μm and not exceeding 100 μm on their surface. Therefore, it appears logical that an in vitro model should represent humid rather than submersed conditions.

19.3.1. Measuring Drug Transport Across Epithelial Barriers in Submersed Conditions: Ussing Chamber and Transwell®-Like Systems

The concept of characterizing epithelial barrier properties and transport processes was originally developed using tissues that are exposed to an aqueous environment at both the apical and the basolateral side. This is the physiologically normal situation for, for example, gall or urine bladder epithelium, intestinal epithelium, and the skin of amphibians. Because these tissues grow as sheets or tubes, it was relatively easy to isolate them from animals and mount such biopsies into appropriate apparatus to perform diffusion experiments. Typically, the epithelium acts as a diffusing barrier, its apical and basolateral side facing different compartments, which can be filled with buffer and will act as donor or receptor, when transport of a solute is measured from one compartment to the other. As reference to the pioneering work of Hans Ussing, who introduced the concept of bioelectrical measurements and transport experiments at epithelial tissues, such setups are often referred to as Ussing chambers, although their design might be quite different to the original [75].

The intention to study transport processes at pulmonary epithelia, however, raised two particular problems: (i) the apical side of these epithelia is typically in contact with air rather than with a liquid and (ii) in order to maximize the surface area, the lungs have a complex treelike structure, ending in millions of tiny alveolar bubbles. The total surface area of the human alveolar epithelium is almost half of that of the intestines (100–120 m^2), with its macroscopic appearance resembling a sponge, and it is virtually impossible to use such a tissue for transport experiments in a diffusion-chamber setup.

As a first compromise, scientists started to work with the lungs of amphibians, such as the bull frog, which consist of only one single alveolus that can be isolated and mounted between the compartments of an Ussing chamber. Experiments of that type provided important first information about physiological transport processes at the alveolar epithelium [76] and were also adopted by some pharmaceutical scientists to study pulmonary drug absorption. Yamamoto et al. [92], for example, investigated the permeability of insulin across Xenopus pulmonary membrane, using a modified Ussing chamber. Simultaneously, they measured the effects of various absorption enhancers, such as sodium caprate, on the membrane's integrity. While the cumbersome work with amphibian lung tissue was more and more abandoned, once methods became available to grow bronchial or alveolar epithelial cells on permeable filter inserts (such as Corning's Transwell® system or analogues of it), the Ussing chamber technique is still used by quite a few researchers to study drug transport processes at pulmonary epithelial barriers. In the same way as it is established working with Caco-2 cells, it is relatively straightforward to determine P_{app} coefficients of drugs or to evaluate effects of various additives and excipients on permeation processes and tissue integrity using epithelial cells of the lung (see Chaps. 10 and 11). Control of the membrane integrity by TEER measurements during the transport experiments is a clear advantage of the Ussing chamber technique. Nevertheless, the submersion of the epithelial barrier is necessary for doing so. Direct administration of aerosols to the epithelium is thus not possible. Another disadvantage of the system is that in vertical diffusion chambers, which comprise the vast majority of commercially

available systems, particle sedimentation within the transport medium will make it impossible to interpret the data.

19.3.2. Setups Allowing to Measure Drug Transport Across Pulmonary Epithelia Interfacing Air

As addressed earlier, application of drug particles in a physiologically relevant manner can solely be conducted as aerosol deposition on air-interfaced growing cell cultures. Typically used submersed cell culture monolayers are covered with liquid layers of several millimeters. For several respiratory epithelial cell types, such as Calu-3, 16HBE14o-, and hAEpC, the possibility of air-interfaced culture (AIC) was confirmed [77]. Under AIC conditions the cells are cultivated without a fluid-filled apical compartment, and fluid homeostasis across the epithelial barrier is maintained by a complex network of ion, protein, and water movement. The resulting liquid layer is thin enough to enable interaction between cells, particles, and/or gas. Moreover, coculture approaches with endothelial cells [78], dendritic cells [79], or macrophages [80] were conducted, in order to increase the complexity and the explanatory power of these models. In conclusion, suitable cell culture systems representing an air rather than liquid interface of pulmonary epithelia are available [81–83].

When studying deposition and subsequent absorption of aerosolized drugs using pulmonary cell culture models, pharmaceutical scientists may take advantage of the experience gathered in environmental toxicology. However, it must be kept in mind that those studies are typically focused on the exposure of xenobiotics/particles over a prolonged period of time, while the focus of pharmaceutically motivated studies is to evaluate efficacy and safety of medicines, that is, to administer single and relatively high doses of drugs and excipients.

19.3.2.1. Longtime, Low-Dose Deposition in Environmental Toxicology

Cultex system [84]: The recently published aerosol deposition system CULTEX [84] allows continuous exposure of lung cell monolayers to complex atmospheres. The device was developed as a research tool in environmental lung toxicology. CULTEX enables treatment of epithelial cells, cultivated on permeable filter inserts, with aerosols. Subsequent to the impingement, in vitro assays and permeability measurement can be carried out [85]. By controlling pO_2, pCO_2, and humidity, cells and lung slices stay viable for at least 48 h. The apparatus setup is shown in Figure 19.2a: pulmonary cell monolayers are placed on membranes inside a deposition chamber. The CULTEX system is entirely made of glass, facilitating the housing of three vessels with cell culture inserts, and the temperature of these vessels is controlled. Nutrient medium is directed to the cell culture insert vessels via a tubing system. The device allows sampling of medium for biological analysis in the course of the experiment, for example, for assessment of transported drug amount. The test aerosol is drawn into the deposition chamber using negative pressure (ΔP). The analysis of several aerosol parameters, including particle concentrations, can be performed online, parallel to the cell exposure.

Aufderheide et al. [84] performed experiments with human lung cells that were directly exposed to diesel exhaust. In contrast to other exposure concepts for complex mixtures, this experimental setup facilitates a direct and reproducible contact between the cell monolayer and the test atmosphere. This could be achieved by following improvements: (i) a strict separation of the medium

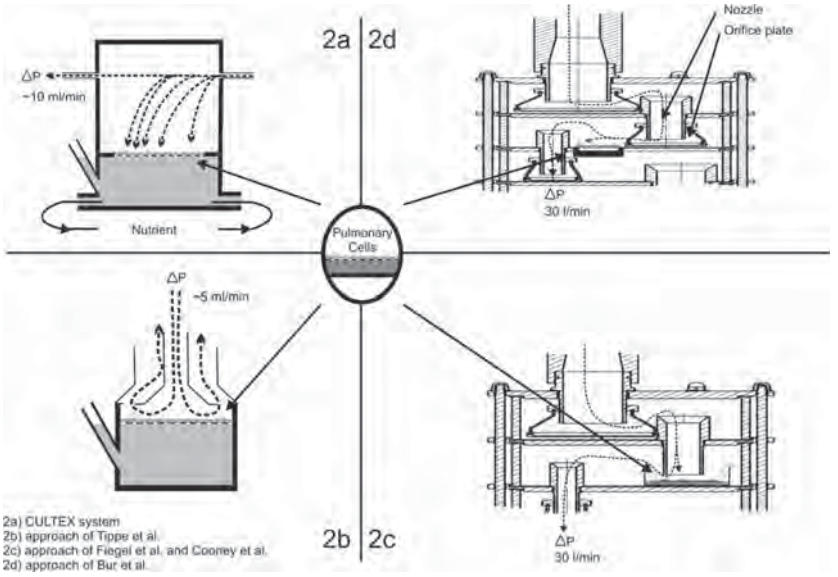

Figure 19.2 Schematic depiction of the described approaches for deposition and subsequent cell–aerosol interaction measurement. Figure 19.2a based on Ref. [84], Figure 19.2b on Ref. [86], and Figure 19.2c on Refs. [54, 89, 90], respectively. Doted lines represent airflow.

and gas supply; (ii) the application of cell culture membranes with small pore size (0.4 μm), thus preventing accidental donor fluid contact with the aerosol during the exposure; and (iii) a transport of the test atmosphere directly to the apical side of the cells.

Results clearly indicated effects of diesel exhausts from different engine operating conditions on the cells already after 1 h of exposure. However, a 1-h continuous exposure to a therapeutic aerosol is probably of limited clinical relevance. To study the effect of drugs administered by typical pharmaceutical single-dose inhalers (MDI, DPI) effective deposition of one "puff" within the time of one breathing maneuver (~5 s) is probably more relevant.

Approach of Tippe et al. [86]: Another setup developed for environmental toxicology-related questions was described by Tippe et al. [86]. A commercially available perfusion unit (MINUCELL, Bad Abbach, Germany) was adapted to study the biological effects of fine and ultrafine particles on cells [87]. The radially symmetric stagnation point flow arrangement deposits particles uniformly and quantifiable onto a cell layer. Because of the low flow velocity over the membrane (~5 ml/min), mechanisms of particle deposition are mostly convective transport and diffusion. For cell exposure, Anodisc® membranes (Whatman, Maidstone, UK) with 47 mm diameter and a pore size of 0.2 μm were used. These membranes remain completely plane after humidification, which is important for homogeneous supply with nutrient medium from the basolateral compartment and for homogeneous particle deposition during exposure. Confluent A549 alveolar epithelial cells were used in the chamber system. Ultrafine carbonaceous model particles with a count median mobility diameter of about 95 $\pm$ 5 nm were delivered. After 6 h, 87 $\pm$ 23 ng/cm^2 of particles were deposited homogeneously on the cell surface with

a deposition efficiency of 2%. Compared to pharmaceutical aerosols, where typically doses up to 500 μg are deposited at rates between 10% and 70% within 5 s, this approach is probably of limited value. Nevertheless, after appropriate modifications the aerosol deposition system of Tippe et al. may possibly be adopted to address pharmaceutical questions.

19.3.2.2. High-Dose, Short Time Application of Aerosols on Models of the Blood–Air Barrier

The pharmacopoeiae describe different aerosol classification devices for metered bolus inhalation. Particularly, the multistage liquid impinger (MSLI) shows good correlation to the in vivo deposition and is the most used impingement system in pharmaceutical research. Because of the high air flow rate in the device (30 l/min), only impaction and sedimentation as deposition mechanism are simulated sufficiently; however, diffusion, the major deposition process in the deep lung in vivo, is not reflected correctly. A thin liquid layer on the stages mimics the fluid layer in the lung and avoids rebounce of particles after initial impaction. In addition, an Anderson cascade impactor can be used for deposition studies, but the missing water layer can make particle rebound an issue.

Approach of Schreier et al. [88] *and Cooney* [90]: Schreier et al. [88] used an Anderson cascade impactor to generate a simulation aerosol system for quasi-realistic gene transfection. The system consisted of a PARI nebulizer, a controller for temperature and humidity, and an Andersen cascade impactor, in which the stages were seeded with pulmonary cells (2CFSMEo-). Cell viability retains over 95% subsequent to the deposition experiments. A fluorescent dye was used to visualize aerosol distribution and to monitor cellular uptake. Additionally, the majority of a gene product was delivered to stages 1 through 5, which are corresponding to the in vivo area from the pharynx down to the terminal bronchi. A corresponding, although very low, transfection of cells was found, with the majority of transfected cells on stages 4 and 5. This system was the first example for a modification of an Anderson cascade impactor to facilitate deposition of pharmaceutical aerosols on pulmonary epithelial cell cultures and has inspired further experimental approaches in this direction.

A further "proof of principle" was conducted by Cooney et al. who demonstrated the feasibility of the Andersen cascade impactor as a cell compatible deposition device [90]. Permeability coefficients of fluorescent isothiocyanate-labeled dextrans after impaction as aerosols on Calu-3 cells were calculated. Deposition did not negatively affect the cell monolayer integrity.

Approach of Fiegel et al. [89]: A crucial question for pulmonary drug delivery is the effect of aerosol impaction on cell viability. In contrast with the slow air streams applied in environmental toxicology setups (only a few milliliter per minute), pharmaceutical setups are working with high air velocities between 30 and 60 l/min. At such high air velocities even small particles have high impaction forces and hence might induce cell-damaging effects. In a study from Fiegel et al., the integrity of Calu-3 cell monolayers impinged with polymeric large porous particles was investigated by means of measurement of the TEER and transport of the paracellularly transported marker, fluorescein-sodium. Filter inserts containing cell monolayers were placed directly under the second

stage nozzle of an Astra-type MSLI, as shown in Figure 19.2c. Microparticles were aerosolized onto the monolayers for 30 s at 30 l/min via a Spinhaler device. The filter insert was then returned to a cell culture plate and returned to an incubator. Light microscopy images of the monolayers confirmed that particles were not aggregated when deposited on the monolayers, which showed homogeneously dispersed particles across the entire surface of the monolayer. In addition, SEM images of both AIC and liquid-covered condition (LCC) grown monolayers impinged with microparticles revealed no apparent damage. Although there was no detectable effect on TEER values, monolayers grown under LCC conditions showed an increased flux of fluorescein-sodium, when compared to the AIC monolayers. Mucus staining of the cell monolayers exhibited positive staining only for Calu-3 cells grown under AIC conditions. This adherent mucus gel layer may protect cells against microparticle impingement like a protective coating, thus cushioning the landing of the particles. In contrast, mucus produced by cells grown under LCC conditions easily dissolves into the apical fluid as it is removed by aspiration of the supernatant prior to particle impinging. Therefore, microparticles landing directly on the surface of cells grown under LCC conditions may cause damage to the barrier properties.

In all three described setups, deposition on the inserts was ∼50% or less of the amount usually anticipated on an equal sized area without cell culture inserts. This difference may be explained by the changed distance between orifice plate and impaction surface in the cell culture insert. The collection efficacy of an impactor/impinger decreases with an increase in the ratio of the orifice plate to impaction plate distance over jet width. Observations indicate that although the inserts are not at an optimal distance from the orifice plate and are not exactly at the same position compared to the original collection stage, the cell culture inserts do function as an impaction surface for particles in a relevant size range.

Approach of Bur et al.: A serious disadvantage of the previously described approaches may be seen in the changed aerodynamics and deposition patterns of the originally "cell free" impactors/impingers following modifications with cell culture inserts. In order to overcome this issue, the adaptation of an MSLI to fit filter grown epithelial cell monolayers is currently being further improved. It appears pivotal that the cell culture inserts do not disturb the air stream in the stage, and as a consequence deposition efficacy and deposition pattern is not altered compared to a nonmodified MSLI. To achieve this, two holes per stage were drilled into the bottom of the stages 2 and 3, housing the inverted cell culture inserts. Consequently, cultivation of pulmonary cells on the bottom side of a cell culture insert was necessary. In this "upside down" cell culture, no significant differences have been found concerning barrier properties. The inner diameter of the drilled wholes exactly matches the external diameter of commercially available Transwell® filter inserts, as can be seen in Figure 19.2d. Since the cell culture insert extends into the lower stage, and thus might affect aerodynamic properties in this particular stage, the filter was encapsulated with stainless steel. The MSLI was operated with a vacuum pump with an air flow rate of 30 or 60 l/min. Time of inhalation was managed by a testing unit for dry powder inhalations allowing adjustment of the duration of inhalation and therefore controlling the cumulative amount of air. First

experiments with the model substance sodium-fluorescein suggest successful deposition of aerosols on cell monolayers.

19.3.3. Critical Evaluation of the State of the Art and Further Needs

In order to characterize pharmaceutical aerosols in vitro, particularly with regard to the cellular response on the deposition, various experimental setups are reported in literature. Devices having their roots in environmental toxicology provide promising starting points for further development of in vitro devices, which must allow application of single high-dose aerosols. Attempts have been made combining commercial impinger and impactor systems with pulmonary cell culture models. However, the high deposition rate used in these systems can only be achieved by applying high flow rates, hence allowing impaction and sedimentation as impingement mechanism and excluding diffusion. Thus, these devices are mainly suitable for modeling deposition in the upper airways. The currently most effective experimental approach is based upon the principle of the air–liquid exposure technique in the MSLI during deposition.

An ideal in vitro model for the characterization of aerosol formulations would incorporate cell types from various regions of the lung (tracheal, bronchial, and alveolar) and would facilitate simulation of deposition mechanisms by impaction, sedimentation, and diffusion of a high-metered single-bolus inhalation. In the future, such systems may reduce the need for animal studies and may offer to correlate in a predictive way the results from such in vitro tests to clinical bioavailability data after pulmonary drug delivery in vivo.

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