European Journal of Pharmaceutics and Biopharmaceutics 141 (2019) 210–220

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

In vitro investigation on the impact of airway mucus on drug dissolution and absorption at the air-epithelium interface in the lungs

Emanuela Cingolani[a], Safar Alqahtani[a], Robyn C Sadler[b], David Prime[b], Snjezana Stolnik[a], Cynthia Bosquillon[a,*]

[a] School of Pharmacy, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom
[b] GlaxoSmithKline, David Jack R&D Centre, Park Road, Ware SG12 0DP, Hertfordshire, United Kingdom

ARTICLE INFO

Keywords:
Mucus
Drug dissolution
Drug permeability
Aerosol
In vitro model

ABSTRACT

Although the mucus layer is the first biological barrier encountered by inhaled drugs upon their deposition in the upper airways, its potential impact on drug dissolution and absorption in the lung has hardly been investigated. Bio-relevant in vitro models were therefore used to assess the role of airway mucus in the fate of drug particles at the air-epithelium interface. Salbutamol and indomethacin were used as model Biopharmaceutics Classification System (BCS) class III and class II drugs, respectively. Dry powders were reproducibly aerosolised using a PennCentury™ Dry Powder Insufflator onto multiple air-liquid interfaced layers of the broncho-epithelial cell line Calu-3 or thin layers of porcine tracheal mucus mounted onto Transwells® inserts, as well as on empty Transwells®. Comparison of the permeation profiles of the two drugs indicated that mucus acted as a barrier for salbutamol transport but increased that of indomethacin, suggesting it facilitates the dissolution of poorly soluble drugs. In presence of Calu-3 layers, the permeability of salbutamol was even more restricted while indomethacin transport was enhanced further. This study demonstrates mucus distinctly affects the absorption characteristics of drugs with different physico-chemical properties. Hence, drug-mucus interactions should be considered during the development of inhaled drugs.

1. Introduction

Pressurised metered dose inhalers (pMDI) and dry powder inhalers (DPI) are extensively used in the management of chronic respiratory diseases. The aerosolised particles they deliver to the patients' airways have first to dissolve upon contact with the lung mucosa for the active compound to exert a therapeutic effect. Particle dissolution is then followed by drug permeation across the pulmonary epithelium. This allows inhaled bronchodilators to reach their pharmacological target on the underlying smooth muscles but can also result in high drug concentrations in blood and potential systemic side effects.

Due to the complex architecture of the airways and the heterogeneity between the central and peripheral areas of the lungs, the parameters affecting particle dissolution and drug absorption at the air-lung interface remain unclear to date [1], which contributes to the current difficulties faced by the pharmaceutical industry in designing safe and effective drug formulations for inhalation [2].

A range of in vitro methods have been described to investigate and predict the fate of drug particles following their deposition in the lung tissue. Intense efforts have particularly been focussed on the development of particle dissolution set-ups relevant to the airway environment [3–6] as well as the optimisation of simulated lung fluids to be used in inhaled drug dissolution assays [7].

The interplay between dissolution and absorption processes at the air interface in the lung has been investigated by aerosolising drug particles at the surface of layers of the human broncho-epithelial cell line Calu-3 cultured in air-liquid conditions and measuring the drug transepithelial transport over time [9–14]. A major advantage of the Calu-3 model is that it provides a physiologically sound representation of the native bronchial epithelium in which both the cellular and non-cellular (i.e., the mucus secretions) components are featured [15], allowing their cumulative impact on particle fate to be accounted for.

On the other hand, although aerosolised particles first come into contact with the mucus layer upon deposition in the upper airways, the potential role of airway mucus secretions in the dissolution and absorption of inhaled drugs has generally been overlooked and to date, only a very limited number of studies have investigated drug particle interactions with airway mucus. Gentamicin sulfate [16] or ketoprofen lysinate [17] were deposited as inhalation dry powders onto a layer of, respectively, an artificial cystic fibrosis mucus or cystic fibrosis sputum

* Corresponding author at: School of Pharmacy, Boots Science Building, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom.
E-mail address: cynthia.bosquillon@nottingham.ac.uk (C. Bosquillon).

https://doi.org/10.1016/j.ejpb.2019.05.022
Received 20 March 2019; Received in revised form 22 May 2019; Accepted 23 May 2019
Available online 30 May 2019

formed at the surface of the diffusion membrane of modified Franz cells. For both drugs, permeation into the receiver compartment was delayed in presence of mucous secretions. It is however noteworthy that the thickness of the mucus layers used in those studies (i.e., 3 mm) was orders of magnitude higher than in physiological conditions. More recently, the dissolution of budesonide and fluticasone propionate particles has been monitored upon their aerosolisation onto layers of a mucus simulant made of polyethelyne oxide and phospholipids [18]. It is nevertheless unclear to which extent this mimicks the physico-chemical properties of airway mucus.

The aim of this work was to probe the role of airway mucus in drug particle fate in the lung using bio-relevant in vitro models. Two therapeutic molecules with different physico-chemical properties were selected for the study: (i) the inhaled bronchodilator salbutamol sulfate, which, according to the oral Biopharmaceutics Classification System, is a high solubility but low permeability drug (BCS class III drug); (ii) the non-steroidal anti-inflammatory drug (NSAID) indomethacin which is, in contrast, a low solubility/high permeability molecule (BCS class II drug). Although currently not administered by the pulmonary route, inhalation of indomethacin and other NSAIDs has been considered [19–21]. The drug permeation profiles were compared after their deposition as inhalation dry powders onto mucus-producing ALI Calu-3 layers or layers of porcine tracheal mucus in order to define the contribution of airway mucus in particle dissolution and drug absorption at the air-epithelium interface in the lung.

2. Materials and methods

2.1. Materials

Micronised salbutamol sulfate and indomethacin dry powders with a median particle size by volume (DV_{50}) ≤ 3 μm were kindly provided by GlaxoSmithKline (GSK; Ware, UK). Information on the particle size distribution was provided by GSK.

Acetic acid was purchased from Fisher Scientific (Loughborough, UK). Isopropanol (IPA), HPLC grade methanol (MeOH) and acetonitrile (AcN) were obtained from Fisher Scientific (Loughborough, UK). HPLC grade water was produced with a Purelab Ultra ELGA system.

Unless specified, other chemicals and cell culture reagents were from Sigma-Aldrich (St. Lewis, MO).

2.2. Deposition system

The system assembled to deposit drug particles onto cell or mucus layers formed onto the membrane of Transwell® inserts consisted of a vacuum glass desiccator and a PennCentury™ Dry Powder Insufflator – Model DP-4 (Penn-Century, Inc., Wyndmoor, PA) without the intratracheal tube. The insufflator was fitted with a PennCentury Air Pump™ – Model AP-1 (Penn-Century. Inc. Wyndmoor, PA) and secured in the desiccator vacuum port via a Fisherbrand™ rubber stopper (Ø = 21 mm, Fig. 1A).

The deposition surface of the desiccator had an internal diameter of 18.4 cm, and the distance between the port entrance of the desiccator and the deposition surface was 20.0 cm (Fig. 1B). Individual 0.4 μm pore size, 1.12 cm^2 surface area polyester Transwell® cell culture inserts (Co-Star Corning, Corning, UK) placed in the centre of petri dish lids (Ø = 3.5 cm) were arranged at the extremity of the internal support of the desiccator at a distance of 7.35 cm from the centre of the spray (Fig. 1C).

2.3. Particle size distribution

Transwell® inserts were arranged in the desiccator chamber according to the geometry showed in Fig. 1C. The polyester membrane was removed from all inserts and replaced with clean glass coverslips (1.2 cm in diameter, VWR, Germany). Two mg of micronised salbutamol sulfate or indomethacin dry powder were sprayed onto the coverslips by actuating the PennCentury™ insufflator twice. The surface of each coverslip was scanned under the Imstar – smart imaging system for life optical microscope (IMSTAR SA, Paris, France) fitted with a X20 objective, acquiring 20 × 20 images (448.8 × 332 μm) 1 μm apart in both the vertical and the horizontal directions over the surface of each coverslip.

The acquired images were batch processed using the public domain ImageJ software (National Institute of Health, US). The average between the major (M) and minor (m) diameters of the best ellipsis fitting the particle was used to describe the particle size (diameter, D).

A frequency distribution was drawn out of the set of diameters (D) by dividing the range of sizes from 0 μm to the maximum D calculated into bins (bin size: 0.4 μm). Each bin contained the number of diameters lying within the size range covered by the bin. The resulting distribution was normalised for number of particles in each bin (frequency %): 100% was associated with the bin containing the highest number of particle diameters, whereas 0% was associated with empty bins.

2.4. Dose deposited on the Transwell® inserts

The PennCentury™ dry powder insufflator was loaded with 2.0 mg of micronized salbutamol sulfate or indomethacin which were sprayed until the device was emptied (typically one or two actuation(s)) onto Transwell® inserts in which the polyester membrane had been removed and replaced with clean glass coverslips (1.2 cm in diameter, VWR, Germany).

Salbutamol sulfate or indomethacin was recovered by washing the coverslip surface four times with 50 μL of MeOH or AcN respectively. The organic solvent was evaporated down using a JOUAN centrifugal evaporator RCT90 for 20 min, and samples were reconstituted with 1 mL of fresh MeOH or AcN (HPLC grade). The samples were analysed quantitatively by HPLC-UV. Experiments were performed in triplicate.

2.5. HPLC-UV methods

HPLC-UV analysis was performed using an Agilent Hewlett Packard HPLC series 1100 fitted with a UV microcell for UV–Vis detection.

Salbutamol sulfate samples were analysed using a SUPELCOSIL™ LC-18-DB (5 μm, 150 mm × 4.6 mm; Supelco™ analytical; Bellafonte, USA) column. The injection volume was 100 μL, and the analysis was carried out at a flow rate of 1.0 mL min^{-1} using 0.1% (w/v) sodium dodecyl sulfate (SDS) in water and MeOH (25:75) as the mobile phase. The chromatographic runs were monitored at 276 nm (linear range: 0.01–10 μg mL^{-1}, y = 22.70x; R^2 = 0.9999).

Indomethacin samples were analysed using an ACE3 C18 (3 μm, 150 mm × i.d. 2.1 mm) column. The injection volume was 10 μL, the flow rate was 0.2 mL min^{-1} and the mobile phase 0.2% v/v H_3PO_4 in H_2O (pH = 2.8) and AcN (20:80). The chromatographic runs were monitored at 318 nm (linear range: 0.05–10 μg mL^{-1}; y = 29.66×, R^2 = 0.9984).

2.6. Cell culture and maintenance

Calu-3 cells (passages 29–39) were cultured in the same conditions as described by Grainger et al [15]. Briefly, they were maintained in Dulbecco's Modified Eagle's Medium: F-12 (DMEM:F-12) containing 10% (v/v) foetal bovine serum (non-USA origin, sterile filtered), 1% (v/v) non-essential amino acid, 1% (v/v) L-glutamine and 1% (v/v) penicillin-streptomycin solutions at 37 °C in a humidified 5% CO_2 atmosphere. When confluence reached approximately 90%, cells were passaged using 0.25% trypsin/0.1% ethylenediaminetetraacetic acid (EDTA) and seeded at a seeding density of 1 × 10^5 cells/mL onto the Transwell® cell culture inserts with 0.5 and 1.5 mL of medium in the apical and basolateral chamber, respectively. The culture was turned into air-liquid interface (ALI) conditions 24 h after seeding by removing

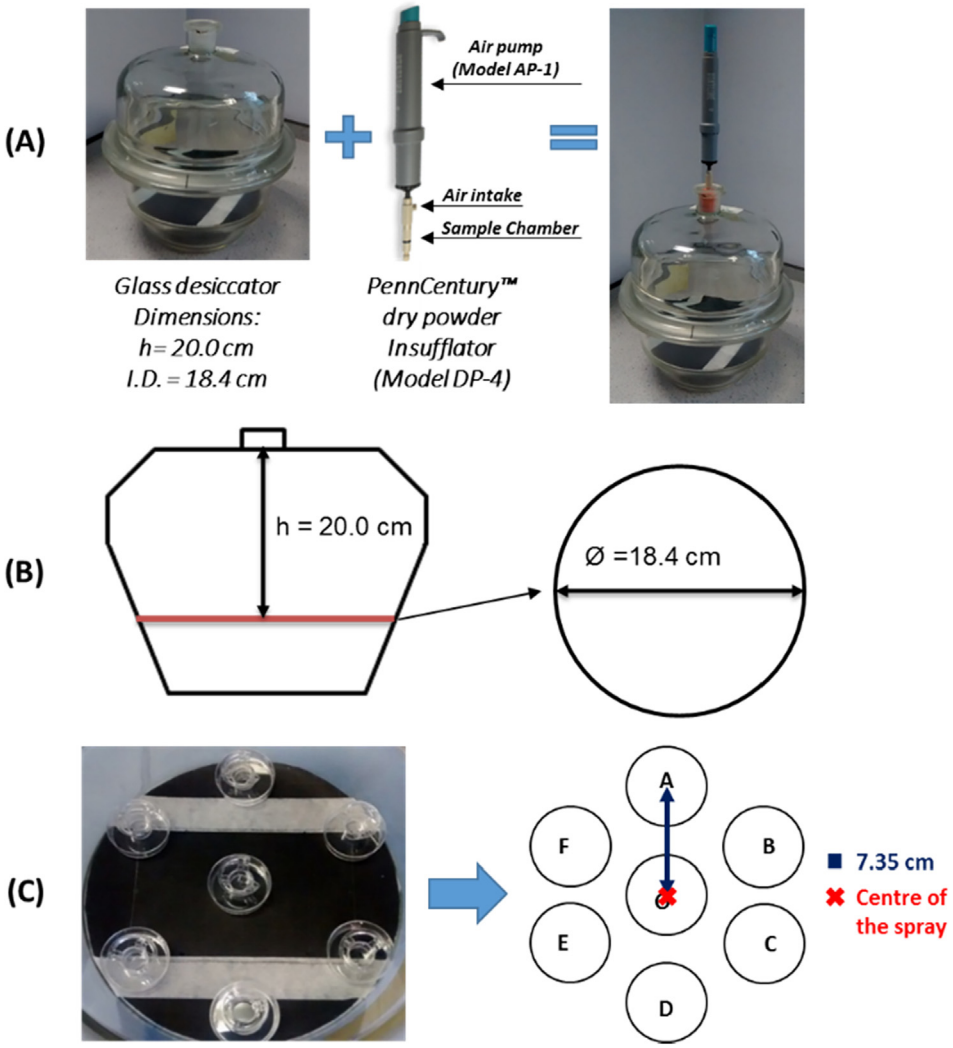


Fig. 1. Representation of the deposition system. (A) elements composing the deposition system and their assembling; (B) dimensions of the desiccator chamber; (C) arrangement of individual Transwell® inserts in the desiccator chamber. The distance from the centre of the spray was 7.35 cm.

the medium from both the apical and the basolateral chambers and adding 500 µL of fresh pre-warmed medium in the basolateral chamber only. Medium was changed every 2–3 days thereafter. The Calu-3 layers were left to differentiate for 21 days. Only layers exhibiting a transepithelial electrical value (TEER) above 500 Ω cm^2 were used for experimentation.

2.7. Cell layer integrity after exposure to dry powder sprays

On day 21 after seeding, three inserts were left in the incubator and used as reference, whereas medium was removed from the basolateral chamber in all other inserts.

Three inserts at a time were transferred to the desiccator chamber and placed in positions B, C and E (Fig. 1C). The cell layers were either (i) left in the chamber for a time period corresponding to a spray (about 30 s) but not exposed to any spray (blank), (ii) exposed to a spray of only air or (iii) exposed to a spray of salbutamol sulfate after loading of 2.0 mg of the drug into the PennCentury™ device.

After exposure, layers were placed into a clean 12-well plate and prepared for TEER measurement. Briefly, 0.5 and 1.5 mL of pre-warmed Hanks's Balanced Salt Solution (HBSS) was added to the apical and basolateral Transwell® chamber, respectively. The cell monolayers were left to equilibrate for 30 min at 37 °C in a 5% CO_2 humidified atmosphere. The TEER of each cellular monolayer was measured using a voltohmmeter EVOM, equipped with STX-2 chopstick electrodes (World Precision Instruments, Sarasota, FL, USA).

Following TEER measurements, the apical to basolateral (A → B) permeability of the paracellular marker lucifer yellow VS dilithium salt (LY) was monitored for one hour. Briefly, 300 µL of 100 µM LY solution was placed in the apical chamber and 900 µL of fresh pre-warmed HBSS was placed in the basolateral chamber. Samples (100 µL) were withdrawn from the basolateral chamber after 30 and 60 min and transferred to a black 96 well plate (Nunc F96, Scientific Laboratory Supplies, Nottingham, UK). Samples (100 µL) were taken from the apical side at the beginning (time zero) and at the end of the experiment. Samples were analysed for LY content using a Tecan (SPARK 10 M) plate-reader and fluorescence was monitored at λ_{em} = 437 nm and λ_{ex} = 535 nm. A flux < 2.0% was considered as an indicator of a tight and intact monolayer [4,5].

The apparent permeability (P_{app}) of LY was calculated as per Eq. (1):

$$P_{app} = \left(\frac{dQ}{dt}\right) \cdot \frac{1}{AC_0} \quad (1)$$

where dQ/dt is the transport (or permeability) rate, A is the surface area (1.12 cm^2) and C_0 corresponds to LY concentration in the donor chamber at time zero.

2.8. Transepithelial transport studies

The transepithelial transport of salbutamol and indomethacin was investigated after the Calu-3 cell layers were exposed to the drugs either in solution or as dry powders delivered using the deposition system described above.

On day 20, i.e., the day prior to the transport studies, the TEER of the Calu-3 cell layers was measured in HBSS. For permeability measurements in solutions, 200 µL of a 5.0 µg/mL solution corresponding to 1.0 µg dose of drug in HBSS was placed in the apical chamber and 900 µL of fresh pre-warmed HBSS (37 °C) was placed in the basolateral compartment. Due to the low water solubility of indomethacin, a 1 mg/mL stock solution of indomethacin in 100 mM Na_2CO_3 was prepared and diluted accordingly in HBSS.

For the dry powder transport experiments, four Transwell® inserts were transferred to positions B, C, E and F in the chamber (Fig. 1C). The PennCentury™ Dry Powder Insufflator was loaded with 2.0 mg of dry powder and 5.0 mL of ambient air was used to produce the spray. The inner and outer plastic surfaces were carefully wiped to remove the drug particles that had not deposited at the surface of the cell layers. The inserts were then transferred to a clean 12 well plate and 500 µL of fresh pre-warmed HBSS was placed in the basolateral chamber.

Cell layers exposed to either the dry powder spray or the drug solution were kept at 37 °C in a 5% CO_2 humidified atmosphere for the duration of the experiment.

Samples (200 µL) were withdrawn from the basolateral side after 5, 10, 15, 20, 30, 60, 120, 180 and 240 min, and 200 µL of fresh pre-warmed HBSS was used to restore the initial basolateral volume. After 4 h, the apical side was washed twice with 100 µL of fresh HBSS and samples were collected for analysis of the residual apical drug concentration. TEER measurements and a LY permeability assay were then carried out as described above.

Afterwards, cells were lysed with 500 µL of IPA:DMSO (1:1), harvested and centrifuged at 14,000 rpm for 5 min. The supernatant was collected and analysed for drug content. The initial dose deposited on each layers was calculated back by adding up the drug content found in the two Transwell® chambers and that associated with the cells at the end of the transport studies.

For experiments when the drug was applied in solution, the P_{app} was calculated as per Eq. (1). Only cell layers with final TEER values above 400 Ω cm^2 were included in the study. Experiments were performed in duplicate (N = 2), using four Transwell® inserts per replicate (n = 4).

2.9. Mucus collection and cleaning

Batches of six tracheas isolated from healthy adult pigs, both males and females, were obtained from a local abattoir. Each trachea (approximate length: 15–20 cm) was cut open longitudinally and the edges were pinned on a support. The mucus was gently scraped off the trachea surface with a spatula and pooled together in plastic tubes. Samples were stored at −20 °C until further use.

The mucus was thawed at room temperature before cleaning. Aliquots were diluted 1:10 with 0.1 M NaCl and stirred at 4 °C for 30 min. The suspension obtained was centrifuged at 14,000 rpm at 4 °C for 15 min. The supernatant containing blood residues and debris was discarded. If the mucus did not look clean, the process was repeated until visibly blood-free and clear samples were obtained. The cleaned mucus was stored at −20 °C until further use.

2.10. Rheological characterisation of porcine tracheal mucus

The rheological characterisation of the pig mucus was performed using a Modular Compact Cone-Plate Rheometer MCR302 (Anton Paar GmbH, Germany). The cone used was a CP25-1 with diameter of 24.972 mm, angle of 1.002°, truncation of 51 µm.

Oscillatory tests were carried out in order to obtain information about the mechanical properties of the mucus. Measurements of the storage modulus (G′) and loss modulus (G″) as function of strain γ (0.1–100%) were carried out at an angular frequency of 10 rad/s, collecting 5 points per decade. Oscillatory frequency sweeps were performed within the linear viscoelastic (LVE) region, under a strain of 0.1%, and G′ and G″ moduli were measured between 0.1 and 100 Hz. Viscosity measurements were collected with a shear rate increasing logarithmically from 10 to 100 s^{-1}.

Mucus samples were analysed in triplicate and all measurements were carried out at 37 °C. Rheoplus analysis software (Anton Paar GmbH, Germany) was used for data processing.

2.11. Scanning electron microscopy

Cryo-SEM was used to visualise the internal structure of porcine tracheal mucus following the cleaning steps described above. Five µL of mucus was placed onto a brass rivet in a Cryo-SEM sample stage and covered by a second rivet. The samples were rapidly frozen in nitrogen slush before being transferred to a sample-prep stage (under vacuum) in a Quorum 3010T cryogenic SEM preparation system. The tops of the rivets were knocked off to fracture the sample. This was followed by sublimation at −90 °C for 10 min to remove water from the sample surface. The samples were sputter coated using the in-chamber Pt source at 10 mA for 60 s. Samples were then transferred into the SEM chamber (under vacuum) and imaged using an accelerating voltage of 10 kV on an FEI Quanta 200 3D FIB-SEM. The working distance was ca.15 mm, which was then varied to ensure that the best images were obtained, while the spot size was 4 mm.

2.12. Coating of Transwell inserts with porcine tracheal mucus

In order to study particle dissolution and drug permeation characteristics in contact with tracheal mucus in similar conditions as in the Calu-3 model, mucus layers were formed at the surface of Transwell® inserts. Various volumes of mucus ranging from 1.0 µL to 12 µL were initially tested in order to determine the lowest quantity needed to obtain a complete and even coating of the semipermeable membrane of the Transwell® inserts.

The selected volume of mucus was re-suspended in a solution of 10% Alcian blue (3% acidic acid in dd-H_2O) to a total volume of 300 µL. The suspension obtained was transferred into a Transwell® insert housed in a 12-well cell culture plate. The plate was centrifuged at 1500 rpm for 15 min (Multifuge 3S, Heraeus). The supernatant was removed and images of the inserts surface coated with stained mucus were acquired using a Leica microscope MZ16 fitted with a Leica EC3 colour camera using an objective X5. Since complete coating of the Transwell® membrane was only achieved when 12 µL of mucus was used (Fig. 2), that volume was used in subsequent experiments.

2.13. Drug permeation across mucus layers

Twelve µL of mucus was resuspended in 0.1 M NaCl for a final volume of 300 µL. The mucus suspension obtained was transferred into four Transwell® inserts housed in a 12-well cell culture plate. The plate was centrifuged at 1500 rpm for 15 min. The supernatant was removed and 500 µL of HBSS was placed in the basolateral chamber. The plate was stored overnight to allow the mucus to stabilise and the excess of water to evaporate.

Drug transport across Transwell® inserts either coated with mucus or left empty was measured following aerosolisation of salbutamol sulfate or indomethacin micronised dry powders in the same conditions as described above. In the experiments without mucus on the Transwell® membrane, a small volume of HBSS was not added in the donor compartment. Development of a Transwell® based dissolution test for inhalation powders has indeed shown that the presence of fluid in the apical chamber was not critical and even decreased the reproducibility

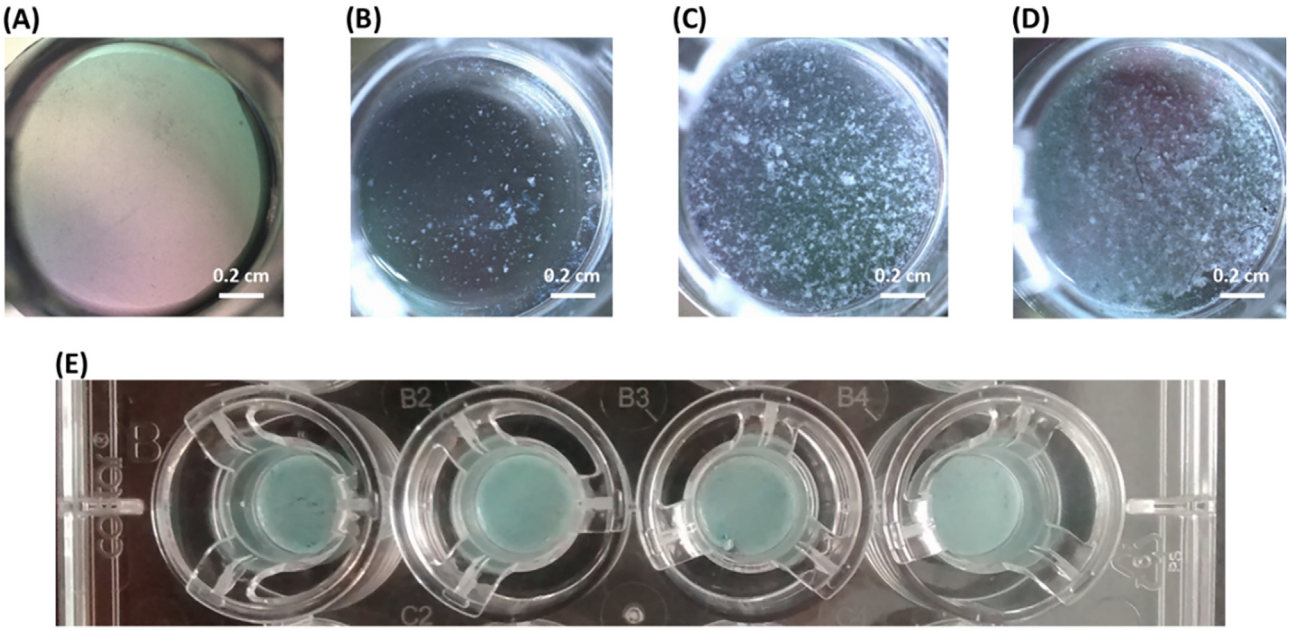


Fig. 2. Optimisation of the coating of Transwell® inserts with porcine tracheal mucus (A) Empty insert; Insert coated with (B) 1.0 µL, (C) 5.0 µL, (D) 10.0 µL or (E) 12.0 µL of porcine tracheal mucus stained with Alcian Blue.

of the dissolution profile for some poorly soluble drugs [22].

At the end of the 4 h permeability studies, the apical side of the empty Transwells® was washed twice with 100 µL of fresh HBSS and samples were collected for analysis of the residual apical drug. The mucus layers were washed and removed from the insert with 200 µL of HBSS. The suspension obtained was vortexed for 1 min and centrifuged for 5 min at 14,000 rpm. The supernatant was collected for quantification of the residual non-permeated drug.

Experiments were performed in duplicate (N = 2), using four Transwell® inserts per replicate (n = 4). All samples were processed and analysed using HPLC-MS/MS.

2.14. HPLC-MS/MS drug analysis

The HPLC system consisted of an Agilent Hewlett Packard series 1100 coupled with a Micromass Quattro Ultima Pt mass spectrometer (Waters, Milford, USA) equipped with an electrospray ion source operated in positive mode for both salbutamol and indomethacin. An ACE3 C18 (3 µm, 150 mm × i.d. 2.1 mm) column fitted with a C18 guard cartridge was used for all analysis.

Salbutamol sulfate in HBSS samples were diluted 1:1 with MeOH (HPLC grade) vortexed for 1 min and centrifuged at 5000 rpm for 5 min at 4 °C. The supernatant was diluted 1:1 with phase A consisting of an aqueous solution containing 0.1% v/v formic acid, ammonium formate 20 mM (pH 3.8), and 50 µL of the resulting solution was injected in the HPLC-MS/MS system for quantification. Samples were run at 0.2 mL min^{-1} isocratically using a mixture of phase A and MeOH (50:50) as mobile phase. Salbutamol was detected in multi reaction monitoring (MRM) mode at m/z 240.1 → 148.1. MS parameters were as follows: capillary voltage, 3.5 kV; cone voltage, 35 V; source temperature, 125 °C; desolvation temperature, 350 °C; collision energy, 20 kV.

Indomethacin in HBSS samples were diluted 1:1 with the mobile phase (AcN:phase A, 50:50), vortexed for 1 min and centrifuged at 5,000 rpm for 5 min at 4 °C. The supernatant (40 µL) was injected in the HPLC-MS/MS system and run at 0.3 mL min^{-1} isocratically using a mixture of phase A and acetonitrile (20:80) as mobile phase. Indomethacin was detected in multi reaction monitoring (MRM) mode at m/z 359.03 → 139.11 and 359.03 → 111. MS parameters were as follows: capillary voltage, 3.5 kV; cone voltage, 37 V; source temperature, 125 °C; desolvation temperature, 350 °C; collision energy, 22 kV.

2.15. Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 6.02.

Unpaired *t*-test (multiple comparisons) and ANOVA one-way analysis (with Tukey's multiple comparison test) were carried out to compare two or more than two groups, respectively. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Spray particle size distribution and dose deposited

The median diameter of both salbutamol sulfate and indomethacin particles deposited on Transwell® inserts after an aerosol spray was between 2 and 5 µm (Table 1). Nevertheless, large aggregates landed on the inserts, indicating that the shear forces created within the PennCentury™ device were not capable of completely disaggregating the dry powders.

The insert positioned in the centre of the chamber (position O) received the largest particles with maximum diameters over 100 µm for both drugs, likely due to larger particles falling perpendicularly onto the Transwell® by inertial impaction while smaller particles were able to follow the airflow and deposited in peripheral areas by sedimentation.

As expected from the particle size distribution data, the insert positioned underneath the aerosolization device received much higher doses than the other Transwell® (Fig. 3A and B).

When only the six inserts placed at the periphery of the desiccator chamber (A, B, C, D, E and F) were considered, 0.85 ± 0.09 µg of salbutamol sulfate and 0.93 ± 0.31 µg of indomethacin were delivered per insert with a satisfactory reproducibility (Fig. 3C).

3.2. Calu-3 cell layer integrity after exposure to a dry powder spray

The integrity of ALI Calu-3 cell layers after exposure to the micronised dry powder spray produced by the PennCentury™ Dry Powder

Table 1
Particle size distributions of salbutamol sulfate (left) and indomethacin (right) dry powder after deposition onto Transwell® inserts arranged in the dessicator chamber.

	Salbutamol sulfate							Indomethacin						
Position	A	B	C	D	E	F	O	A	B	C	D	E	F	O
Min [μm]	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Max [μm]	79.15	41.55	51.75	43.1	84.55	47.6	106.6	56.80	48.60	58.80	31.90	63.20	33.95	141.80
DV_{10} [μm]	0.85	1.10	1.20	1.00	1.15	1.00	1.30	0.90	1.10	0.90	1.10	0.80	0.90	1.00
DV_{50} [μm]	2.10	2.80	3.05	2.65	2.55	2.68	2.80	2.85	3.95	2.65	4.50	2.30	3.25	3.10
DV_{90} [μm]	6.23	7.75	8.80	9.02	6.30	8.46	5.75	10.00	12.64	9.22	13.10	7.70	10.81	8.30
Mean [μm]	3.40	3.92	4.39	4.29	3.54	4.34	3.45	4.36	5.65	4.07	6.04	3.53	4.78	4.13
St. Dev [μm]	5.11	3.79	4.36	5.16	4.18	5.40	3.54	4.39	5.27	4.05	5.31	3.65	4.61	3.73

Insufflator was assessed by monitoring the TEER and the apical to basolateral (A → B) transepithelial flux of the paracellular marker LY.

In Fig. 4A, the TEER "before exposure" refers to the value recorded 24 h before the experiments as performing TEER measurements on the experimental day would have caused the removal of the mucus layer at the surface of the cell layers [15] and possibly an increase in the apical fluid volume. The TEER value dropped for all layers subjected to an air or dry powder puff but nevertheless remained above 500 Ω cm^2 in all cases (Fig. 4A).

The transport of LY across the cell layers was lower than 2% of the dose applied in all conditions tested (Fig. 4B). Although this increased after the cell layers were exposed to the dry powder spray, the corresponding P_{app} was calculated as $3.4 \pm 0.5 \times 10^{-7}$ cm/s, which is a largely accepted value for a low molecular weight paracellular marker in Calu-3 layers [23].

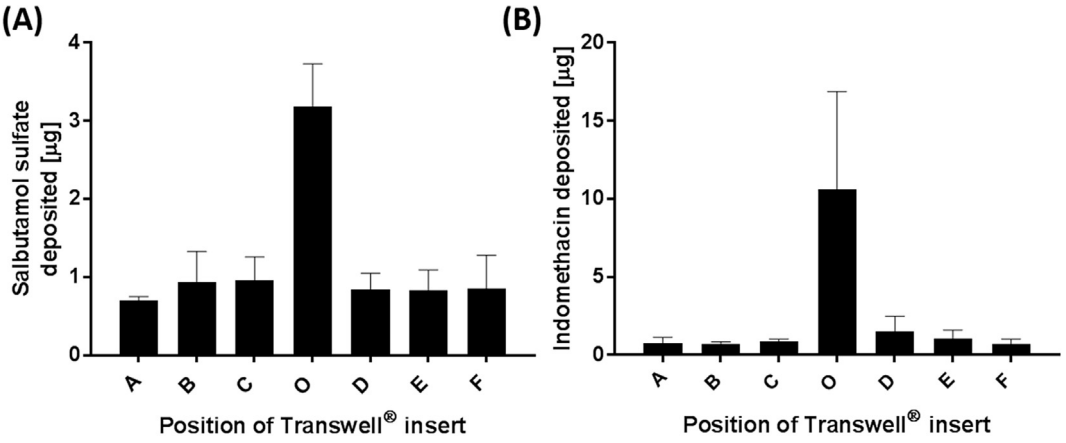
Combined together, the data obtained from the TEER measurements and the LY assay confirmed that the cell layers maintained their barrier properties after exposure to a powder spray. It is noteworthy that this was not the case when the cell layers had been maintained in submerged conditions for 48 h prior to aerosol exposure (data not shown), suggesting that the mucus layer formed at the air-epithelium interface in ALI cultures protected the underlying cell layers from physical shocks caused by particle landing.

3.3. Transepithelial drug transport

The A → B transepithelial transport of salbutamol sulfate and indomethacin was compared after the drugs were applied onto Calu-3 cell layers in solution or as dry powder sprays.

The percentage of salbutamol transported followed a linear trend reaching 0.95 ± 0.03% in 4 h when the cell layers were exposed to a solution of the drug (Fig. 5A). The P_{app} was determined as $1.1 \pm 0.1 \times 10^{-6}$ cm/s, which is in line with the value reported by Haghi et al. after application of a 0.01 mM salbutamol solution [12]. After aerosol delivery, the drug absorption profile featured an initial burst with 25% of the dose absorbed across the cell layers in the first 5 min, then followed a linear trend over the first 30 min and finally slowly reached a plateau at 79 ± 4% of the dose deposited transported in 4 h (Fig. 5B).

When indomethacin was applied to the cell layers in solution, drug concentrations in the basolateral compartment of the Transwell® increased linearly for the first 120 min and eventually plateaued out when about 85% of the dose applied had permeated through the cell layers (Fig. 5C). The P_{app} calculated over the linear range of the



(C)

Drug	Position	Average dose deposited [μg]	CV [%]
Micronised Salbutamol Sulfate	A, B, C, D, E, F	0.85 ± 0.09	10.79
Micronised Indomethacin	A, B, C, D, E, F	0.93 ± 0.31	33.93

Fig. 3. Dose delivered onto Transwell® inserts for a Penn-Century insufflator loading dose of 2 mg. (A) Doses of salbutamol sulfate delivered onto each insert; (B) Doses of indomethacin delivered onto each insert; (C) average doses of salbutamol sulfate and indomethacin delivered onto inserts positioned at the same distance from the centre of the spray. Data are presented as mean ± SEM.

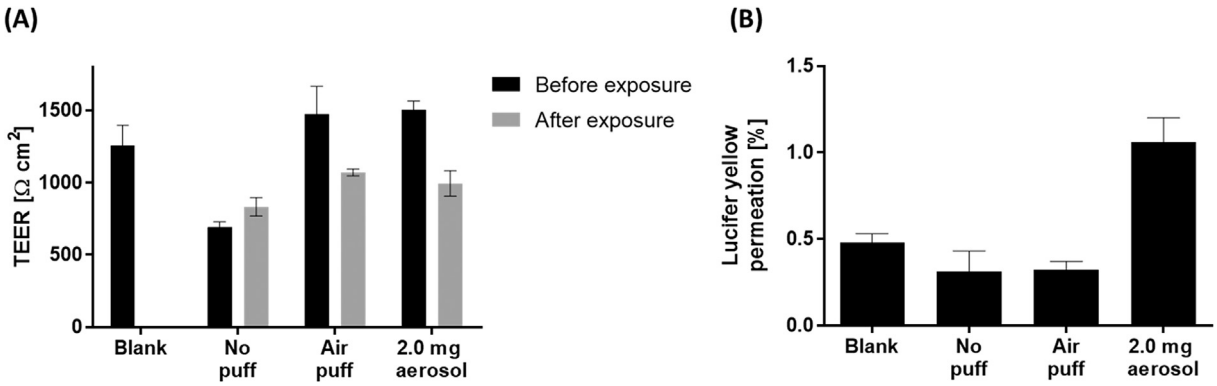


Fig. 4. Calu-3 cell layer integrity after exposure to a salbutamol sulfate dry powder spray produced by the PennCentury™ Dry Powder Insufflator. (A) TEER values; (B) Lucifer yellow transepithelial transport [%] measured over 60 min. Blank: layers kept in the incubator; No puff: layers kept in the desiccator chamber for approximately 30 s but not exposed to the spray; Air puff: layers moved to the desiccator chamber and exposed to a 5.0 mL of ambient air puff; 2.0 mg aerosol: layers moved to the desiccator chamber and exposed to salbutamol aerosol produced with the PennCentury™ device loaded with 2.0 mg of dry powder. Data are presented as mean ± SEM (N = 2, n = 4).

absorption profile (up to 120 min) was 2.5 ± 0.5 × 10^{-5} cm/s, in line with its expected high permeability across biological membranes. As observed with salbutamol, a rapid absorption of indomethacin across Calu-3 layers was measured over the 5 min following delivery of the dry powder. This was followed by a linear increase in the drug transported across the cell layers over 60 min and a final plateau between 60 and 240 min (Fig. 5D).

For both drugs investigated, the transport rate across the cell layers was higher following aerosol delivery than for the corresponding solution. The time needed for 50% of the initial dose to cross the cell layer (t_{50}) was respectively 53 ± 8 min or > 240 min for salbutamol and 27 ± 3 or 84 ± 6 min for indomethacin (Fig. 5 & Table 2).

Table 2
Permeation half-lives t_{50} [min] across empty Transwell® inserts, mucus layer and Calu-3 cell layers after deposition of salbutamol sulfate and indomethacin dry powders.

Drug	t_{50} [min]		
	Transwell® membrane	Mucus layer	Calu-3 layer
Salbutamol sulfate	< 5	≈5	53 ± 8
Indomethacin	88 ± 9	49 ± 7	27 ± 3

3.4. Characterisation of mucus collected from pig tracheas

The rheological properties of tracheal porcine mucus following the cleaning step were compared with those of the native mucus by

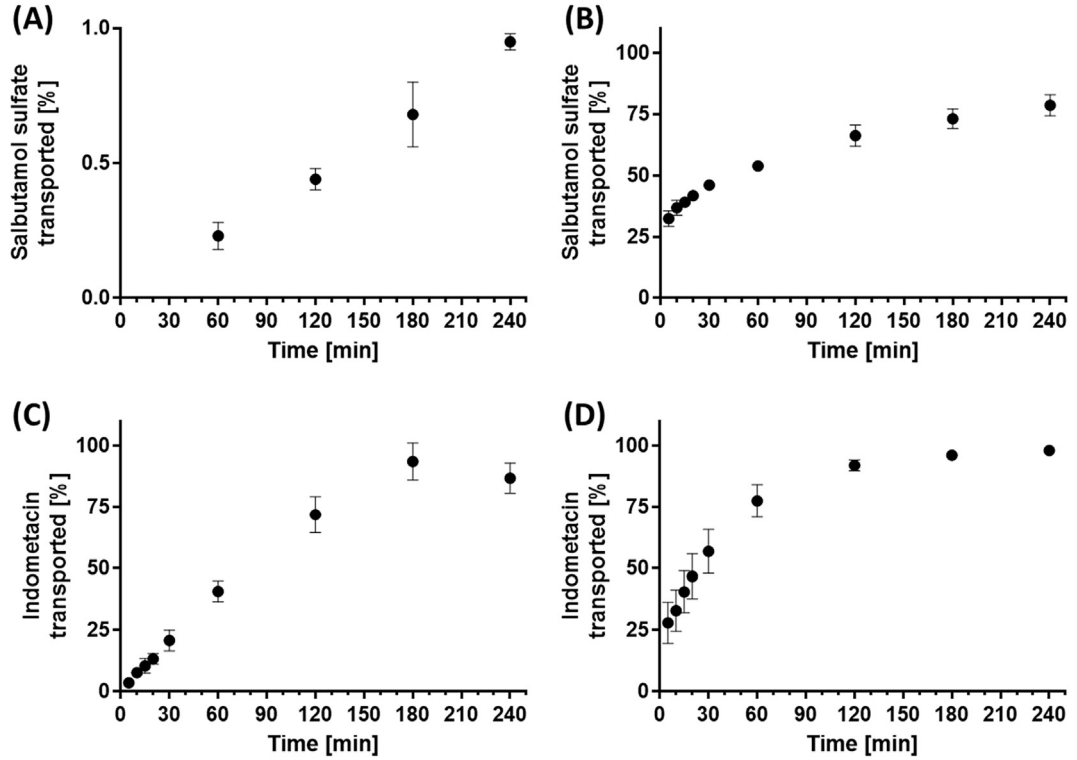


Fig. 5. Apical to basolateral (A → B) transport of (A) & (B) salbutamol sulfate or (C) & (D) indomethacin across Calu-3 layers. Drugs were applied either (A) & (C) in solution or (B) & (D) as dry powders at a dose corresponding to 1.0 µg. Data are presented as mean ± SEM (N = 2, n = 4).

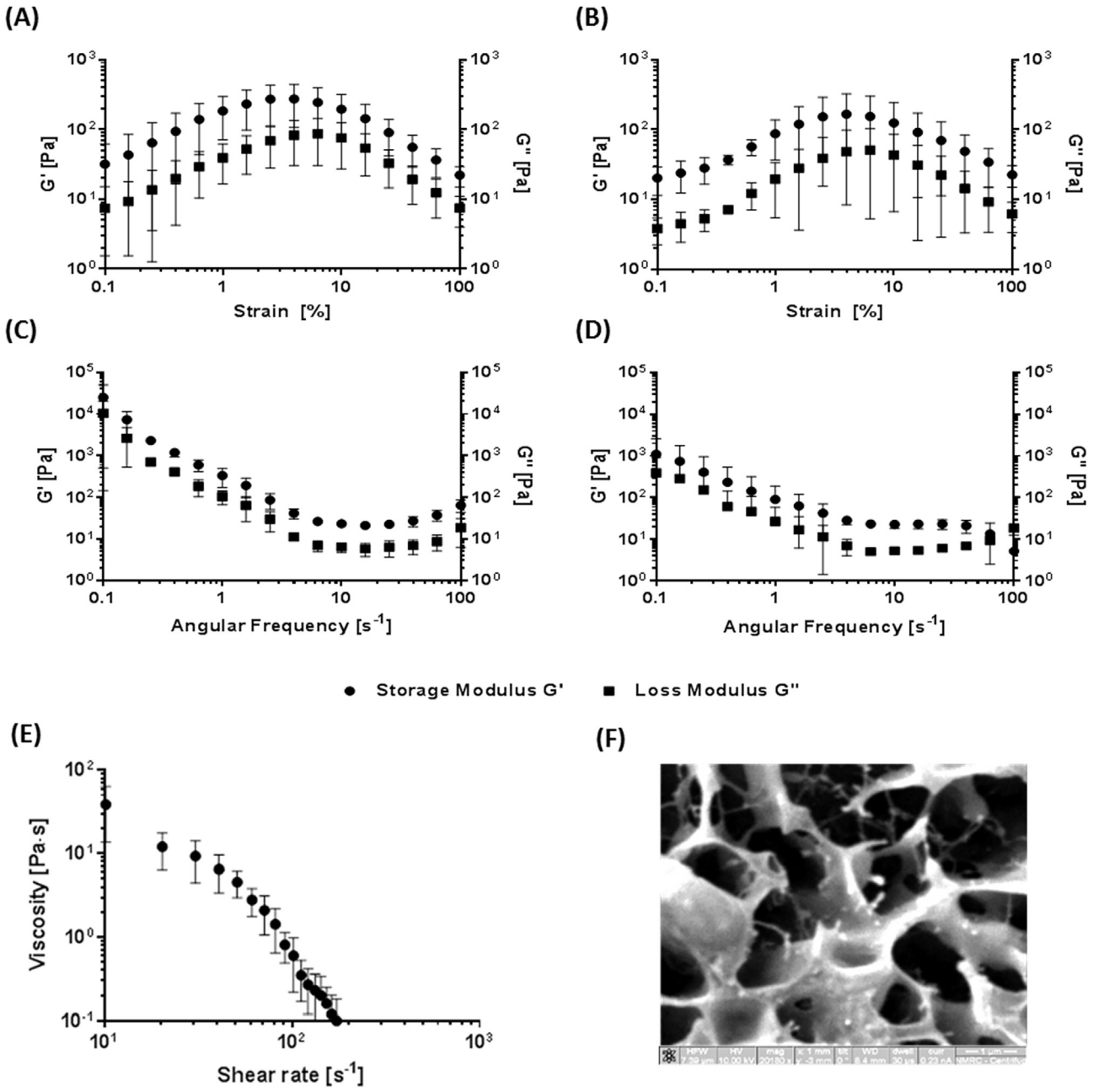


Fig. 6. Characterisation of porcine tracheal mucus. (A-D) macro-rheology of native and cleaned pig mucus. Storage (G′) and loss (G″) moduli of native (A) and cleaned mucus (B) measured as a function of deformation (amplitude sweeps experiments). Frequency = 10 rad/s. Storage (G′) and loss (G″) moduli of native (C) and cleaned mucus (D) measured as a function of frequency (frequency sweeps experiments). Strain = 0.1%; (E) viscosity of clean mucus as a function of shear stress. (A–E) Results are showed as MEAN ± SD (N = 3), (F) Cryo-SEM image of porcine tracheal mucus following the cleaning steps. The scale bars represent 1 μm and the magnification is ~×20,000. The image resolution was enhanced using the Luminance HDR® software.

performing oscillatory measurements. Oscillatory tests provide information about two key parameters, namely the storage modulus (G′) which defines the elastic behaviour of the samples and the loss modulus (G″) which characterises its viscous behaviour.

Similarly to human airway mucus [17,24,25], the mucus samples under investigation displayed G′ > G″ across a broad range of frequencies with the resulting G′ and G″ curves being almost parallel (Fig. 6A–D), which is typical of cross-linked gels. Both native and cleaned mucus showed the same viscoelastic behaviour, although the values for G′ and G″ were higher for the former than for the later, probably due to the different level of hydration between the two samples as well as the presence of blood cells in the raw sample.

Furthermore, the viscosity of mucus samples after the cleaning step decreased with an increase in the shear rate showing a shear-thinning behaviour typical of non-Newtonian fluids (Fig. 6E).

Finally, SEM visualisation of the internal structure of cleaned mucus revealed the characteristic mesh-like structure of the mucin network exhibiting an heterogeneous pore size distribution (Fig. 6F) [25].

Taken together, those data demonstrate that the cleaning process did not significantly modify the properties of porcine tracheal mucus.

3.5. Drug interactions with mucus

In order to better understand the role of airway mucus in particle dissolution and drug absorption in the lungs, both salbutamol sulfate and indomethacin powders were sprayed onto empty Transwell® or

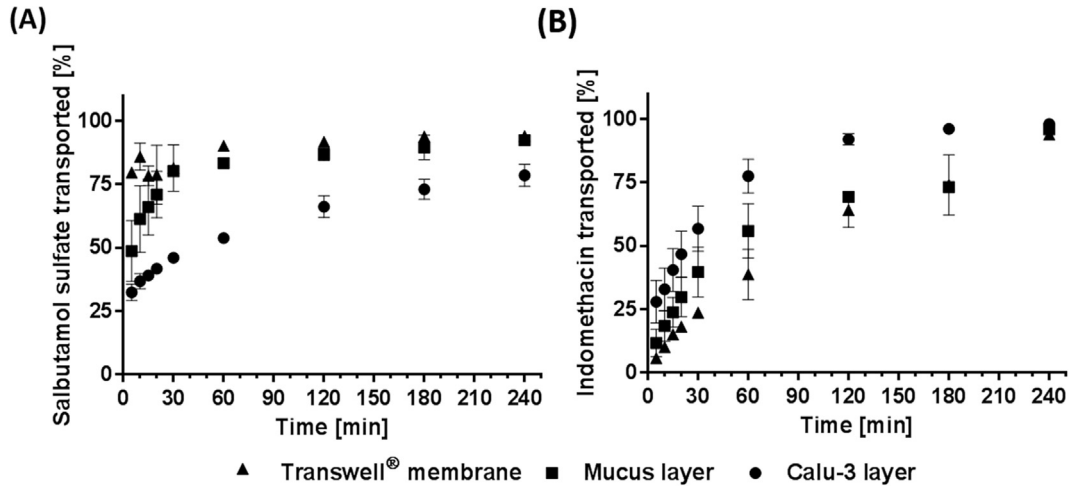


Fig. 7. Comparison of apical to basolateral (A → B) drug permeation profiles across empty Transwell® inserts, porcine tracheal mucus and Calu-3 layers after deposition as dry powders. (A) salbutamol sulfate; (B) indomethacin. Data across Calu-3 layers are those presented in Fig. 5. Data are presented as mean ± SEM (N = 2, n = 4).

Transwell® coated with porcine tracheal mucus.

As expected, salbutamol sulfate quickly permeated across the semi-permeable membrane with 79.8 ± 0.1% of the dose deposited measured in the basolateral chamber after 5 min (Fig. 7A). Conversely, indomethacin slowly diffused through the membrane and 88 ± 9 min were needed for 50% of the delivered dose to reach the receiver compartment (Fig. 7B and Table 2).

After delivery of salbutamol sulfate particles at the surface of mucus layers, 49 ± 8% of the initial dose applied had permeated into the basolateral chamber within the first 5 min (Fig. 7A and Table 2). Thereafter, the % of drug transported increased linearly over 30 min and then reached a plateau at ≈ 95% in 4 h. In contrast, indomethacin slowly diffused across the mucus layer. Its transport profile showed a linear trend over the first 30 min followed by a decrease in the permeation rate over the subsequent two hours until a plateau was reached at ≈90% of the dose deposited after 4 h (Fig. 7B).

Interestingly, when comparing the drug transport rate across Calu-3 cell layers, mucus layers and empty Transwell® inserts, an opposite trend was observed between the two drugs investigated (Fig. 7 and Table 2). The A → B permeation of salbutamol sulfate across the Transwell® membrane was slowed down by the presence of mucus and the effect was even more pronounced when Calu-3 cells were cultured on the inserts. In the case of indomethacin, the permeation rate across empty Transwell® inserts was the lowest measured but this was enhanced when the membrane was coated with mucus. However, diffusion into the aqueous phase of the Transwell® basolateral chamber seemed to remain a limiting step. When Calu-3 cell layers were added to the system, indomethacin transport was promoted further, probably due to partitioning of the drug into the phospholipidic bilayer of the cellular membrane.

4. Discussion

The design of dry powder formulations for inhalation providing optimal pulmonary drug retention and systemic absorption profiles remains a significant challenge to date. A major underlying cause is the lack of understanding of the factors affecting drug dissolution and permeation processes in the pulmonary tissue. Surprisingly, although the mucus layer lining the upper airways is the first biological barrier encountered by drugs depositing in the central part of the lungs, its role on particle fate in the lungs has hardly been explored.

The aim of this work was therefore to investigate in vitro the impact of airway mucus on drug dissolution and permeation at the air-epithelium interface. The inhaled bronchodilator salbutamol sulfate (a BCS class III drug, i.e., exhibiting a high solubility but a low permeability) and the anti-inflammatory drug indomethacin (a low solubility/high permeability BCS class II compound) were delivered as dry powders onto ALI Calu-3 layers, mucus covered Transwell® inserts or clean Transwell® using a bespoke aerosolisation system. Comparison of the permeation profiles across the three barriers showed that mucus hindered salbutamol transport but facilitated that of indomethacin. Furthermore, the presence of an epithelial cell layer underneath the mucus had, respectively, an additional negative or positive effect on the drug permeability.

A simple and low-cost deposition system based on a PennCentury™ Dry Powder Insufflator was first assembled to aerosolise drug particles at the surface of differentiated Calu-3 cells or porcine tracheal mucus covering the semi-permeable membrane of Transwell® inserts. More sophisticated apparatus have previously been employed to expose bronchial epithelial cell layers to drug particle sprays. Those include a Twin Stage Liquid Impinger [12,14,26], a Multistage Liquid Impinger [8,9], a cascade centripeter impactor [10], a modified version of the Andersen cascade impactor [13,27] or a specifically designed Pharmaceutical Aerosol Deposition Device on Cell Cultures (PADDOC) [11]. In comparison, no mechanism was available in the system described herein to eliminate particles in the non-respirable size range before they reached the biological layers, which resulted in large powder aggregates as well as particles with a 3–5 μm geometric diameter depositing onto them. It was nevertheless deemed suitable for studying drug particle fate at the air-epithelium interface in vitro as consistent and controlled clinically-relevant doses of drugs could be delivered in a narrow particle size range (3–5 μm) on up to six Transwell® inserts geometrically arranged underneath the powder spray. Furthermore, the permeability characteristics of salbutamol sulfate and indomethacin could be differentiated following aerosol delivery to Calu-3 layers. Similarly, distinct absorption profiles were obtained for salbutamol and budesonide or salbutamol and beclomethasone dipropionate after their exposure to the same cell culture model as inhalation dry powders using either a Multistage Liquid Impinger [8,9,11] or a modified Andersen cascade impactor [13].

For both drugs investigated in this study, transport across Calu-3 layers was more efficient after aerosol deposition than when they were applied in solution according to a conventional drug permeability measurement protocol. Similar observations have been reported for salbutamol, budesonide and salmeterol xinafoate in previous studies [8–12]. The faster transport rate upon dry powder exposure can be explained by an enhanced drug diffusion promoted by the high concentration gradient across the cell layers resulting from the dissolution

of drug particles in the small volume of Calu-3 lining fluid. In salbutamol case, the Organic Cation Transporters that have been shown to transport the drug across Calu-3 layers [12] could contribute to its increased transepithelial flux in a powder form since those carriers use the concentration gradient as a driving force. The initial burst absorption following particle deposition was more pronounced in our study than previously observed with salbutamol or budesonide inhalation powders in Calu-3 layers with similar barrier properties [8,12]. Much higher doses were delivered to the cell layers in those studies, which might have resulted in drug concentrations in the Calu-3 lining fluid approaching saturation. A slower drug absorption immediately after particle deposition might have therefore resulted from a delayed particle dissolution at the surface of the cell layers. In contrast, our transport data suggests a rapid solubilisation of drug particles in the Calu-3 apical medium despite the initial presence of large aggregates.

Interestingly, even though it is poorly soluble in water, indomethacin was rapidly and completely absorbed across the cell layers, indicating that particle dissolution was indeed not a rate-limiting step in the absorption process. Similarly, ~60% of budesonide [9] and ~44–50% of beclomethasone dipropionate [13], two inhaled drugs with a low solubility in water, were transferred across Calu-3 layers in 4 h when they were delivered as inhalation powders. All three drugs are highly permeable and the fast absorption of the fraction dissolved in the Calu-3 lining fluid is likely to promote particle dissolution. Furthermore, it has been demonstrated that mucus-like secretions accumulate on the apical surface of Calu-3 layers when these are differentiated in ALI conditions [15] and those could also play a role in drug dissolution.

In order to investigate the role of particle interactions with mucus in isolation, we developed an *in-vitro* model consisting of Transwell® inserts coated with a thin layer of porcine tracheal mucus. Native porcine tracheal mucus was used as a surrogate for human airway secretions due to a relative ease of access as well as reported similarities between pig and human mucosal secretions [28]. In addition, crude mucus collected from animal tissues provides a closer representation of the mucus barrier in vivo as compared to other in vitro models such as purified mucin or synthetic mucus since components other than mucin (e.g. lipids and proteins) are also present [28]. Similar systems to measure drug or particle diffusion across gastro-intestinal mucus have been previously described [29,30] but the mucus layer formed onto the Transwell® membrane was generally too thick (i.e., 400–900 μm) to represent the airway mucus (~10 μm in the upper airways [1]). Attempts were made to coat the surface of Transwell® membranes with the lowest possible amount of porcine tracheal mucus. However, full coverage was only achieved using a mucus volume of 12 μL (Fig. 2), which corresponds to a ~100 μm thick layer. Although this represents 10 times the mucus layer lining the upper airways, the fluids covering Calu-3 cells grown in ALI conditions have been estimated to amount to a similar volume (taking the surface area of the Transwell® membrane into account) [31], which allows a direct comparison of drug transport characteristics following particle deposition onto mucus or ALI Calu-3 layers.

The permeation of salbutamol sulfate and indomethacin across bare Transwell® inserts, mucus layers and Calu-3 cell layers followed an opposite order, indicating that the biological materials were acting as barriers in the case of salbutamol, but conversely enhanced indomethacin permeability. These observations were expected for salbutamol since the drug is readily soluble but exhibits a low permeability across biological membranes and its positive charge at physiological pH favours binding to the negatively charged mucin network by electrostatic interactions [32]. The effect of mucus on indomethacin transport was more surprising considering lipophilic drugs have been shown to have a high affinity for mucus [32]. However, as alluded above, the proteinic and lipidic components of the mucus probably facilitate the particle dissolution process and thus, increase the drug concentration gradient between the two Transwell® chambers, which promotes absorption. Moreover, indomethacin is an acidic drug which bears a negative charge at neutral pH. Its diffusion into the receiver compartment might therefore be further enhanced by electrostatic repulsions between the drug and the mucin fibres. A greater permeation of acidic drugs, such as ibuprofen across gastric mucus was indeed measured with an increase in pH, and this was attributed to the drug higher ionisation degree [33]. Indomethacin transport was the highest when Calu-3 layers were included in the in vitro model, which indicates that partitioning into the cell membrane can counterbalance potential solubility issues in conditions mimicking the airway environment.

The behaviour of indomethacin at the air-epithelium interface, i.e., rapid and complete absorption despite its low aqueous solubility, could not be predicted by the oral BCS, which supports the recent call for the establishment of a BCS specifically tailored to the inhaled route [34]. Our data, together with those reported previously with budesonide [8], are in line with increasing evidence that low solubility in water does not limit drug absorption in the lungs to the same extent as it does in the gastro-intestinal tract. In fact, only drugs that are classified as insoluble in water, such as certain inhaled corticosteroids, exhibit a dissolution limited absorption profile in vivo after pulmonary delivery [34]. This absence of impact of low water solubility on the pulmonary absorption of most inhaled drugs is generally attributed to the solubility enhancing properties of the lung surfactant found in the lower airways and alveoli [35]. Our data indicates that the mucus layer might also promote the solubilisation of drug particles depositing in the upper airways. The role of mucus in particle solubilisation might nevertheless have been overestimated in our model as the volume of secretions produced at the surface of the cell layers or added to the Transwells® inserts were proportionally much higher than that covering the airway epithelium in vivo. As expected, the mucus used in our study was less viscous than sputum collected from cystic fibrosis patients [17]. To which extent secretions from diseased lungs might differently affect drug particle fate in the airways requires further investigation.

5. Conclusion

This in vitro study is the first to specifically investigate the role of airway mucus in the interplay between drug dissolution and absorption at the air-epithelium interface in the lungs using physiologically-relevant models. The absorption characteristics of two drugs with different physico-chemical properties; i.e., salbutamol sulfate and indomethacin, were distinctly affected by a mucus layer, which indicates that drug-mucus interactions should be considered during the development of inhaled drugs. As in previous studies, a dissolution limited absorption across bronchial epithelial layers was not apparent for the low solubility/high permeability drug investigated. Our data suggests the dissolution of poorly water soluble drugs in the lung may be enhanced by contact with mucus components and further driven by partitioning into cell membranes. Hence, an accurate estimation of drug solubility in the lung might only be achieved in vitro with dissolution methods incorporating a biorelevant model of the airway mucosa such as ALI Calu-3 layers.

Acknowledgments

The authors thank Joon Han (Dillon) Goh for his help with the development of the mucus layer model used in this study.

This work was funded by the Engineering and Physical Science Research Council (EPSRC, UK), the University of Nottingham and GlaxoSmithKline. E. Cingolani was sponsored by the EPSRC Centre for Doctoral Training in Targeted Therapeutics & Formulation Science (grant n. EP/I01375X/1).

Note: all raw data created during this research are openly available from the corresponding author.

References

[1] J.S. Patton, J.D. Brain, L.A. Davies, J. Fiegel, M. Gumbleton, K.J. Kim, et al., The particle has landed – characterizing the fate of inhaled pharmaceuticals, J. Aerosol. Med. Pulm. Drug Deliv. 23 (2010) S71–S87.

[2] B. Forbes, B. Asgharian, L.A. Dailey, D. Ferguson, P. Gerde, M. Gumbleton, L. Gustavsson, C. Hardy, D. Hassall, R. Jones, R. Lock, J. Maas, T. McGovern, G.R. Pitcairn, G. Somers, R.K. Wolff, Challenges in inhaled product development and opportunities for open innovation, Adv. Drug. Deliv. Rev. 63 (2011) 69–87.

[3] T. Riley, D. Christopher, J. Arp, A. Casazza, A. Colombani, A. Cooper, M. Dey, J. Maas, J. Mitchell, M. Reiners, N. Sigari, T. Tougas, S. Lyapustina, Challenges with developing in vitro dissolution tests for orally inhaled products (OIPs), AAPS PharmSciTech. 13 (2012), https://doi.org/10.1208/s12249-012-9822-3.

[4] S. May, B. Jensen, M. Wolkenhauer, M. Schneider, C.M. Lehr, Dissolution techniques for in vitro testing of dry powders for inhalation, Pharm. Res. 29 (2012) 2157–2166.

[5] B. Forbes, P. Bäckman, D. Christopher, M. Dolovich, B.V. Li, B. Morgan, In vitro testing for orally inhaled products: developments in science-based regulatory approaches, AAPS J. 17 (2015) 837–852.

[6] S.P. Velaga, J. Djuris, S. Cvijic, S. Rozou, P. Russo, G. Colombo, A. Rossi, Dry powder inhalers: an overview of the in vitro dissolution methodologies and their correlation with the biopharmaceutical aspects of the drug products, Eur. J. Pharm. Sci. 113 (2018) 18–28.

[7] A. Kumar, W. Terakosolphan, M. Hassoun, K.K. Vandera, A. Novicky, R. Harvey, P.G. Royall, E.M. Bicer, J. Eriksson, K. Edwards, D. Valkenborg, I. Nelissen, D. Hassall, I.S. Mudway, B. Forbes, A biocompatible synthetic lung fluid based on human respiratory tract lining fluid composition, Pharm. Res. 34 (2017) 2454–2465.

[8] M. Bur, B. Rothen-Rutishauser, H. Huwer, C.-M. Lehr, A novel cell compatible impingement system to study in vitro drug absorption from dry powder aerosol formulations, Eur. J. Pharm. Biopharm. 72 (2009) 350–357.

[9] M. Bur, H. Huwer, L. Muys, C.-M. Lehr, Drug transport across pulmonary epithelial cell monolayers: effect of particle size, apical liquid volume and deposition technique, J. Aerosol Med. Pulm. Drug Deliv. 23 (2010) 119–127.

[10] R.C. Sadler, D. Prime, P.K. Burnell, G.P. Martin, B. Forbes, Integrated in vitro experimental modelling of inhaled drug delivery: deposition, dissolution and absorption, J. Drug. Del. Sci. Tech. 21 (2011) 331–338.

[11] S. Hein, M. Bur, T. Kolb, B. Muellinger, U.F. Schaefer, C.-M. Lehr, The Pharmaceutical Aerosol Deposition Device on Cell Cultures (PADDOCC) in vitro system: design and experimental protocol, Altern. Lab. Anim. ATLA. 38 (2010) 285–295.

[12] M. Haghi, D. Traini, M. Bebawy, P.M. Young, Deposition, diffusion and transport mechanism of dry powder microparticulate salbutamol, at the respiratory epithelia, Mol. Pharm. 9 (2012) 1717–1726.

[13] M. Haghi, D. Traini, P. Young, In vitro cell integrated impactor deposition methodology for the study of aerodynamically relevant size fractions from commercial pressurised metered dose inhalers, Pharm. Res. 31 (2014) 1779–1787.

[14] C.I. Grainger, M. Saunders, F. Buttini, R. Telford, L.L. Merolla, G.P. Martin, S.A. Jones, B. Forbes, Critical characteristics for corticosteroid solution metered dose inhaler bioequivalence, Mol. Pharm. 9 (2012) 563–569.

[15] C.I. Grainger, L.L. Greenwell, D.J. Lockley, G.P. Martin, B. Forbes, Culture of Calu-3 cells at the air interface provides a representativie model of the airway epithelial barrier, Pharm. Res. 23 (2006) 1482–1490.

[16] P. Russo, M. Stigliani, L. Prota, G. Auriemma, C. Crescenzi, A. Porta, R.P. Aquino, Gentamicin and leucine inhalable powder: what about antipseudomonal activity and permeation through cystic fibrosis mucus? Int. J. Pharm. 440 (2013) 250–255.

[17] M. Stigliani, M.D. Manniello, O. Zegarra-Moran, L. Galietta, L. Minicucci, R. Casciaro, E. Garofalo, L. Incarnato, R.P. Aquino, P. Del Gaudio, P. Russo, Rheological properties of cystic fibrosis bronchial secretion and in vitro drug permeation study: the effect of sodium bicarbonate, J. Aerosol. Med. Pulm. Drug. Deliv. 29 (2016) 337–345.

[18] P. Gerde, M. Malmlöf, L. Havsborn, C.O. Sjöberg, P. Ewing, S. Eirefelt, K. Ekelund, DissolvIt: an in vitro method for simulating the dissolution and absorption of inhaled dry powder drugs in the lungs, Assay Drug Dev Technol. 15 (2017) 77–88.

[19] J. Tamaoki, J. Nakata, K. Nishimura, M. Kondo, K. Aoshiba, K. Kawatani, A. Nagai, Effect of inhaled indomethacin in asthmatic patients taking high doses of inhaled corticosteroids, J. Allergy. Clin. Immunol. 105 (2000) 1134–1139.

[20] A.A. Onischuk, T.G. Tolstikova, I.V. Sorokina, N.A. Zhukova, A.M. Baklanov, V.V. Karasev, G.G. Dultseva, V.V. Boldyrev, V.M. Fomin, Anti-inflammatory effect from indomethacin nanoparticles inhaled by male mice, J. Aerosol. Med. Pulm. Drug. Deliv. 21 (2008) 231–243.

[21] M. Stigliani, R.P. Aquino, P. Del Gaudio, T. Mencherini, F. Sansone, P. Russo, Non-steroidal anti-inflammatory drug for pulmonary administration: design and investigation of ketoprofen lysinate fine dry powders, Int. J. Pharm. 448 (2013) 198–204.

[22] S. May, S. Kind, B. Jensen, M. Wolkenhauer, M. Schneider, C.-M. Lehr, Miniature dissolution testing of powders for inhalation, Dissolution Technologies (2015) dx. doi.org/10.14227/DT220315P40.

[23] B. Forbes, C. Ehrhardt, Human respiratory epithelial cell culture for drug delivery applications, Eur. J. Pharm. Biopharm. 60 (2005) 193–205.

[24] A.L. Innes, S.D. Carrington, D.J. Thornton, S. Kirkham, K. Rousseau, R.H. Dougherty, W.W. Raymond, G.H. Caughey, S.J. Muller, J.V. Fahy, Ex vivo sputum analysis reveals impairment of protease-dependent mucus degradation by plasma proteins in acute asthma, Am. J. Respir. Crit. Care. Med. 180 (2009) 203–210.

[25] X. Murgia, B. Loretz, O. Hartwig, M. Hittinger, C.M. Lehr, The role of mucus on drug transport and its potential to affect therapeutic outcomes, Adv. Drug. Deliv. Rev. 124 (2018) 82–97.

[26] M. Haghi, D. Traini, D.S. Postma, M. Bebawy, P.M. Young, Fluticasone uptake across Calu-3 cells is mediated by salmeterol when deposited as a combination powder inhaler, Respirology. 18 (2013) 1197–1201.

[27] H.X. Ong, D. Traini, C.Y. Loo, L. Sarkissian, G. Lauretani, S. Scalia, P.M. Young, Is the cellular uptake of respiratory aerosols delivered from different devices equivalent? Eur. J. Pharm. Biopharm. 93 (2015) 320–327.

[28] A.C. Groo, F. Lagarce, Mucus models to evaluate nanomedicines for diffusion, Drug Discov. Today. 19 (2014) 1097–1108.

[29] A.C. Groo, K. Mircheva, J. Bejaud, C. Ailhas, I. Panaiotov, P. Saulnier, T. Ivanova, F. Lagarce, Development of 2D and 3D mucus models and their interactions with mucus-penetrating paclitaxel-loaded lipid nanocapsules, Pharm. Res. 31 (2014) 1753–1765.

[30] H. Friedl, S. Dünnhaupt, F. Hintzen, C. Waldner, S. Parikh, J.P. Pearson, M.D. Wilcox, A. Bernkop-Schnürch, Development and evaluation of a novel mucus diffusion test system approved by self-nanoemulsifying drug delivery systems, J. Pharm. Sci. 102 (2013) 4406–4413.

[31] C.I. Grainger, L.L. Greenwell, G.P. Martin, B. Forbes, The permeability of large molecular weight solutes following particle delivery to air-interfaced cells that model the respiratory mucosa, Eur. J. Pharm. Biopharm. 71 (2009) 318–324.

[32] H.H. Sigurdsson, J. Kirch, C.M. Lehr, Mucus as a barrier to lipophilic drugs, Int. J. Pharm. 453 (2013) 56–64.

[33] L.R. Shaw, W.J. Irwin, T.J. Grattan, B.R. Conway, The influence of excipients on the diffusion of ibuprofen and paracetamol in gastric mucus, Int. J. Pharm. 290 (2005) 145–154.

[34] J.E. Hastedt, P. Bäckman, A.R. Clark, W. Doub, A. Hickey, G. Hochhaus, P.J. Kueh, C-M. Lehr, P. Mauser, J. McConville, R. Niven, M. Sakagimi, J.G. Weers, Scope and relevance of a pulmonary biopharmaceutical classification system AAPS/FDA/USP Workshop March 16–17th, 2015 in Baltimore, MD, AAPS Open (2016) 2:1 DOI: 10.1186/s41120-015-0002-x.

[35] S.C. Das, P.J. Stewart, The influence of lung surfactant liquid crystalline nanostructures on respiratory drug delivery, Int. J. Pharm. 514 (2016) 465–474.