Mucin dispersions as a model for the oromucosal mucus layer in in vitro and ex vivo buccal permeability studies of small molecules

Marxen, Eva; Mosgaard, Mette Dalskov; Pedersen, Anne Marie Lynge; Jacobsen, Jette

Published in:
European Journal of Pharmaceutics and Biopharmaceutics

Link to article, DOI:
10.1016/j.ejpb.2017.09.016

Publication date:
2017

Document Version
Peer reviewed version

Mucin dispersions as a model for the oromucosal mucus layer in *in vitro* and *ex vivo* buccal permeability studies of small molecules

Eva Marxen[a,1], *Mette Dalskov Mosgaard*[a,1,2], *Anne Marie Lynge Pedersen*[b] *and Jette Jacobsen*[a]

[a]Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, Copenhagen, Denmark, eva.marxen@sund.ku.dk, jette.jacobsen@sund.ku.dk

[b]Department of Odontology, Faculty of Health and Medical Sciences, University of Copenhagen, Nørre Allé 20, Copenhagen, Denmark, amlp@sund.ku.dk

[1] These authors contributed equally to this work.

[2]Present address: Department of Micro- and Nanotechnology, Technical University of Denmark, Ørsteds Plads, Kgs. Lyngby, Denmark, medmo@nanotech.dtu.dk

Corresponding author:

Associate Prof. Jette Jacobsen

Department of Pharmacy, University of Copenhagen

Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark

Email: jette.jacobsen@sund.ku.dk

Phone: +4535336299

Abstract

The mucus layer is believed to play a part in drug permeation across the oral mucosa. Human freeze-dried saliva (HFDS) and porcine gastric mucin (PGM) was evaluated as model for mucus layer per se or in conjunction with *in vitro* and *ex vivo* buccal permeability models.

Four small molecules (nicotine, mannitol, propranolol, caffeine) showed decreased permeability across mucin dispersions, compared to controls, and a greater effect was seen with HFDS than with PGM. Permeability of propranolol and caffeine across filter-grown TR146 cells was decreased by the presence of mucin, whereas no effect was found on nicotine and mannitol. Incubation of porcine buccal mucosa with mucin dispersions for 24 h compromised the integrity of the tissue, whereas 30 min incubation did not affect tissue integrity. Tissue incubation with mucin dispersions did not decrease nicotine permeability. For the studied model drugs, it is concluded that mucin dispersions constitute a minor barrier for drug diffusion compared to the epithelium.

Keywords

Mucus, permeability, barrier, buccal drug delivery, drug diffusion, absorption, small molecules

Abbreviations

BSA	Bovine serum albumin
HBSS	Hanks' balanced salt solution
HFDS	Human freeze-dried saliva
Log P	Logarithm of partition coefficient
Log D	Logarithm of distribution coefficient
MW	Molecular weight
P_{app}	Apparent permeability coefficient
PBS	Phosphate buffered saline
PGM	Porcine gastric mucin
SD	Standard deviation
TEER	Transepithelial electrical resistance

1 Introduction

Over the last years the interest in oromucosal drug delivery has increased due to advantages with this administration route. The harsh environment of the gastrointestinal tract is avoided and hepatic first pass metabolism is circumvented. Furthermore, the oral cavity is easily accessible for rapid self-administration and the formulation can quickly be removed in case of adverse events [1]. The epithelial surface of a mucosal membrane is covered by a mucus layer. The mucus layer has multiple physiological functions such as lubrication, hydration, and tissue protection. Mucus is a complex viscoelastic network, mainly consisting of water (95-99%) and mucins (1-5%) [2]. Mucins are glycoproteins which may be susceptible to changes in salt concentration or temperature, which can affect the mucin network and thereby the barrier properties of the mucus layer. It is believed that drug permeation through the mucus layer is affected by interactions and entanglement with the mucin network and by the unstirred water layer that mucus constitutes [3-5]. Mucus could retard drug diffusion by interacting with the drug and by decreasing diffusion rate due to higher viscosity. Reversely, mucus may increase solubility of the drug, and consequently increase the drug concentration adjacent to the epithelium.

Only few oromucosal formulations are on the market, and this may be due to lack of standardized *in vitro* methods to evaluate and optimize drug delivery systems [6]. Several models for assessing drug permeability through oral mucosa are known, however, they lack the mucus layer. Permeability across excised mucosal tissue can be studied in side-by-side diffusion cells such as modified Ussing chambers or Franz cells [7]. To the authors' knowledge it has not been confirmed that the mucus layer on the epithelium is intact after handling the excised tissue [8]. Filter grown cell cultures, such as TR146 cells derived from human buccal carcinoma have also been used to model oral mucosa [9, 10]. However, these cells do not produce mucus. Cell cultures that produce a mucus layer, such as HT29-MTX cells derived from human colon adenocarcinoma, form a single cell layer, and thus are

not suitable for modeling the oral mucosa, consisting of multiple cell layers [11]. Setups for studying drug transport across a mucus layer have previously been designed for Ussing chambers [12, 13]. However, they are either not suitable for liquid mucus or require several preparation steps for each replicate. Thus, there is a need for a simple setup containing mucus that can be used for high throughput studies.

Mucin can be obtained from mucus collected by gentle scraping of a mucosal membrane, from saliva, or from gastric fluid in animals or humans. Porcine gastric mucin (PGM) is commercially available as a crude mixture of mucin. Despite the gastric origin, PGM is commonly used to mimic mucus in the oral cavity [14, 15]. However, PGM may differ significantly from mucin found in the human saliva and on the oral mucosa, due to the difference in species and place of origin [16]. Furthermore, PGM has been through processing steps that may alter the properties of the molecules, thus there may be a need for a mucin source that is more similar to native oromucosal mucus.

Currently, oromucosal drug delivery is more feasible for small molecules, since they can diffuse the epithelial cell layers more easily than larger molecules. Therefore, four small molecules with different physicochemical properties were chosen as model drugs for this study; nicotine (MW = 162.2 g/mol; log $D_{6.8}$ = 0.30, calculated from log P = 1.43 and pKa = 7.9) [17], mannitol (MW = 182.2 g/mol; log P = -3.1) [18], propranolol (MW = 259.3 g/mol; log $D_{6.8}$ = 1.20) [19], and caffeine (MW = 194.2 g/mol; log P = -0.07) [20].

The aim of this study was to implement a mucin dispersion mimicking the mucus layer into *in vitro* and *ex vivo* permeability models and study the barrier properties of the mucin dispersion using small molecules. Thus, the permeability of nicotine, mannitol, propranolol and caffeine across a mucin dispersion, TR146 cells and porcine buccal mucosa was studied. The TR146 cells and the porcine buccal mucosa were incubated with mucin dispersions prior to the permeability experiments. Furthermore, the suitability of PGM and human freeze-dried saliva (HFDS) as sources of mucin was

evaluated. Implementation of a mucus layer will improve the predictability of the currently used permeability models, and this has to the authors' knowledge not previously been done.

2 Materials and methods

2.1 Materials

Potassium dihydrogen phosphate, calcium chloride, sodium hydrogen carbonate, sodium chloride and ortho-Phosphoric acid 85% were purchased from Merck KGaA (Darmstadt, Germany). Sodium phosphate monobasic anhydrous was obtained from Amresco (Solon, OH, USA). Potassium chloride was obtained from Riede-de Haën (Seelze, Germany). Hanks' balanced salt solution (HBSS) (10x), +$CaCl_2$, +$MgCl_2$ and 7.5% sodium bicarbonate was purchased from Gibco® life technologies (Grand Island, NY, USA). Nicotine bitartrat dihydrate was kindly donated from Fertin Pharma (Vejle, Denmark). Pearlitol® 160 (mannitol) was obtained from Roqette Pharma (Lestrem, France). Caffeine was purchased from VWR (Leuven, Belgium). [^{14}C]-mannitol (57.1 mCi/mmol), [^{3}H]-nicotine (80.4 Ci/mmol), [^{14}C]-caffeine (54.9 mCi/mmol), [^{3}H]-propranolol (18.6 Ci/mmol) and Ultima Gold™ liquid scintillation fluid were purchased from Perkin Elmer Inc. (Waltham, USA). Falcon 12-well tissue culture plates and cell culture inserts (polyethylene terephthalate membrane, 0.9cm^2 area, 0.4 mm pore size) were obtained from Becton Dickinson Labware (Franklin Lakes, NJ, USA). Bovine serum albumin (BSA), Mucin from porcine stomach, type II (PGM), (±)-propranolol hydrochloride, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Alcian blue 8GX, MTS-4-fluroescein, phenazine methosulfate and silicone oil were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Human freeze-dried saliva (HFDS) from pooled saliva samples (dialyzed and free of minerals) was kindly donated by the Department of Odontology, University of Copenhagen (Copenhagen, Denmark). The saliva was centrifuged at 2000 g for 10 min, dialyzed at 5°C for two days and finally lyophilized. Dialysis tubing visking, cellulose, type 36/32 inch, thickness 0.02 mm, width 44 mm, MWCO 14,000 was from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Deionized water was

collected from Milli-Q water system, SG Ultra Clear 2002 from Evoqua Water Technologies LLC (Warrendale, PA, USA).

2.2. Methods

2.2.1 Preparation of mucin dispersions

A buffer, named saliva buffer, containing 5 mM KH_2PO_4, 15 mM KCl, 1 mM $CaCl_2$ and 5 mM $NaHCO_3$ was prepared and adjusted to pH 6.8 [21]. PGM and HFDS were dispersed in saliva buffer and exposed to slow stirring at 5°C overnight. The concentrations of mucin varied in the experiments and are stated in the respective sections below.

2.2.2 Comparison of mucin sources

The mucin sources, PGM and HFDS, were visually compared in dry solid form. Furthermore, the pH of the two dispersions was measured. The viscosity of PGM and HFDS dispersions (2% w/v) were determined as described by [22]. Briefly, an AR-G2 plate and cone rheometer (TA instruments-Waters, New Castle, USA) was used with a 40 mm aluminum steel plate in diameter. A gap of 500 µm was selected (630 µL sample) and all the measurements were conducted at 37 °C. A protective casing, custom made at the Department of Pharmacy, University of Copenhagen (Denmark) was attached to the fixed heating plate and silicone oil (500 µL) was placed around the sample to prevent evaporation. The sample was equilibrated for 5 min before measurements were conducted. A steady state flow test to determine the viscosity was performed (shear rates 0.001-1000 s^{-1}, three consecutive measurements of 10 s with <5% variance). Four measurements were conducted per decade within a maximum time for each shear rate of 2 min (discarded if equilibrium was not reached within 2 min). TA Instruments Rheology Advantage Software (TA Instruments-Waters) was used to generate rheology data.

2.3 Permeability studies

2.3.1 Permeability across an isolated mucin dispersion

The new permeability device consisting of a test sample compartment in a tailor-made slider was developed for the modified Ussing chambers (Physiologic Instruments Inc., San Diego, CA, USA) to enable drug permeation study through e.g. an isolated mucus layer.

The new permeability device consists of three parts as shown in Fig. 1; a cylinder placed between a two piece slider. Dialysis membranes were rinsed in cold water and then soaked three times 5 min in 200 mL 100 °C hot water. The prepared dialysis membranes were placed between the slider and the cylinder (positions shown with A in Fig. 1) and held in place by joining the parts. The mucus dispersion was then added with a syringe through a small hole in the slider and cylinder. The diffusion area was 0.50 cm^2 and the thickness of the mucin layer (cylinder length) was 6.0 mm. The composition of the receptor and donor fluid is given in Table 1. The permeability of nicotine, mannitol, propranolol and caffeine was studied across saliva buffer, 2% (w/v) PGM dispersion or 2% (w/v) HFDS dispersion.

The study was conducted in modified Ussing chambers as previously described by Holm et al. [23]. Briefly, 2.0 mL of donor and receptor fluid was added to the respective compartments. Stirring was ensured by supplying hydrated atmospheric air, and the temperature was kept at 36±1°C. Receptor samples of 100 µL were taken from the receptor compartment at 5, 10, 20 and 30 min and then every 30 min up to 5 h. From the donor compartment, 100 µL was taken in triplicates at the start and end of the experiment. After sampling the compartments were replenished. At the end of the experiment a 100 µL sample was taken from the mucin dispersions or saliva buffer. Drug content was determined in the dialysis membranes by rinsing off excess drug and placing the dialysis membrane in a scintillation vial for measurement.

2.3.2 Permeability across TR146 cells

The TR146 cell line was provided by Imperial Cancer Research Technology (London, UK) and cultivated and grown on filters as previously described [24]. On the first day of the experiment filter-grown cells aged 25-27 days were washed on the apical side and the growth medium was changed on the basolateral side. The cells were incubated on the apical side with 200 µL 4% (w/v) PGM or HFDS dispersion. Cells incubated with saliva buffer were used as a control. The cells were incubated at 37°C in 5% CO_2/95% air at 98% humidity for 24 h.

On the second day of experiment, the initial transepithelial electrical resistance (TEER) was measured on the control cells (Endohm and voltmeter EVOM from World Precision Instruments (Sarasota, FL, USA)). The composition of donor and receptor fluids is shown in Table 2. 1600 µL receptor medium was added to the basolateral side and 220 µL donor solution was added to 200 µL mucin dispersion or saliva buffer on the apical side. The experiment was conducted at 37°C using a thermostatic horizontal shaker, 100 rpm, (Edmund Bühler, swip Type KL-2) (Hechingen, Germany). 100 µL samples were taken from the basolateral side at time 10 min, 30 min, 45 min, 60 min and then every 30 min up to 240 min, and the compartment was replenished with receptor fluid. At 10 min a donor sample of 20 µL and at 240 min three donor samples of 20 µL were taken. The first samples were taken at 10 min to allow the donor fluid to mix with the mucin dispersion. After the permeability experiment the cells were washed twice with HBSS buffer on both apical and basolateral side, TEER was measured and a MTS-PMS viability test was conducted as described by Eirheim et al. [25].

2.3.2.1 Mucin attachment to cell surfaces

After 24 h incubation the mucin dispersions or saliva buffer was gently removed and the cells were washed in 0.1 M phosphate buffered saline (PBS) pH 6.8. 200 µL Alcian blue solution (1% (v/v) Alcian blue and 3% (v/v) acetic acid in water) was added to the apical side and the setup was shaken (100 rpm) for 5 min. The Alcian blue solution was gently removed and the cells were washed twice in PBS pH 6.8. The cells were than examined under an Olympus BH2 light microscope (Olympus,

Tokyo, Japan) and representative pictures were taken with an AxioCam ERc5s (Zeiss, Jena, Germany).

2.3.3 Permeability across porcine buccal mucosa

Porcine buccal mucosa was obtained from healthy experimental control pigs (approx. 30 kg Danish Landrace/Yorkshire x Durox (D-LY)). Immediately after euthanization of the pigs the cheeks were excised using a scalpel and placed in ice cold PBS pH 7.4. Within 3 h the excised cheeks were frozen in 40% (w/v) glycerol and 20% (w/v) sucrose in PBS pH 7.4, and on the day of experiment the tissue was thawed as described by Marxen et al. [26]. The buccal mucosa was trimmed with surgical scissors and sliced to a thickness of 792 µm ± 88 µm (n=36) using a Stadie-Riggs tissue slicer (Thomas Scientific, Swedesboro, NJ, USA). The buccal mucosa was mounted on slider P2405 from physiologic instruments Inc. (San Diego, CA, USA) (exposed area 0.40 cm^2). The sliders were placed in the upper compartment of a desiccator, the epithelium facing upwards. The lower compartment of the desiccator was filled with NaCl saturated water to ensure high humidity. 100 µL saliva buffer, 5% (w/v) PGM or HFDS dispersion was added to the apical surface of the tissue and incubated at 37 °C for 24 h, 30 min or the tissue was used immediately. When incubating for 24 h, the sliders were placed in PBS pH 7.4 to keep the basolateral side of the tissue moist.

The compositions of the donor and receptor solutions are presented in Table 1. The permeability experiment was conducted as described in Section 2.3.1, with few changes: After the experiment, the tissue was dissolved in approximately 1.0 mL concentrated phosphoric acid, heated to 70°C and a 100 µL sample was taken for quantification of the radiolabeled model drugs.

2.4 Quantitative analysis

2 mL Ultima Gold™ liquid scintillation fluid was added to all samples before whirl-mixing. Quantitative analysis of [^{3}H]-nicotine, [^{14}C]-mannitol, [^{3}H]-propranolol and [^{14}C]-caffeine was performed by liquid scintigraphy using a Tri-Carb 2910TR Liquid Scintillation Analyzer (Perkin Elmer, Waltham, MA, USA).

2.5 Data analysis

Accumulated amount (Q, mol) of nicotine, mannitol, propranolol and caffeine appearing in the receptor compartment was plotted as a function of time (t). Steady state flux (J_{SS}, mol s^{-1} cm^{-2}) was calculated as the slope of the linear section of this curve (R^2 above 0.99 for isolated mucin layer and porcine buccal mucosa; R^2 above 0.95 for TR146 cells), using Equation 1, where A (cm^2) is the area of diffusion.

$$J_{SS} = \frac{dQ}{dt} * \frac{1}{A} \qquad \text{Equation 1}$$

Steady state flux was obtained at different time intervals: Isolated mucin layer (90-210 min); porcine buccal mucosa (90-300 min); TR146 cells (45-150 min for nicotine and caffeine, 120-240 for propranolol and mannitol).

The apparent permeability coefficient (P_{app}) was calculated from Fick's first law of diffusion ($J_{SS} = P_{app} \cdot \Delta C$). When sink conditions are upheld, Fick's first law can be simplified to Equation 2, under the assumption that $C_{donor} >> C_{receptor}$. Thus P_{app} (cm s^{-1}) was calculated from Equation 2, where $C_{0,donor}$ (mol cm^{-3}) is the initial donor concentration.

$$P_{app} = \frac{Jss}{C_{0,donor}} \qquad \text{Equation 2}$$

The total recovery (% of initial drug added) of the four model drugs was the sum of the accumulated drug amount (Q) at the end of the experiment in receptor compartment, donor compartment and drug amount in isolated mucin dispersion, saliva buffer, or porcine buccal mucosa, respectively. In the

cell studies the filter grown TR146 cells were used for viability testing, and thus the drug retained in cells was not quantified. Due to low recovery of propranolol, an additional experiment was performed to quantify the amount of propranolol in filter and cells.

2.6 Statistical analysis

Data in this study are presented as means with standard deviations (SD) unless otherwise stated. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used to determine statistically significant difference between three or more means. An unpaired *t*-test was used to compare two means. Both tests were performed assuming equal variance and normal distribution of data. GraphPad Prism 7 for Windows, from GraphPad Software Inc. (La Jolla, CA, USA) was used for all statistical calculations. *P*-values below 0.05 were considered statistically significant.

3 Results and discussion

3.1 Comparison of mucin sources

The mucin sources appeared very different in solid dried forms. PGM is a fine brownish powder whereas HFDS has a bulky white fibrous structure. The pH of PGM dispersion after stirring overnight was 4.4 ± 0.3 (n=9) and the pH of HFDS dispersion was 6.6 ± 0.4 (n=9). A *t*-test showed that the pH of PGM dispersion was significantly different from pH of the HFDS dispersion, which was expected since PGM is gastric mucin, and thus originates from an acidic environment, whereas HFDS is derived from saliva with an average pH of 6.8. In the present studies the mucin dispersions were adjusted to pH 6.8 to mimic pH in the oral cavity. Mucin contains acidic functional groups, including sialic acid [27]. Adjusting pH will affect the degree of ionization of the acidic groups, thus a change in pH will likely affect the interactions between mucin strands, causing enlargement or compaction of the mucin network. Therefore, it is advantageous to use a mucin-type with an innate pH as close

255 to pH 6.8 as possible, thus HFDS would be a more suitable source for mucin used to mimic mucus in
256 the oral cavity.
257 The viscosity was determined to compare the shear thinning properties and viscosities of the two
258 types of mucin dispersion.
259 "The viscosity of a mucin dispersion demonstrates the degree of entanglement of mucin, and it is
260 known that increased viscosity correlates with decreased permeability through the dispersion [28].
261 The viscosities as a function of shear rate was determined for a 2% (w/v) PGM dispersion and a 2%
262 (w/v) HFDS dispersion. The results are presented in Fig. 2. The HFDS dispersion had a higher
263 viscosity than the PGM dispersion throughout the whole shear rate range (Fig. 2). Furthermore, a
264 decrease in viscosity with increasing shear rates (shear thinning) was observed for the HFDS
265 dispersion whereas the PGM dispersion had a constant viscosity at shear rates above 1 s^{-1}. At shear
266 rates below 1 s^{-1} several of the measurements on PGM gave negative values and were excluded. It
267 has been estimated that the movements in the oral cavity during speaking and swallowing correspond
268 to shear rates of approximately 1-160 s^{-1} [29, 30]. The rheology measurements of the PGM dispersion
269 was considered acceptable, since the measurements above 1 s^{-1} shear rate was consistent, and this is
270 the range of shear rate of interest in oromucosal drug delivery. It is known that saliva and mucus
271 exhibit shear thinning properties [29], thus, based on the rheology results presented in Fig. 2 showing
272 HFDS to exhibit shear thinning unlike PGM, it appeared that HFDS was more similar to native mucus
273 and saliva. The observed differences may be due to differences between mucin from porcine gastric
274 fluid and human oral mucin [16]. Before the rheology measurements, pH was adjusted to 6.8, which
275 may affect protein conformation and interactions [31]. PGM originates from a gastric environment
276 with pH 1-2, and thus may be more affected by the pH increase compared to HFDS. Furthermore, it
277 is unknown what processing steps PGM has been subjected to, and the processing may have affected
278 the mucin molecules in a manner that decreased the gel forming properties. Measures of pH and

viscosity indicate that the HFDS is a more suitable mucin source than PGM, in terms of mimicking saliva and the mucus layer in the oral cavity. However, PGM, but not HFDS, is commercially available."

3.2 Permeability studies

3.2.1 Permeability across an isolated mucin dispersion

The P_{app} was determined for the four model drugs permeating across the 2% (w/v) PGM, 2% (w/v) HFDS dispersions and the saliva buffer. The results are presented in Fig. 3, and the P_{app} values are given in Table 3. The P_{app} of both nicotine and mannitol across the saliva buffer was significantly higher than the P_{app} across the PGM dispersion, which in turn was higher than P_{app} across the HFDS dispersion (Fig. 3a and b). The P_{app} of propranolol across the saliva buffer was significantly higher than the P_{app} across the PGM dispersion and the HFDS dispersion. However, no significant difference was observed between the PGM and the HFDS dispersions (Fig. 3c). For caffeine the P_{app} across the saliva buffer and the PGM dispersion was significantly higher than the P_{app} across the HFDS dispersion. No significant difference was observed between the PGM dispersion and the saliva buffer (Fig. 3d).

Overall, it appears that the mucin dispersions act as a barrier to drug diffusion, and that HFDS constitutes a larger barrier than PGM. The diffusion of a drug is dependent on the molecular size of the drug and the viscosity of the medium. As described in Section 3.1 the viscosity of HFDS was higher, especially at low shear rates. The mucin dispersions are unstirred in this setup, thus the difference in viscosity may be an explanation for the decreased permeability found across HFDS. However, the mucin dispersions are not simply an unstirred water layer as they contain glycoproteins that potentially interact with the drug molecule. Several studies have shown the ability of mucin to

interact with a broad range of molecules [32, 33]. A detailed study of interactions between the model drugs and mucin is beyond the scope of this study.

The cylinder containing the mucus dispersions or the saliva buffer was 6.0 mm thick. The thickness of the mucus layer in the oral cavity shows regional variations, and has been determined to be approximately 50 µm in the buccal area [34]. That is approximately 100 times thinner than in this setup and it is likely that the permeability differences found will be negligible compared to the barrier exerted by the epithelium.

3.2.2 Permeability across TR146 cells

The TR146 cells were incubated with 4% (w/v) of PGM, 4% (w/v) HFDS dispersion or saliva buffer for 24 h, before determining P_{app} of the four model drugs across the cells.

The results are presented in Fig. 4, and the exact values are given in Table 3. No significant difference was seen between the P_{app} of nicotine across the cells incubated with saliva buffer, PGM dispersion and HFDS dispersion (Fig. 4a). The P_{app} of mannitol across the PGM dispersion was significantly lower than P_{app} across the cells incubated with saliva buffer or the HFDS dispersion. However, no significant difference was seen between P_{app} of mannitol across the saliva buffer and HFDS dispersion (Fig. 4b). The P_{app} of propranolol and caffeine across the PGM and HFDS dispersion was significantly lower than the P_{app} across saliva buffer. However, no significant difference was observed between the PGM and HFDS dispersions (Fig. 4c and d).

The effects from the mucin dispersion on nicotine permeability across an isolated layer (Section 3.2.1), is not confirmed when the TR146 cells are present, indicating that the cell layers constitute a larger barrier to nicotine, than the one provided by the mucin dispersions. Also the differences found between HFDS and PGM dispersions, for propranolol and caffeine disappeared, which indicates that the effect shown with a 6.0 mm mucin dispersion layer is larger than the barrier exerted by the mucus layer *in vivo*. Moreover, the findings indicate that certain interactions between the drug molecules

and mucin did take place, as nicotine appeared less affected by mucin dispersions than the other model drugs. P_{app} for propranolol was decreased by 47% and 58% in the presence of PGM and HFDS dispersions, respectively, suggesting a higher degree of interaction with propranolol compared to the other model drugs. In accordance with previous findings we found that mucus constituted an increasing barrier to drug diffusion with increasing lipophilicity of the diffusing drug [11, 35]. This substantiates the need of a model as the one presented here, to determine whether the mucins affect the permeability of a drug molecule of interest.

3.2.2.1 Integrity and viability of TR146 cells

Following the permeability study TEER and MTS/PMS tests were used to determine the integrity and viability, respectively, of the TR146 cell layers. TEER values are an indication of electron transport particularly via the paracellular pathway. The initial TEER value of the TR146 cells was determined to be 145±43 Ω cm^2 (n=19) after 24 h incubation with saliva buffer. After the 4 h permeability study, the TEER value for the TR146 cells incubated with PGM dispersion was 339±100 Ω cm^2 (n=13), with HFDS dispersion was 196±31 Ω cm^2 (n=11) and with saliva buffer was 157±59 Ω cm^2 (n=14). The TEER value for PGM incubated cells were significantly higher (p<0.05) than the initial TEER value, and no significant differences were found between HFDS and buffer incubated cells and the initial TEER value. The initial TEER value of 145 Ω cm^2 indicate that the integrity of the TR146 cells was maintained after 24 h of incubation with saliva buffer, which is supported by Sander et al. who reported an initial TEER value of 151±38 Ω cm^2 (n=119) on filter-grown TR146 cells. Furthermore, the integrity of the cell layers is not compromised during the permeability study. The TEER values were increased for the cells incubated with PGM indicating that incubation with mucin dispersions decreased electron transport. The findings are in line with Pontier et al. who showed that TEER values were increased after the HT29-MTX cells had intrinsically produced a mucus layer from mucus secreting goblet cells [36].

The integrity of the TR146 cell layers was supported by the relatively low P_{app} of mannitol. Mannitol is routinely used as a marker for integrity of the cell layers, in particular the paracellular pathway. Jacobsen et al. showed that mannitol permeability across TR146 cell layers grown for 30 days was $5.2 \cdot 10^{-6}$ cm/s, and since the mannitol permeability found in this study was between 1.4 and $3.9 \cdot 10^{-6}$ cm/s (Table 3), the integrity of the cell layers seems to be maintained [24].

The MTS/PMS assay measures dehydrogenase activity in cells as a measure of cell viability. The dehydrogenase activity after a 4 h permeability study in TR146 cells incubated with saliva buffer was assumed to be 100% viable and the cellular viability of the TR146 cells incubated with PGM and HFDS dispersions were determined relative to the cells incubated with saliva buffer. According to Nielsen and Rassing [37], TR146 cells were not sensitive towards pH changes in the range 5.5-9.0, or to osmolality changes in the range approximately 100-400 mOsm, thus it was assumed that the saliva buffer would not affect viability of the TR146 cells. After 24 h incubation with mucin dispersion and a 4 h permeability study the viability in cells exposed to nicotine and mannitol was 106% ± 4% (PGM) and 102% ± 2% (HFDS), and in cells exposed to propranolol and caffeine the viability was 107% ± 2% (PGM) and 114% ± 21% (HFDS).

MTS/PMS assays are often used to measure cellular toxicity of drugs. However, in this study it was mainly used as a measure of the impact of incubation with PGM and HFDS dispersions compared to saliva buffer (control). The selected concentrations of the model drugs have been validated in previous studies, thus not expected to be toxic for the TR146 cells [10, 17, 38]. In this study the viability of the cells did not decrease after exposure to the mucin dispersions, compared to incubation with saliva buffer. The high integrity and viability supports the feasibility of the *in vitro* TR146 cell model to study the effect of mucin on drug permeation.

3.2.2.2 Mucin attachment to cell surfaces

After incubation with mucin dispersions the attachment of mucin to the TR146 cells was qualitatively examined by staining the cells with Alcian blue dye. Representative images of the stained cells are presented in Fig. 5. Alcian blue stains the negatively charged groups in mucin at physiological pH [39].

The TR146 cells incubated with saliva buffer (Fig. 5, left) showed scattered blue spots on the cell layer surface. The staining of the TR146 cells incubated with PGM dispersion resembles the cells incubated with saliva buffer solution. However, a few more densely stained areas were found on the cells (Fig. 5, middle). The TR146 cells incubated with HFDS dispersion displayed larger stained areas (Fig. 5, right). The Alcian blue stained cell surfaces clearly indicated a larger amount of mucin attached to the cell surface when incubating with the HFDS dispersion. The attachment could be due to interactions between the cell surface and mucin. The small amount of blue stains present on the TR146 cells incubated with saliva buffer could indicate lack of washing during the staining procedure or that other glycoproteins attached to the epithelial cell surface interacts with Alcian blue [40].

3.2.3 Permeability across porcine buccal mucosa

It was attempted to reintroduce a mucus layer to excised porcine buccal mucosa by incubating the tissue with 5% (w/v) mucin dispersions. Due to limitations in tissue supply only nicotine and mannitol were studied.

Initially, the tissue was incubated with saliva buffer or PGM for 24 h to allow time for interactions between cell-bound mucin and mucin from the dispersions. The studies of mannitol (Fig. 6, b) indicated that the integrity of the porcine buccal mucosa was compromised after 24 h incubation compared to non-incubated tissue, for which mannitol amount was below the quantification limit (10x noise/background). The lost integrity after 24 h incubation could be ascribed to hydration of the tissue, which was further implied by visual swelling of the tissue after 24 h incubation and the permeability study [41].

Secondly, porcine buccal mucosa was incubated for only 30 min with mucin dispersions or saliva buffer. The choice of 30 min was based on previous studies allowing buccal mucosa to equilibrate for 30 min, prior to the experiment [42]. As expected, after 30 min incubation with PGM, HFDS or saliva buffer the permeated amount of mannitol was below the quantification limit (Fig. 6b), indicating that tissue integrity was not compromised. Accordingly, the effect of mucin dispersions on mannitol permeability across buccal mucosa cannot be determined.

The P_{app} values of nicotine after different incubation times were not affected by the possible loss of tissue integrity, hence the P_{app} values of nicotine after 24 h incubation was not significantly higher than P_{app} for non-incubated tissue (Fig. 6a). The P_{app} of nicotine after incubation for 30 min in PGM dispersion was significantly higher than the P_{app} found with no incubation, 24 h in both saliva buffer and PGM dispersion and after 30 min in HFDS dispersion. No significant difference was observed between any of the other treatments. The significantly higher P_{app} value after incubation for 30 min in PGM dispersion could indicate an enhancing effect on nicotine permeability. However, since none of the findings from Section 3.2.1 and 3.2.2 indicate an enhancing effect of the PGM dispersion this is believed to be coincidental.

Nicotine is more lipophilic than mannitol and permeates through both the transcellular pathway and the paracellular pathway [43]. At pH 6.8, 93% of the nicotine will be mono-protonated. However, the non-ionized form passing via the transcellular pathway contributes more to the apparent permeability of nicotine than the mono-protonated form following the paracellular pathway [17, 43]. As a result, the possible decrease in barrier integrity for mannitol has insignificant effect on the permeation of nicotine, as it predominantly follows the transcellular pathway.

In Section 3.2.1 and Fig. 3 it is shown that PGM and HFDS decreases drug permeation across an isolated mucin dispersion. This finding was not supported from the results with porcine buccal mucosa. Except for 30 min incubation with PGM dispersion, which increased nicotine permeability,

no other treatments significantly affected the permeability of nicotine compared to the permeability across non-incubated tissue. This could be explained by lack of interactions between the tissue and the applied mucin. The extent of interaction could be decreased by damage of the mucosal surface from handling, such as freezing, thawing and slicing of the tissue. Bio-incompatibility between the tissue and the applied mucin could also affect the degree of interaction. Another possible explanation for the lack of effect on nicotine permeability could be that the epithelium constitutes a much larger barrier to nicotine, hence a possible effect from the mucin dispersions become negligible in comparison.

3.2.4 Total drug recovery

After the permeability studies the total recovery of the four model drugs was determined. The results are presented in Table 4. For nicotine, mannitol and caffeine the recoveries were acceptable, whereas, the recovery of propranolol was lower.

The relatively poor recovery for propranolol is likely due to adsorption to the Ussing chamber walls and cell inserts. It has previously been shown that diazepam, a lipophilic small molecule, adsorbed to Ussing chamber walls during transport studies [44]. In the cell studies the recovery was initially measured without adding propranolol content in the filter-grown cells, as these were used in viability testing; however, the recovery of propranolol was only 56.5% (Table 4). Therefore, an additional experiment with propranolol was performed, where the propranolol content of the filter and cells was added to the recovery resulting in a propranolol recovery of 91.4% (Table 4). It is likely that propranolol, due to its lipophilic nature, was mostly located in the lipophilic cell layers. Other studies have also shown that approximately 30% of propranolol was located in filter-grown TR146 cells after a permeability study [24].

4 Conclusions

The pH of the HFDS dispersion and its shear thinning properties is more similar to native oral mucus than PGM, thus HFDS is more suitable for mimicking the mucus layer in the oral cavity.

A new one compartment device has been applied to study drug permeability across an isolated mucin dispersion or saliva buffer. Four model drugs showed decreased permeability across mucin dispersions and a greater effect was seen with HFDS than with PGM. The effect on drug permeation could be caused by differences in viscosity between the mucin dispersions and the saliva buffer or by interactions between drug molecules and mucin.

TR146 cells were subjected to mucin dispersions 24 h prior to a permeability study. This model may become a promising *in vitro* method to study drug permeation across a mucus layer in conjunction with a multi-layered epithelium. The integrity and viability of the TR146 cells were maintained during 24 h incubation and a subsequent permeability study. Permeability of propranolol and caffeine was decreased by the presence of mucin, however, this was not shown for nicotine and mannitol. The HFDS attached to the TR146 cell surface to a higher extent than PGM, however, the strength and mechanism behind the attachment needs further studies.

Incubation of porcine buccal mucosa with mucin dispersions for 24 h caused compromised integrity of the tissue. Mannitol permeability across non-incubated tissue and tissue incubated for 30 min was too low to be quantified. Tissue incubation with mucin dispersions did not decrease nicotine permeability, indicating that the epithelium constitute the main barrier for nicotine diffusion across porcine buccal mucosa. Further studies are needed to determine whether it is possible to reintroduce mucin molecules on the tissue surface.

Acknowledgements

The Nicotine Science Center, Fertin Pharma A/S, Vejle, Denmark is thankfully acknowledged for their funding of this project. The authors gratefully acknowledge Imperial Cancer Research Technology (London, UK) for providing the TR146 cell line. Helle Kristensen is thanked for

providing the schematic drawing of the slider. Assistant Professor Andrew Richard Williams is acknowledged for his kind donation of whole cheek specimens from experimental control pigs.

References

[1] A.H. Shojaei, Buccal mucosa as a route for systemic drug delivery: a review, J. Pharm. Pharmaceut. Sci. 1 (1998) 15-30.

[2] V.F. Patel, F. Liu, M.B. Brown, Advances in oral transmucosal drug delivery, J. Control. Release. 153 (2011) 106-116.

[3] P.G. Bhat, D.R. Flanagan, M.D. Donovan, The limiting role of mucus in drug absorption: Drug permeation through mucus solution, Int. J. Pharm. 126 (1995) 179-187.

[4] T. Loftsson, Drug permeation through biomembranes: cyclodextrins and the unstirred water layer, Pharmazie. 67 (2012) 363-370.

[5] J. Griessinger, S. Dunnhaupt, B. Cattoz, P. Griffiths, S. Oh, S. Borros i Gomez, M. Wilcox, J. Pearson, M. Gumbleton, M. Abdulkarim, I. Pereira de Sousa, A. Bernkop-Schnurch, Methods to determine the interactions of micro- and nanoparticles with mucus, Eur. J. Pharm. Biopharm. 96 (2015) 464-476.

[6] V.F. Patel, F. Liu, M.B. Brown, Modeling the oral cavity: in vitro and in vivo evaluations of buccal drug delivery systems, J. Control. Release. 161 (2012) 746-756.

[7] H. Sohi, A. Ahuja, F.J. Ahmad, R.K. Khar, Critical evaluation of permeation enhancers for oral mucosal drug delivery, Drug Dev. Ind. Pharm. 36 (2010) 254-282.

[8] E. Meng-Lund, E. Marxen, A.M. Pedersen, A. Mullertz, B. Hyrup, R. Holm, J. Jacobsen, Ex vivo correlation of the permeability of metoprolol across human and porcine buccal mucosa, J. Pharm. Sci. 103 (2014) 2053-2061.

[9] J. Jacobsen, E.B. Nielsen, K. Brondum-Nielsen, M.E. Christensen, H.B. Olin, N. Tommerup, M.R. Rassing, Filter-grown TR146 cells as an in vitro model of human buccal epithelial permeability, Eur. J. Oral Sci. 107 (1999) 138-146.

[10] H.M. Nielsen, M.R. Rassing, TR146 cells grown on filters as a model of human buccal epithelium: IV. Permeability of water, mannitol, testosterone and beta-adrenoceptor antagonists. Comparison to human, monkey and porcine buccal mucosa, Int. J. Pharm., 194 (2000) 155-167.

[11] I. Behrens, P. Stenberg, P. Artursson, T. Kissel, Transport of lipophilic drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells, Pharm. Res. 18 (2001) 1138-1145.

[12] D.A. Norris, P.J. Sinko, Effect of size, surface charge, and hydrophobicity on the translocation of polystyrene microspheres through gastrointestinal mucin, J. Appl. Polym. Sci. 63 (1997) 1481-1492.

[13] Physiologic Instruments, Sliders for Snapwell Chambers - P2319 in, http://www.physiologicinstruments.com/Sliders_for_Snapwell_Chambers_P2319_p/p2319.htm. Accessed 31 March 2017.

[14] E. Meng-Lund, C. Muff-Westergaard, C. Sander, P. Madelung, J. Jacobsen, A mechanistic based approach for enhancing buccal mucoadhesion of chitosan, Int. J. Pharm., 461 (2014) 280-285.

[15] B.J. Teubl, M. Absenger, E. Frohlich, G. Leitinger, A. Zimmer, E. Roblegg, The oral cavity as a biological barrier system: design of an advanced buccal in vitro permeability model, Eur. J. Pharm. Biopharm. 84 (2013) 386-393.

[16] P.A. Nielsen, E.P. Bennett, H.H. Wandall, M.H. Therkildsen, J. Hannibal, H. Clausen, Identification of a major human high molecular weight salivary mucin (MG1) as tracheobronchial mucin MUC5B, Glycobiology. 7 (1997) 413-419.

[17] H.M. Nielsen, M.R. Rassing, Nicotine permeability across the buccal TR146 cell culture model and porcine buccal mucosa in vitro: effect of pH and concentration, Eur. J. Pharm. Sci. 16 (2002) 151-157.

[18] G.M. Grass, S.A. Sweetana, In vitro Measurement of Gastrointestinal Tissue Permeability Using a New Diffusion Cell, Pharmaceutical research, 5 (1988) 372-376.

[19] S. Amores, J. Lauroba, A. Calpena, H. Colom, A. Gimeno, J. Domenech, A comparative ex vivo drug permeation study of beta-blockers through porcine buccal mucosa, Int. J. Pharm., 468 (2014) 50-54.

[20] U. Kulkarni, R. Mahalingam, S.I. Pather, X. Li, B. Jasti, Porcine buccal mucosa as an in vitro model: relative contribution of epithelium and connective tissue as permeability barriers, J. Pharm. Sci. 98 (2009) 471-483.

[21] H. Whelton, Introduction: The Anatomy and Physiology of Salivary Glands, in: W. Edgar, D. O'Mullane (Eds.) Saliva and oral health, British Dental Association, London, 1996, pp. 1-8.

[22] K.D. Madsen, C. Sander, S. Baldursdottir, A.M. Pedersen, J. Jacobsen, Development of an ex vivo retention model simulating bioadhesion in the oral cavity using human saliva and physiologically relevant irrigation media, Int. J. Pharm., 448 (2013) 373-381.

[23] R. Holm, E. Meng-Lund, M.B. Andersen, M.L. Jespersen, J.J. Karlsson, M. Garmer, E.B. Jorgensen, J. Jacobsen, In vitro, ex vivo and in vivo examination of buccal absorption of metoprolol with varying pH in TR146 cell culture, porcine buccal mucosa and Gottingen minipigs, Eur. J. Pharm. Sci. 49 (2013) 117-124.

[24] J. Jacobsen, B. van Deurs, M. Pedersen, M.R. Rassing, TR146 cells grown on filters as a model for human buccal epithelium: I. Morphology, growth, barrier properties, and permeability, Int. J. Pharm. 125 (1995) 165-184.

[25] H.U. Eirheim, C. Bundgaard, H.M. Nielsen, Evaluation of different toxicity assays applied to proliferating cells and to stratified epithelium in relation to permeability enhancement with glycocholate, Toxicol. In Vitro. 18 (2004) 649-657.

[26] E. Marxen, M.C. Axelsen, A.M. Pedersen, J. Jacobsen, Effect of cryoprotectants for maintaining drug permeability barriers in porcine buccal mucosa, Int. J. Pharm. 511 (2016) 599-605.

[27] A. Allen, W.J. Cunliffe, J.P. Pearson, L.A. Sellers, R. Ward, Studies on gastrointestinal mucus, Scand. J. Gastroenterol. Suppl. 93 (1984) 101-113.

[28] B. Brodin, B. Steffansen, C.U. Nielsen, Passive diffusion of drug substances: the concepts of flux and permeability., in: B. Brodin, B. Steffansen, C.U. Nielsen (Eds.) Molecular biopharmaceutics: Aspects of drug characterisation, drug delivery and dosage form evaluation, PharmaPress Ltd. (2010) 135-151.

[29] R.G. Schipper, E. Silletti, M.H. Vingerhoeds, Saliva as research material: biochemical, physicochemical and practical aspects, Arch. Oral Biol. 52 (2007) 1114-1135.

[30] R.T. Balmer, S.R. Hirsch, The non-newtonian behavior of human saliva, Biorheology. 74 (1978) 125-129.

[31] X. Cao, R. Bansil, K.R. Bhaskar, B.S. Turner, J.T. LaMont, N. Niu, N.H. Afdhal, pH-dependent conformational change of gastric mucin leads to sol-gel transition, Biophys. J. 76 (1999) 1250-1258.

[32] A.W. Larhed, P. Artursson, J. Grasjo, E. Bjork, Diffusion of drugs in native and purified gastrointestinal mucus, J. Pharm. Sci. 86 (1997) 660-665.

[33] J.-J. Niibuchi, Y. Aramaki, S. Tsuchiya, Binding of antibiotics to rat intestinal mucin, Int. J. Pharm. 30 (1986) 181-187.

[34] J. Lee, S.K. Lee, Y.W. Choi, The effect of storage conditions on the permeability of porcine buccal mucosa, Arch. Pharm. Res. 25 (2002) 546-549.

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

[36] C. Pontier, J. Pachot, R. Botham, B. Lenfant, P. Arnaud, HT29-MTX and Caco-2/TC7 monolayers as predictive models for human intestinal absorption: role of the mucus layer, J. Pharm. Sci. 90 (2001) 1608-1619.

[37] H.M. Nielsen, M.R. Rassing, TR146 cells grown on filters as a model of human buccal epithelium: III. Permeability enhancement by different pH values, different osmolality values, and bile salts, Int. J. Pharm. 185 (1999) 215-225.

[38] J.A. Nicolazzo, B.L. Reed, B.C. Finnin, The effect of various in vitro conditions on the permeability characteristics of the buccal mucosa, J. Pharm. Sci. 92 (2003) 2399-2410.

[39] A. Beduneau, C. Tempesta, S. Fimbel, Y. Pellequer, V. Jannin, F. Demarne, A. Lamprecht, A tunable Caco-2/HT29-MTX co-culture model mimicking variable permeabilities of the human intestine obtained by an original seeding procedure, Eur. J. Pharm. Biopharm. 87 (2014) 290-298.

574 [40] J. Maury, C. Nicoletti, L. Guzzo-Chambraud, S. Maroux, The filamentous brush border
575 glycocalyx, a mucin-like marker of enterocyte hyper-polarization, Eur. J. Biochem. 228 (1995) 323-
576 331.

577 [41] C.A. Squier, B.K. Hall, In-vitro permeability of porcine oral mucosa after epithelial separation,
578 stripping and hydration, Archives of oral biology. 30 (1985) 485-491.

579 [42] V.H. Deneer, G.B. Drese, P.E. Roemele, J.C. Verhoef, A.H.L. Lie, J.H. Kingma, J.R.
580 Brouwers, H.E. Junginger, Buccal transport of flecainide and sotalol: effect of a bile salt and
581 ionization state, Int. J. Pharm. 241 (2002) 127-134.

582 [43] L.L. Chen, D.J. Chetty, Y.W. Chien, A mechanistic analysis to characterize oramucosal
583 permeation properties, Int. J. Pharm. 184 (1999) 63-72.

584 [44] E. Meng-Lund, J. Jacobsen, L. Jin, C. Janfelt, R. Holm, A. Mullertz, J.A. Nicolazzo, Azone(R)
585 decreases the buccal mucosal permeation of diazepam in a concentration-dependent manner via a
586 reservoir effect, J. Pharm. Sci. 103 (2014) 1133-1141.

587