



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

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

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17 **Abstract**

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

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

29 **Keywords**

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

31

32 **Abbreviations**

33	BSA	Bovine serum albumin
34	HBSS	Hanks' balanced salt solution
35	HFDS	Human freeze-dried saliva
36	Log P	Logarithm of partition coefficient
37	Log D	Logarithm of distribution coefficient
38	MW	Molecular weight
39	$P_{app}$	Apparent permeability coefficient
40	PBS	Phosphate buffered saline
41	PGM	Porcine gastric mucin
42	SD	Standard deviation
43	TEER	Transepithelial electrical resistance

## 44 **1 Introduction**

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

59 Only few oromucosal formulations are on the market, and this may be due to lack of standardized *in*  
60 *vitro* methods to evaluate and optimize drug delivery systems [6]. Several models for assessing drug  
61 permeability through oral mucosa are known, however, they lack the mucus layer. Permeability  
62 across excised mucosal tissue can be studied in side-by-side diffusion cells such as modified Ussing  
63 chambers or Franz cells [7]. To the authors' knowledge it has not been confirmed that the mucus layer  
64 on the epithelium is intact after handling the excised tissue [8]. Filter grown cell cultures, such as  
65 TR146 cells derived from human buccal carcinoma have also been used to model oral mucosa [9,  
66 10]. However, these cells do not produce mucus. Cell cultures that produce a mucus layer, such as  
67 HT29-MTX cells derived from human colon adenocarcinoma, form a single cell layer, and thus are

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

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

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

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

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

## 94 **2 Materials and methods**

### 95 **2.1 Materials**

96 Potassium dihydrogen phosphate, calcium chloride, sodium hydrogen carbonate, sodium chloride and  
97 ortho-Phosphoric acid 85% were purchased from Merck KGaA (Darmstadt, Germany). Sodium  
98 phosphate monobasic anhydrous was obtained from Amresco (Solon, OH, USA). Potassium chloride  
99 was obtained from Riede-de Haën (Seelze, Germany). Hanks' balanced salt solution (HBSS) (10x),  
100 +CaCl<sub>2</sub>, +MgCl<sub>2</sub> and 7.5% sodium bicarbonate was purchased from Gibco® life technologies (Grand  
101 Island, NY, USA). Nicotine bitartrat dihydrate was kindly donated from Fertin Pharma (Vejle,  
102 Denmark). Pearlitol® 160 (mannitol) was obtained from Roquette Pharma (Lestrem, France). Caffeine  
103 was purchased from VWR (Leuven, Belgium). [<sup>14</sup>C]-mannitol (57.1 mCi/mmol), [<sup>3</sup>H]-nicotine (80.4  
104 Ci/mmol), [<sup>14</sup>C]-caffeine (54.9 mCi/mmol), [<sup>3</sup>H]-propranolol (18.6 Ci/mmol) and Ultima Gold™  
105 liquid scintillation fluid were purchased from Perkin Elmer Inc. (Waltham, USA). Falcon 12-well  
106 tissue culture plates and cell culture inserts (polyethylene terephthalate membrane, 0.9cm<sup>2</sup> area, 0.4  
107 mm pore size) were obtained from Becton Dickinson Labware (Franklin Lakes, NJ, USA). Bovine  
108 serum albumin (BSA), Mucin from porcine stomach, type II (PGM), (±)-propranolol hydrochloride,  
109 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Alcian blue 8GX, MTS-4-fluorescein,  
110 phenazine methosulfate and silicone oil were all purchased from Sigma-Aldrich (St. Louis, MO,  
111 USA). Human freeze-dried saliva (HFDS) from pooled saliva samples (dialyzed and free of minerals)  
112 was kindly donated by the Department of Odontology, University of Copenhagen (Copenhagen,  
113 Denmark). The saliva was centrifuged at 2000 g for 10 min, dialyzed at 5°C for two days and finally  
114 lyophilized. Dialysis tubing visking, cellulose, type 36/32 inch, thickness 0.02 mm, width 44 mm,  
115 MWCO 14,000 was from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Deionized water was

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

## 118 **2.2. Methods**

### 119 2.2.1 Preparation of mucin dispersions

120 A buffer, named saliva buffer, containing 5 mM  $\text{KH}_2\text{PO}_4$ , 15 mM KCl, 1 mM  $\text{CaCl}_2$  and 5 mM  
121  $\text{NaHCO}_3$  was prepared and adjusted to pH 6.8 [21]. PGM and HFDS were dispersed in saliva buffer  
122 and exposed to slow stirring at 5°C overnight. The concentrations of mucin varied in the experiments  
123 and are stated in the respective sections below.

### 124 2.2.2 Comparison of mucin sources

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

### 138 2.3 Permeability studies



### 139 2.3.1 Permeability across an isolated mucin dispersion

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

143 The new permeability device consists of three parts as shown in Fig. 1; a cylinder placed between a  
144 two piece slider. Dialysis membranes were rinsed in cold water and then soaked three times 5 min in  
145 200 mL 100 °C hot water. The prepared dialysis membranes were placed between the slider and the  
146 cylinder (positions shown with A in Fig. 1) and held in place by joining the parts. The mucus  
147 dispersion was then added with a syringe through a small hole in the slider and cylinder. The diffusion  
148 area was 0.50 cm<sup>2</sup> and the thickness of the mucin layer (cylinder length) was 6.0 mm. The  
149 composition of the receptor and donor fluid is given in Table 1. The permeability of nicotine,  
150 mannitol, propranolol and caffeine was studied across saliva buffer, 2% (w/v) PGM dispersion or 2%  
151 (w/v) HFDS dispersion.

152 The study was conducted in modified Ussing chambers as previously described by Holm et al. [23].  
153 Briefly, 2.0 mL of donor and receptor fluid was added to the respective compartments. Stirring was  
154 ensured by supplying hydrated atmospheric air, and the temperature was kept at 36±1°C. Receptor  
155 samples of 100 µL were taken from the receptor compartment at 5, 10, 20 and 30 min and then every  
156 30 min up to 5 h. From the donor compartment, 100 µL was taken in triplicates at the start and end  
157 of the experiment. After sampling the compartments were replenished. At the end of the experiment  
158 a 100 µL sample was taken from the mucin dispersions or saliva buffer. Drug content was determined  
159 in the dialysis membranes by rinsing off excess drug and placing the dialysis membrane in a  
160 scintillation vial for measurement.

161

### 162 2.3.2 Permeability across TR146 cells

163 The TR146 cell line was provided by Imperial Cancer Research Technology (London, UK) and  
164 cultivated and grown on filters as previously described [24]. On the first day of the experiment filter-  
165 grown cells aged 25-27 days were washed on the apical side and the growth medium was changed on  
166 the basolateral side. The cells were incubated on the apical side with 200  $\mu$ L 4% (w/v) PGM or HFDS  
167 dispersion. Cells incubated with saliva buffer were used as a control. The cells were incubated at  
168 37°C in 5% CO<sub>2</sub>/95% air at 98% humidity for 24 h.

169 On the second day of experiment, the initial transepithelial electrical resistance (TEER) was measured  
170 on the control cells (Endohm and voltmeter EVOM from World Precision Instruments (Sarasota, FL,  
171 USA)). The composition of donor and receptor fluids is shown in Table 2. 1600  $\mu$ L receptor medium  
172 was added to the basolateral side and 220  $\mu$ L donor solution was added to 200  $\mu$ L mucin dispersion  
173 or saliva buffer on the apical side. The experiment was conducted at 37°C using a thermostatic  
174 horizontal shaker, 100 rpm, (Edmund Bühler, swip Type KL-2) (Hechingen, Germany). 100  $\mu$ L  
175 samples were taken from the basolateral side at time 10 min, 30 min, 45 min, 60 min and then every  
176 30 min up to 240 min, and the compartment was replenished with receptor fluid. At 10 min a donor  
177 sample of 20  $\mu$ L and at 240 min three donor samples of 20  $\mu$ L were taken. The first samples were  
178 taken at 10 min to allow the donor fluid to mix with the mucin dispersion. After the permeability  
179 experiment the cells were washed twice with HBSS buffer on both apical and basolateral side, TEER  
180 was measured and a MTS-PMS viability test was conducted as described by Eirheim et al. [25].

#### 181 2.3.2.1 Mucin attachment to cell surfaces

182 After 24 h incubation the mucin dispersions or saliva buffer was gently removed and the cells were  
183 washed in 0.1 M phosphate buffered saline (PBS) pH 6.8. 200  $\mu$ L Alcian blue solution (1% (v/v)  
184 Alcian blue and 3% (v/v) acetic acid in water) was added to the apical side and the setup was shaken  
185 (100 rpm) for 5 min. The Alcian blue solution was gently removed and the cells were washed twice  
186 in PBS pH 6.8. The cells were then examined under an Olympus BH2 light microscope (Olympus,

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

### 189 2.3.3 Permeability across porcine buccal mucosa

190 Porcine buccal mucosa was obtained from healthy experimental control pigs (approx. 30 kg Danish  
191 Landrace/Yorkshire x Durox (D-LY)). Immediately after euthanization of the pigs the cheeks were  
192 excised using a scalpel and placed in ice cold PBS pH 7.4. Within 3 h the excised cheeks were frozen  
193 in 40% (w/v) glycerol and 20% (w/v) sucrose in PBS pH 7.4, and on the day of experiment the tissue  
194 was thawed as described by Marxen et al. [26]. The buccal mucosa was trimmed with surgical scissors  
195 and sliced to a thickness of  $792 \mu\text{m} \pm 88 \mu\text{m}$  (n=36) using a Stadie-Riggs tissue slicer (Thomas  
196 Scientific, Swedesboro, NJ, USA). The buccal mucosa was mounted on slider P2405 from  
197 physiologic instruments Inc. (San Diego, CA, USA) (exposed area  $0.40 \text{ cm}^2$ ). The sliders were placed  
198 in the upper compartment of a desiccator, the epithelium facing upwards. The lower compartment of  
199 the desiccator was filled with NaCl saturated water to ensure high humidity. 100  $\mu\text{L}$  saliva buffer,  
200 5% (w/v) PGM or HFDS dispersion was added to the apical surface of the tissue and incubated at 37  
201  $^{\circ}\text{C}$  for 24 h, 30 min or the tissue was used immediately. When incubating for 24 h, the sliders were  
202 placed in PBS pH 7.4 to keep the basolateral side of the tissue moist.

203 The compositions of the donor and receptor solutions are presented in Table 1. The permeability  
204 experiment was conducted as described in Section 2.3.1, with few changes: After the experiment, the  
205 tissue was dissolved in approximately 1.0 mL concentrated phosphoric acid, heated to  $70^{\circ}\text{C}$  and a  
206 100  $\mu\text{L}$  sample was taken for quantification of the radiolabeled model drugs.

207

208

## 209 2.4 Quantitative analysis

210 2 mL Ultima Gold™ liquid scintillation fluid was added to all samples before whirl-mixing.  
211 Quantitative analysis of [<sup>3</sup>H]-nicotine, [<sup>14</sup>C]-mannitol, [<sup>3</sup>H]-propranolol and [<sup>14</sup>C]-caffeine was  
212 performed by liquid scintigraphy using a Tri-Carb 2910TR Liquid Scintillation Analyzer (Perkin  
213 Elmer, Waltham, MA, USA).

## 214 2.5 Data analysis

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

$$220 \quad J_{ss} = \frac{dQ}{dt} * \frac{1}{A} \quad \text{Equation 1}$$

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

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

$$228 \quad P_{app} = \frac{J_{ss}}{C_{0,donor}} \quad \text{Equation 2}$$

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

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

## 235 **2.6 Statistical analysis**

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

## 243 **3 Results and discussion**

### 244 3.1 Comparison of mucin sources

245 The mucin sources appeared very different in solid dried forms. PGM is a fine brownish powder  
246 whereas HFDS has a bulky white fibrous structure. The pH of PGM dispersion after stirring overnight  
247 was  $4.4 \pm 0.3$  (n=9) and the pH of HFDS dispersion was  $6.6 \pm 0.4$  (n=9). A *t*-test showed that the pH  
248 of PGM dispersion was significantly different from pH of the HFDS dispersion, which was expected  
249 since PGM is gastric mucin, and thus originates from an acidic environment, whereas HFDS is  
250 derived from saliva with an average pH of 6.8. In the present studies the mucin dispersions were  
251 adjusted to pH 6.8 to mimic pH in the oral cavity. Mucin contains acidic functional groups, including  
252 sialic acid [27]. Adjusting pH will affect the degree of ionization of the acidic groups, thus a change  
253 in pH will likely affect the interactions between mucin strands, causing enlargement or compaction  
254 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  $1 \text{ s}^{-1}$ . At shear  
266 rates below  $1 \text{ s}^{-1}$  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  $1\text{-}160 \text{ s}^{-1}$  [29, 30]. The rheology measurements of the PGM dispersion

269 was considered acceptable, since the measurements above  $1 \text{ s}^{-1}$  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

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

282

## 283 3.2 Permeability studies

### 284 3.2.1 Permeability across an isolated mucin dispersion

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

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

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

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

### 309 3.2.2 Permeability across TR146 cells

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

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

320 The effects from the mucin dispersion on nicotine permeability across an isolated layer (Section  
321 3.2.1), is not confirmed when the TR146 cells are present, indicating that the cell layers constitute a  
322 larger barrier to nicotine, than the one provided by the mucin dispersions. Also the differences found  
323 between HFDS and PGM dispersions, for propranolol and caffeine disappeared, which indicates that  
324 the effect shown with a 6.0 mm mucin dispersion layer is larger than the barrier exerted by the mucus  
325 layer *in vivo*. Moreover, the findings indicate that certain interactions between the drug molecules



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

### 333 3.2.2.1 Integrity and viability of TR146 cells

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

350 The integrity of the TR146 cell layers was supported by the relatively low  $P_{app}$  of mannitol. Mannitol  
351 is routinely used as a marker for integrity of the cell layers, in particular the paracellular pathway.  
352 Jacobsen et al. showed that mannitol permeability across TR146 cell layers grown for 30 days was  
353  $5.2 \cdot 10^{-6}$  cm/s, and since the mannitol permeability found in this study was between 1.4 and  $3.9 \cdot 10^{-6}$   
354 cm/s (Table 3), the integrity of the cell layers seems to be maintained [24].

355 The MTS/PMS assay measures dehydrogenase activity in cells as a measure of cell viability. The  
356 dehydrogenase activity after a 4 h permeability study in TR146 cells incubated with saliva buffer was  
357 assumed to be 100% viable and the cellular viability of the TR146 cells incubated with PGM and  
358 HFDS dispersions were determined relative to the cells incubated with saliva buffer. According to  
359 Nielsen and Rassing [37], TR146 cells were not sensitive towards pH changes in the range 5.5-9.0,  
360 or to osmolality changes in the range approximately 100-400 mOsm, thus it was assumed that the  
361 saliva buffer would not affect viability of the TR146 cells. After 24 h incubation with mucin  
362 dispersion and a 4 h permeability study the viability in cells exposed to nicotine and mannitol was  
363  $106\% \pm 4\%$  (PGM) and  $102\% \pm 2\%$  (HFDS), and in cells exposed to propranolol and caffeine the  
364 viability was  $107\% \pm 2\%$  (PGM) and  $114\% \pm 21\%$  (HFDS).

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

372 3.2.2.2 Mucin attachment to cell surfaces

373 After incubation with mucin dispersions the attachment of mucin to the TR146 cells was qualitatively  
374 examined by staining the cells with Alcian blue dye. Representative images of the stained cells are  
375 presented in Fig. 5. Alcian blue stains the negatively charged groups in mucin at physiological pH  
376 [39].

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

### 386 3.2.3 Permeability across porcine buccal mucosa

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

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

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

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

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

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

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

#### 429 3.2.4 Total drug recovery

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

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

## 443 4 Conclusions

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

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

451 TR146 cells were subjected to mucin dispersions 24 h prior to a permeability study. This model may  
452 become a promising *in vitro* method to study drug permeation across a mucus layer in conjunction  
453 with a multi-layered epithelium. The integrity and viability of the TR146 cells were maintained during  
454 24 h incubation and a subsequent permeability study. Permeability of propranolol and caffeine was  
455 decreased by the presence of mucin, however, this was not shown for nicotine and mannitol. The  
456 HFDS attached to the TR146 cell surface to a higher extent than PGM, however, the strength and  
457 mechanism behind the attachment needs further studies.

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

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