Mucus-PVPA (Mucus Phospholipid Vesicle-Based Permeation Assay): an artificial permeability tool for drug screening and formulation development

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Abstract

The mucus layer covering all mucosal surfaces in our body is the first barrier encountered by drugs before their potential absorption through epithelial tissues, and could thus affect the drugs' permeability and their effectiveness. Therefore, it is of key importance to have *in vitro* permeability models that can mimic this specific environment. For this purpose, the novel mucus phospholipid vesicle-based permeation assay (mucus-PVPA) has been developed and used for permeability screening of drugs and formulations. The model proved to be stable under the chosen conditions and demonstrated the ability to discriminate between compounds with different chemical structures and properties. Overall, a decrease in drug permeability was found in the presence of mucus on top of the PVPA barriers, as expected. Moreover, mucoadhesive (chitosan-coated) and mucopenetrating (PEGylated) liposomes were investigated in the newly developed model. The mucus-PVPA was able to distinguish between the different liposomal formulations, confirming the penetration potential of the tested formulations and the related drug permeability. The mucus-PVPA model appears to be a promising *in vitro* tool able to mimic the environment of mucosal tissues, and could therefore be used for further drug permeability screening and formulation development**.**

Keywords

In vitro model; permeability; mucosal administration; mucus; liposomes; mucoadhesive; mucopenetrating

1. Introduction

The mucus layer covering mucosal epithelia is the first barrier encountered by many drugs and formulations when entering the body. This layer could thus potentially limit the effectiveness of most drug delivery systems (Groo and Lagarce, 2014). Mucus is found on many epithelial surfaces such as the gastrointestinal tract (GI), the respiratory tract, the eye and the female genital tract; its composition, structure and thickness differ according to the different locations in the body (Friedl et al., 2013; Leal et al., 2017; Sigurdsson et al., 2013). The main components of mucus are water, glycoproteins (i.e. mucins), free proteins, salts and lipids (Groo and Lagarce, 2014). An important role is played by mucins, negatively charged glycoproteins (polypeptide backbone with oligosaccharide side chains), which are secreted by mucosal glands and goblet cells (Leal et al., 2017; Sigurdsson et al., 2013). The structure of the mucin gel can hinder the diffusion of drugs (Boegh and Nielsen, 2015) by two main mechanisms, namely the interaction and size filtering (Olmsted et al., 2001).

Transmucosal drug delivery gained increasing attention in the past two decades. Various strategies have been proposed to improve the mucosal permeability of drugs, including mucoadhesive and mucopenetrating systems, such as liposomes (Leal et al., 2017). Therefore, to properly tackle the screening of new drugs and optimization of novel mucosal formulations, it is of key importance to exploit *in vitro* tools comprising mucus to better understand its impact on drug permeation and absorption and to better predict the fate of a drug *in vivo*. Many models have been developed to study the effect of the sole mucus layer on drug permeability, without the presence of an artificial membrane. Some of them comprise the use of native mucus and some others exploit the use of commercially available mucins in different types of media (Khanvilkar et al., 2001; Legen and Kristl, 2001; Matthes et al., 1992). However, it has to be noted that the removal of mucus from its physiological

environment can modify its characteristics (e.g. gel-forming properties) (Kocevar-Nared et al., 1997). Therefore, it becomes challenging to produce a model able to mimic physiological mucus, and the differences between native and reconstituted mucus can lead to variances in the resulting drug permeability. On the other hand, to date, several *in vitro* cell-based (Caco-2 model, Artusson et al., 2001) and artificial models (PVPA model, Flaten et al., 2006b; PAMPA model, Kansy et al., 1998; Permeapad^{TM}, di Cagno et al., 2015; AMI-system, Berben et al., 2017) have been developed for the screening of new drugs and formulations. Some of those models also include the mucus layer, such as mucus-producing cell systems (i.e. Caco2/HT29-MTX co-culture) and cell-based mucosal models with artificial mucus (Boegh et al., 2014; Lechanteur et al., 2017). Unfortunately, the robustness and reproducibility of these mucus-including models are not yet well defined. Therefore, the lack of a reliable artificial *in vitro* model comprising mucus remains a considerable limitation for permeability studies targeting the mucosal administration route.

Among the non-cell-based models, the phospholipid vesicle-based permeation assay (PVPA) has been developed in our group and established in the past decade as a predictive and reliable artificial model for the screening of drugs and optimization of formulations (Flaten et al., 2006b; Flaten et al., 2011; Kanzer et al., 2010; Naderkhani et al., 2014a,b). So far, this model has not taken into account the crucial influence of mucus on the permeation of drugs. Therefore, in this study, the effect of mucus on drug permeability was assessed and the novel mucus-PVPA developed and validated. The permeability of five model drugs (atenolol, ibuprofen, indomethacin, metronidazole and naproxen) was investigated. The drugs were chosen to cover a range of relevant physicochemical properties to challenge the mucus-PVPA's ability to distinguish between drugs with different physicochemical characteristics. Moreover, since nanoparticulate formulations have demonstrated great efficacy in *in vitro* and *in vivo* experiments (Chen et al., 2013; Netsomboom and Bernkop-Schnürch, 2016), a focus

was put on the permeation of three selected drugs (indomethacin, metronidazole and naproxen) from mucoadhesive (chitosan-coated) and mucopenetrating (PEGylated) liposomal formulations, to better understand the influence of the mucus layer on the diffusion of the nanocarriers and permeability of the drugs contained in such delivery systems.

2. Materials and methods

2.1. Materials

Lipoid egg phospholipids E80 (80% phosphatidylcholine), Lipoid soybean lecithin S100 (>94% phosphatidylcholine) and Lipoid PE 18:0/18:0 (PEG 2000) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Acetic acid (≥99.8%), ammonium molybdate, atenolol, calcein, chitosan (low molecular weight, Brookfield viscosity 20,000 cps, degree of deacetylation 92%), chloroform, ethanol (96%, v/v), Fiske-Subbarow reducer, hydrochloric acid, ibuprofen, indomethacin, methanol CHROMASOLV®, metronidazole, mucin from porcine stomach type III (bound sialic acid 0.5-1.5%, partially purified), naproxen, phosphorus standard solution, potassium phosphate monobasic, sodium chloride, sodium hydroxide and sodium phosphate dibasic dodecahydrate were products of Sigma-Aldrich, Chemie GmbH (Steinheim, Germany). Hydrogen peroxide 30% and titriplex® III were purchased from Merck KGaA (Darmstadt, Germany). Acetonitrile for HPLC (gradient grade) was obtained from VWR chemicals (Fontenay-sous-Bois, France) and sulfuric acid was purchased from May&Baker LTD (Dagenham, England). All chemicals employed were of analytical grade.

Plates and Transwell filter inserts (d = 6.5 mm) were products of Corning Inc. (Corning, New York). The nitrocellulose membrane filters (0.65 µm DAWP) were obtained from Millipore

(Billerica, Massachusetts) and the Nucleopore track-etch membrane filters (0.4 and 0.8 µm pore size) were purchased from Whatman (part of GE Healthcare, Oslo, Norway).

2.2. PVPA barriers preparation

The PVPA barriers were prepared by depositing egg-phospholipid liposomes on top of cellulose ester filters by centrifugation followed by a freeze-thaw cycle according to a method previously described (Naderkhani et al., 2014a).

2.3. Mucus barrier

Different concentrations of mucin (10, 20 and 40 mg/mL) were used as a model for the mucus layer. These suspensions were obtained by the hydration of mucin from porcine stomach type III with phosphate buffer saline (PBS) pH 7.40. The viscosity of the mucus was measured at room temperature on HAAKE ViskoTester 7 plus (Thermo, Hafrsfjord, Norway) using spindle TL5. In the *in vitro* permeability studies, the mucin suspension was directly pipetted on top of the PVPA barriers before the addition of the drugs or formulation to be tested. The drug solutions/formulations were carefully added on top of the mucus layer in the donor compartment in order to prevent mixing of the two layers. The division of the two layers was visibly distinct.

2.4. *In vitro* permeability study using the mucus-PVPA

The permeability of different drugs/marker (calcein, CAL; atenolol, ATN; ibuprofen, IBP; indomethacin, IND; naproxen, NPR; metronidazole, MTR; Table 1) was investigated at

room temperature (23-25 °C) in the presence and absence of mucus following the procedure previously described (Naderkhani et al., 2014a). In the experiments performed in the presence of mucus, 50 µL of mucin 10 mg/mL were added, if not stated otherwise, before the careful addition of drug/marker. To maintain sink conditions, the inserts were moved to a new acceptor compartment at certain time intervals for 5 hours. After ended experiment, the electrical resistance was measured to confirm the integrity of the barriers and the samples collected as previouslu described (Flaten et al., 2006 a,b; Naderkhani et al., 2014 a,b)The fluorescent marker calcein was used to monitor the barriers' integrity during the study (Flaten et al., 2006b) and was quantified spectrofluorometrically on POLARstar Galaxy fluorometer (Fluostar, BMG Labtechnologies, Offenburg, Germany) at excitation and emission wavelengths of 485 and 520 nm, respectively. The quantification of indomethacin was carried out by HPLC using a Waters X-select™ CSH ™ C18 (2.5 µm, 3.0x75 mm) XP column preceded by a Waters X-select™ CSH ™ C18 (3.5 µm, 3.0x20 mm) guard cartridge on a Waters e2795 Separation Module connected to a Waters 2489 UV/Visible Detector (Waters, Milford, Massachusetts, USA) at a wavelength of 254 nm. The mobile phase consisted of acetonitrile and MilliQ water (60:40, v/v) with 0.1% glacial acetic acid and the flow rate was set at 0.5 mL/min (retention time 2.8 minutes). Atenolol, ibuprofen, metronidazole and naproxen were quantified spectrophotometrically on SpectraMax 190 Microplate reader (Molecular Devices Corporation, California, USA) at wavelengths of 274, 220, 320 and 270 nm, respectively.

For each compound the experiment was performed at least in triplicates (6 inserts for each parallel) and the apparent permeability coefficient (P_{app}) was calculated with the equation derived from Fick's law for steady state conditions:

$$P_{app}\left(\frac{cm}{s}\right) = \frac{dQ}{dt} \text{ x } \frac{1}{A \text{ x } Cd}$$

where dQ/dt is the slope at the steady-state conditions (nmol/s), A represents the surface area of the PVPA barriers (cm^2) and C_d is the concentration of the compound in the donor compartment (nmol/mL).

As earlier described by our group (Flaten et al., 2006a, b), the concentrations of the drugs investigated in the study were chosen in order to reach a concentration in the acceptor compartment that was below the solubility limits and thus to obtain sink conditions.

Table 1

2.4.1. The effect of temperature, mucus volume and mucin concentration on the permeability of drugs

The permeability of different drugs/marker (Table 1) was measured in the absence and presence of mucus at 37 °C and compared to the one obtained at room temperature (23-25 °C) to evaluate possible changes in permeability due to elevated temperature. Different concentrations of mucin (10, 20 and 40 mg/mL) were tested to estimate their effect on the permeability of the tested compounds. Moreover, different volumes of mucus (mucin 10 mg/mL; mucus volume range: 20-50 µL) were deposited on top of the PVPA barriers, and the permeability of naproxen was measured to assess if the different mucus' volumes would have any effect on the drug's permeability.

2.5. PVPA barriers – mucus interaction

2.5.1. Phospholipid assay

In order to determine any changes in the barriers' integrity caused by the addition of mucus on top of the PVPA barriers, the amount of phospholipids released after the addition of the mucus layer was measured by the modified phosphorus assay (Bartlett, 1959) as previously described by us (Naderkhani et al., 2015).

2.5.2. *In vitro* mucus binding test

The binding potential of the egg-phospholipid liposomes to mucus was evaluated to determine its interaction with the PVPA barriers. The study was conducted as previously described (Jøraholmen et al., 2017). The experiment was carried out in triplicate and the binding efficiency of mucus to the liposomes was calculated according to Jøraholmen and colleagues (2017).

2.6. Preparation of liposomal formulations

Three different types of liposomal formulations containing either indomethacin (IND), metronidazole (MTR) or naproxen (NPR) were prepared to study the effect of the formulation on drug permeability.

Plain liposomes were obtained using the film hydration technique, according to the method described by Berginc and colleagues (Berginc et al., 2014). The liposome dispersion was sonicated for 1 minute using a Sonics high intensity ultrasonic processor (Sonics & Materials Inc., Newtown, Connecticut) (amplitude setting of 500 W/20 kHz processor 40%) to produce a smaller and more homogeneous size distribution. The sonicated liposome dispersion was stored in the refrigerator for at least 2 hours prior to further use.

Chitosan-coated liposomes were prepared from plain liposomes in the absence of unentrapped drug as previously described (Jøraholmen et al., 2014; Naderkhani et al., 2014a). After storage in refrigerator (4-8 °C) overnight, the pH was measured and adjusted to 7.40.

PEGylated liposomes were prepared using Lipoid S100 (200 mg), PEG 2000 (36.3 mg) and the drug (IND, MTR or NPR; 20 mg), following the method described by Jøraholmen and colleagues (Jøraholmen et al., 2017).

2.7. Characterization of liposomal formulations

2.7.1. Entrapment efficiency and recovery

The encapsulated drug (IND, MTR or NPR) in the different liposomal formulations was separated from the unentrapped drug by dialysis using a dialysis tubing with a MWCO 12-14,000 Da (Medicell International Ltd., London, UK). The liposomal dispersions (4.2 mL) were dialyzed against a medium (PBS, pH 7.40) for 6 hours at room temperature. The volume of PBS was adjusted to assure the solubility of the drugs. Aliquots of the dialyzed liposomes were dissolved in MeOH to free the drug contained in the liposomes and compared with the amount of drug in the medium (unentrapped drug) to calculate the entrapment efficiency for the specific drug. Drugs were quantified as previously described in section 2.4.

2.7.2. Size analysis and zeta potential measurements

The diameter of the dialyzed liposomes containing different drugs was determined using a Malvern Zetasizer Nano ZS (Malvern, Oxford, UK). Two samples for each batch of liposomes were analysed and the diameters calculated from the mean of three measurements

for each sample. The liposome dispersions were diluted 1:50 (v/v) in PBS pH 7.40 for plain and PEGylated liposomes, and PBS pH 7.40 and acetic acid 0.1% (1:1 v/v) for the chitosan-coated ones, in order to dilute the formulations in their own preparation media. The polydispersity index (PI) of each batch was measured to assess the population's homogeneity.

All liposomal formulations (plain, chitosan-coated and PEGylated) were diluted 1:10 (v/v) in freshly filtered water (0.2 µm filters) to determine the zeta potential using a Malvern Zetasizer Nano ZS (Malvern, Oxford, UK). The disposable folded capillary cells (DTS1070) were cleaned before the loading of the sample using ethanol and filtered water. Two samples for each batch of formulations were measured in three parallels at room temperature.

2.8. Statistical analysis

Statistical analysis was done using GraphPad Prism 7.0 software. Student's t-test was used to detect significant differences between two sets of data ($p < 0.05$). Comparisons between three or more groups were performed using one-way ANOVA and significance ($p < 0.05$) was found out using the Bonferroni multiple comparison *post hoc* test.

3. Results and discussion

Mucosal tissues, found at various locations in the body, can provide access to both local and systemic drug administration, and are an interesting barrier considering transmucosal delivery (Leal et al., 2017). Moreover, mucosal administration is seen as one of the most convenient, easy and cost-effective routes (Lechanteur et al., 2017). However, the mucus layer covering all mucosal tissues represents a barrier that drugs must overcome to reach

deeper epithelia or become absorbed. Therefore, it is of key importance to develop reliable *in vitro* tools able to evaluate the effect of mucus on drug permeability.

3.1. The effect of mucus on the PVPA barriers

The mucus-PVPA model is expected to provide fast and reliable means to predict/optimize the permeation of drugs once in contact with mucosal surfaces. Unpurified mucin type III from porcine stomach was employed, since this type of mucin has already been exploited in several other studies (Berben et al., 2017; Griffiths et al., 2010; Jøraholmen et al., 2017); the molecular weight and structure of pig mucins resemble human mucins (Groo and Lagarce, 2014). Moreover, its preparation avoids the degradation that occurs with purified mucin type II; the degradation often leads to a different mesh structure and related different rheological properties compared to native mucus (Groo and Lagarce, 2014). To assess whether the mucus-PVPA can provide reliable evidences on drug permeability, the integrity and functionality of the barriers were investigated. The permeability of the hydrophilic marker calcein in the presence of mucus served as a model. Moreover, the effect of different mucus layer thicknesses on the permeability of a model drug as well as characterization of the interaction between mucus and the PVPA barriers were evaluated.

3.1.1. Permeability of a highly hydrophilic marker

The permeability of the hydrophilic marker calcein was investigated in the presence of different mucin concentrations to study their effect on permeability. This fluorescent marker provides information on potential aqueous pathways in the PVPA barrier (Flaten et al., 2006b). Fig. 1 shows that there was no significant change in calcein's P_{app} in the absence or

presence of different concentrations of mucin. Considering mucus' overall hydrophilicity and negative charge, more hydrophilic compounds have exhibited lower affinity for mucus compared to hydrophobic ones (Boegh et al., 2014). Boegh et al. (2014) have compared the permeability of a hydrophilic drug (mannitol) with a hydrophobic one (testosterone) in the presence and absence of biosimilar mucus on top of Caco-2 cell monolayers. The authors have found that the greatest reduction in permeability in the presence of mucus was obtained for the hydrophobic drug compared to the hydrophilic one. In our case, considering calcein chemical properties (Table 1), it was not expected that its permeability should be affected to a great extent by the presence of the mucus layer. Therefore, the lack of changes in permeability in the presence of mucus indicates that calcein is free to diffuse through the mucus layer and to permeate through the PVPA barriers without any considerable interaction with this hydrophilic layer. Moreover, as previously stated, no increase in calcein permeability suggests that the barriers are able to maintain their integrity in the presence of mucus. Furthermore, the electrical resistance remained constant in all of the tested conditions (Fig. 1), also indicating no significant changes in the barriers' integrity. These findings are of significant importance especially when compared to the already established cell-based *in vitro* models including the mucus layer such as the Caco-2/HT29-MTX (Hilgendorf et al., 2000). The major drawback of the Caco-2/HT29-MTX model is related to the decrease in transepithelial electrical resistance (TEER) produced by the introduction of the mucus-producing HT29-MTX goblet cells (Schimpel et al., 2014). In fact, the presence of these cells lead to a leakier Caco-2 cell monolayer, thus suggesting an uncertain relevance in comparison of the permeability between the presence and absence of mucus (Lechanteur et al., 2017). On the contrary, in our case, the addition of mucus on top of the barriers did not cause any change in electrical resistance, enabling us to compare values obtained with and without the addition of the mucus layer.

Fig. 1

3.1.2. Characterization of the interaction between mucus and the PVPA barrier

To assess possible disintegration events taking place in the barrier when exposed to mucus, the release of phospholipids from the PVPA barriers into the donor chamber in the presence of mucus (mucin 10 and 40 mg/mL) was quantified and compared to the release in the presence of PBS pH 7.40 on top of the barriers (control). Results (data not shown) indicated that no significant difference in phospholipid release was found in the presence and absence of mucus. This evidence is in agreement with previous reports on the robustness of the original PVPA barriers (Flaten et al., 2008) and confirms the maintenance of the barriers' integrity and their low degree of interaction with mucus.

To further test the potential interaction between the liposomes in the PVPA barriers and mucus, a mucin binding test was performed. The results obtained (data not shown) confirmed a lack in binding between the two components, especially evident for liposomes with bigger diameter size, comparable to the liposome size on top of the PVPA barrier. This evidence highlights, once again, the lack of changes produced in the PVPA barriers by the mucus layer.

The lack of structural changes in the barriers was also suggested by studies performed using the confocal laser scanning microscopy (CLSM) (results in Supplementary). The PVPA barriers were investigated to visually examine if the mucus layer would interfere with the barrier's integrity. The micrographs of the cross-sectioned PVPA barriers showed that no aqueous channels were present throughout the barriers, thus confirming the intact integrity of the barriers for all the tested conditions, and that calcein was mainly present in the donor side of the PVPA barrier. These findings are in agreement with previous reports from confocal studies on the PVPA barrier integrity (Flaten et al., 2006a; Fischer et al., 2012).

3.1.3. Viscosity, composition and structure of the mucus layer

Since mucin is the major determinant in mucus rheology (Sigurdsson et al., 2013), the viscosity measurements were performed to study the effect of different mucin concentrations (Fig. 2). The tested suspensions exhibited a Newtonian character, with lower viscosity of mucin in concentration of 10 mg/mL compared to the mucin in higher concentrations. The increase in viscosity with increasing mucin concentrations correlates well with the gel-forming effect of mucin (Grießinger et al., 2015; Dawson et al., 2004). Although the *in vivo* mucus layer has been reported to be of non-Newtonian character (viscoelastic with shear-thinning properties), studies have reported that the hydrated mucin type III from porcine stomach exhibits a Newtonian behaviour (Mackie et al., 2017; Boegh and Nielsen, 2015). Moreover, a comparison between the viscosity of human saliva and porcine gastric mucin was proposed by and Park and colleagues (Park et al., 2007). Both human saliva and animal mucin suspensions exhibited similar viscosities with increasing shear rates. Furthermore, an increase in viscosity was found with increasing mucin concentrations, as also found in our analyses.

As previously stated, the composition and concentration of mucin vary in the body depending on the location and function of the mucosal tissue. However, mucin accounts for generally not more than 5% of the mucus components (Griffiths et al., 2010). Even though the differences in viscosity have to be taken into account when developing a new model, they are only one of the factors affecting the diffusion of drugs through the mucus (Shaw et al., 2005). For these reasons, mucin in concentration of 10 mg/mL (viscosity 2.1 mPa*s) was used as a model for mucus in the permeability experiments in this study.

Fig. 2

The mucus-simulating media used in this study was prepared using solely unpurified mucin from porcine stomach type III. Constituents such as lipids, proteins and DNA were not added to keep the mucus-simulating layer as simple as possible and to be a general model for mucus, since the content of the other components can vary according to the different site, different species and the specific physiopathological condition (Lieleg et al., 2010). Our aim was to investigate if the presence of sole mucin would affect the permeation of the drugs through the PVPA barriers, and we concluded that it did. However, as reported by Larhed and colleagues (Larhed et al., 1998), other components can significantly hinder the diffusion of drugs through the mucus layer. In particular, the authors found that lipids had a major role in reducing the diffusion of drugs in native pig intestinal mucus. Moreover, it has to be kept in mind that a model mucus system made only out of mucin cannot be considered entirely equivalent to natural mucus, most likely due to the changes in physico-chemical properties caused by the mucin isolation procedures (Kocevar-Nared et al., 1997).

With regards to mucus structure, scanning electron microscopy images of mucin from porcine stomach type III have been obtained by Teubl and colleagues (Teubl et al., 2013). The authors suggested a structural similarity between mucin from porcine stomach and human salivary mucin fibres. The mucus mesh size was also determined for both samples (pore size up to 0.9 µm for porcine gastric and 0.8 µm for human mucin). These results can be compared to the ones by Bajka and colleagues (Bajka et al., 2015), who have investigated *ex vivo* porcine mucus and who have estimated the main pore diameter of the mucin sheets to be around 200 nm. The different results obtained in these two studies could be traced back to the different sample preparation methods and different sample origin (Huckaby and Lai, 2017). These considerations can give us an estimation on how the mucus layer on the PVPA barriers may look like compared to both human and animal mucus and on how particles/formulations

could diffuse through this layer, together with the pore size of the mucin mesh. However, it has to be taken into consideration the fact that the structure and composition of the mucus layer differs according to different animal species and different sites of the body (Huckaby and Lai, 2017) and that the mucus-PVPA model so far is aimed to be established as an artificial model for mucosal tissues in general.

3.1.4. Permeability study: the effect of the mucus layer thicknesses

To assess possible changes in drug permeability related to different mucus layer thicknesses on top of the PVPA barriers, the permeability of naproxen was measured in the presence of different volumes of mucus (mucin 10 mg/mL). The thickness of the mucus layer has been reported to be around 600 µm in the human stomach and 50-450 µm in the intestine and colon (Fig. 3, black arrow), although this might vary depending on fasted and fed state (Boegh and Nielsen, 2015; Shaw et al., 2005), and the thickness in the respiratory tract, in the female reproductive tract and the ocular mucus layer varies according to the specific site (Huckaby and Lai, 2017; Khanvilkar et al., 2001). For the naproxen permeability experiment, 20, 22, 25 and 50 µL of mucus, respectively, were added on top of the barriers and the thickness of the layer (Fig. 3, shaded area) was calculated from the surface area of the filter support. Results showed that there was a significant difference in naproxen's P_{app} when tested in the presence or absence of mucus (addressed in section 3.2), but there was no significant variation between the different mucus volumes/thicknesses. Therefore, even though the calculated mucus layer thickness for 50 µL of mucin suspension exceeded the physiological range, it was considered the best volume to use. This volume assured that the whole surface area of the barriers will be fully covered with mucus and thus reduced any deviations in the application volume.

Fig. 3

3.2. Permeability of drugs in solution using the mucus-PVPA

Four different model drugs (naproxen, indomethacin, ibuprofen and atenolol) were used both to evaluate whether the additional mucus layer would affect their permeability and to further highlight whether different mucin concentrations (10, 20 and 40 mg/mL) would have an effect on drug permeability. The drugs were chosen to cover a range of relevant physicochemical properties (Table 1)

Fig. 4 shows that for all drugs there was a significant decrease ($p < 0.05$) in permeation with the addition of the mucus layer. This behaviour was to be expected especially for the more lipophilic drugs (naproxen, indomethacin and ibuprofen), whereas a decrease in permeability was not expected for the more hydrophilic atenolol. However, Boegh and colleagues (2014) have previously reported a significant decrease in permeability of the hydrophilic drug mannitol in the presence of a biosimilar mucus layer on Caco-2 cell monolayer, highlighting the fact that mucus can represent a barrier to both hydrophilic and lipophilic drugs. In fact, it has to be taken into account that there are multiple mechanisms taking place during diffusion of drugs through the mucus layer before the permeation process, and that especially mucins' properties can influence mucus' barrier characteristics. Mucins are formed by a polypeptide backbone to which oligosaccharide side chains are attached, resembling the structure of a bottle-brush. These two different regions provide mucins with both a hydrophobic (protein backbone) and hydrophilic (glycosylated regions) nature, which can affect the diffusion of various types of drugs and formulations (Peppas and Huang, 2004). Moreover, in Olmsted et al. (2001) it is suggested that there are two major mechanisms

hindering compounds from diffusing through this layer: i) the interaction filtering, dependant on the electrostatic, hydrophobic forces, hydrogen bonds and specific binding interactions, and ii) the size filtering properties of the mucin mesh. However, the overall hydrophilicity of the mucin gel mostly affects lipophilic compounds, whereas hydrophilic ones tend to be freer to penetrate through (Boegh and Nielsen, 2015). In fact, it has been demonstrated how lipophilic drugs are able to interact with the non-glycosylated regions of the mucin macromolecule (naked protein region), which provide an area for a hydrophobic interaction with the drug. Therefore, the interaction between a lipophilic drug and mucin's hydrophobic region can slow down its diffusion through the mucus layer (Khanvilkar et al. 2001). On the other hand, for the hydrophilic compounds, their ionization can be the driving force of the diffusion through the mucus (Shaw et al., 2005).

In conclusion, the use of differently viscous mucus layers (mucin concentration of 10, 20 or 40 mg/mL) did not lead to differences in permeability of all of the tested drugs (Fig. 4), even though an increase in viscosity could suggest a slowed-down diffusion through mucus and a lower permeability through the barrier. Therefore, since no direct correlation was found between the concentration of mucin in the mucus layer and the drugs' permeability, mucin 10 mg/mL was chosen as the preferred suspension since it was the easiest to handle from a practical point of view.

Fig. 4

Fig. 5 shows the permeabilities of different compounds in the presence and absence of mucus (no mucin or 10 mg/mL mucin suspension, respectively) at room temperature (23-25 °C) and at the physiological temperature (37 °C). The permeability of the fluorescent marker calcein was measured at both temperatures to assure that the barriers would maintain their

integrity in both conditions. In all experiments, the electrical resistance was found to be in the range reported for the barriers with maintained integrity (Flaten et al., 2008). The different P_{apps} of the tested drugs confirmed the ability of the barriers to discriminate between compounds with different chemical structures and properties (Table 1) both for the original PVPA barriers and for the novel mucus-PVPA ones. Although some of the chosen drugs had similar chemical properties, the resulting permeability values were found to be compound-dependent, confirming that multiple forces are responsible for the diffusion and permeation of drugs, and that an *in vitro* screening model should be able to highlight different characteristics, especially in relation to mucus-drug interaction. The permeability of all the tested drugs further increased at 37 °C, most probably due to a more fluid lipid layer of the barriers and potentially a lower viscosity connected to the higher temperature. In general, the addition of mucus on top of the PVPA barriers led to a significant decrease in permeability at both temperatures as earlier discussed and as expected due to the intrinsic characteristic of mucus (Sigurdsson et al., 2013).

Fig 5

However, if all drugs/marker would have behaved identically in presence of the mucus layer compared to its absence, one could conclude that the rate-limiting factor could be the different diffusive pathway between the original PVPA barriers and the mucus-PVPA model. Nevertheless, what we have found in our study was that the permeabilities were linked to the chemical structure and physiochemical properties of the drug/marker and to the possible interactions with the mucus layer. For this reason, we believe that the interaction with this layer, rather than the longer diffusive pathway, is the important factor influencing the permeability of the compounds analysed in this study.

Permeability experiments were also carried out on filters covered with mucus only (without the phospholipid vesicle barrier), in order to assess the contribution of the sole mucus layer on the permeability of the drugs. However, it was found that the filters were not able to hold the mucus in the donor compartment (58.82 ± 2.57 % of the total amount of mucus that was placed on top of the filters was found in the acceptor medium after 5 hours). Due to this, it was not possible to assess the contribution of the mucus layer alone and compare it to the PVPA or mucus-PVPA model.

A correlation between permeability coefficients of model drugs obtained with the PVPA model, other well known models (such as Caco-2 and PAMPA) and the fraction absorbed in humans after oral administration was already assessed in previous studies (Flaten et al., 2006b; Naderkhani et al., 2014b). The novel mucus-PVPA model was still able to correctly classify the different model drugs in the same way the original model did (poorly, moderately and excellently absorbed drugs), even though P_{app} values significantly changed with the addition of mucus compared to its absence.

3.3. Permeability of liposome-associated drugs using the mucus-PVPA

Concerning mucosal administration, nanoparticulate mucoadhesive and mucopenetrating formulations have demonstrated great efficacy in multiple *in vitro* and *in vivo* studies, for both local and systemic drug delivery, confirming their innovative contribution to the pharmaceutical development (Netsomboom and Bernkop-Schnürch, 2016). In particular, liposomes have been established as promising carriers to improve the absorption of poorly absorbed drugs and several liposomal products are already on the market (Allen and Cullis, 2013). Mucoadhesive formulations (e.g. chitosan-coated liposomes) can actively interact with the mucus layer, extending the resident time in the application site and increasing the local

concentration of the drug contained in the delivery systems (Boegh and Nielsen, 2015). On the other hand, mucopenetrating formulations (e.g. PEGylated liposomes) are able to avoid the interaction with the mucus layer, accessing the underlying epithelia in a more effective manner (das Neves et al., 2011; Lai et al., 2011; Mahmood et al., 2017).

The optimal formulation should be able to assure a high drug concentration at the administration site and consequently a concentration gradient, allowing a passive diffusion across the mucus layer. In this study, plain, chitosan-coated and PEGylated liposomes have been chosen as model drug delivery systems to get their diffusive properties be tested on the novel mucus-PVPA model. We have already tested mucoadhesive and plain liposomes on the original PVPA (Naderkhani et al., 2014a). However, we realized the importance of the presence of mucus to optimize the estimation of the penetration potential of nanosystems.

3.3.1. The effect of the delivery system on drug permeability in the mucus-PVPA

The degree of interaction with mucus largely depends on the size and surface properties of the delivery system. It has been reported that by increasing the particle size of a delivery system from 124 to 560 nm the amount transported in time through the mucus layer significantly decreases due to a stronger steric impediment (Sanders et al., 2000). Moreover, Takeuchi et al. (2001) have found that 100 nm liposomes are able to diffuse through the mucus layer to a higher extent compared to bigger ones. However, the surface properties of the delivery system could also dictate its interaction with mucus, making the size the secondary diffusion driving force. It has been demonstrated that nanosystems bearing a positive charge are able to actively interact with the negatively charged mucus layer, producing a mucoadhesion effect (e.g. chitosan-coated particles) (Mackie et al., 2017), whereas slightly negatively charged and neutral systems would favour a higher diffusion

ability thanks to their lack of interaction with such layer (e.g. PEGylated particles) (Griffiths et al., 2010; Jøraholmen et al., 2017; Lieleg et al., 2010). However, the particles that are strongly attracted to mucus would be completely immobilized, whereas excessively negatively charged particles would be repulsed and unable to diffuse through such a layer (Groo and Lagarce, 2014; Lieleg et al., 2010). Lieleg and colleagues have confirmed that particles' mobility through the mucus layer is particularly influenced by their surface charge. They suggested that charged particles can interact via electrostatic interaction with mucin, slowing down their diffusion through the mucus layer (Lieleg et al., 2010). The authors have compared the diffusion through mucus of differently functionalised particles at different pHs, and found out that at neutral pH the diffusion of charged particles was not majorly hindered compared to that of neutral particles, whereas at pH 3 there was a significant difference in the diffusion of neutral and charged formulations. Moreover, according to the results from Lieleg et al., the zeta potential of the PEGyalted particles changed with the different pH conditions (neutral surface potential at pH 3 and negative at pH 7, Lieleg et al., 2010).

In our study, plain, chitosan-coated and PEGylated liposomes were prepared incorporating three different drugs, respectively (Table 2). The size of the liposomes ranged between 100 and 200 nm and the liposome dispersions exhibited a bimodal size distribution with varying polydispersity indexes (PI), depending on the formulation. The zeta potential varied between the different formulations and was dependant on the incorporated drug. However, the coating process led to an increase in zeta potential for the chitosan-coated formulations, as expected (Berginc et al., 2014). It has to be highlighted that the PEGylated formulations exhibited a negative zeta potential for all the drugs incorporated and this characteristic could be of a key importance regarding the mucus-penetrating properties (Groo and Lagarce, 2014). The fact that negatively charged nanocarriers have the characteristics of being mucopenetrating is also supported by the results from Chen et al. (Chen et al., 2013).

Moreover, the surface potential of PEGylated liposomes obtained by Jøraholmen and colleagues, confirms the fact that PEG grafting can produce negatively charged liposomes (Jøraholmen et al., 2017). The entrapment of the three model drugs varied depending on their chemical properties All formulations were prepared according to the methods reported by Jøraholmen and colleagues (Jøraholmen et al., 2014; Jøraholmen et al., 2015; Jøraholmen et al., 2017). The liposomes prepared in our study exhibited comparable characteristics to the ones described in the above-mentioned papers. In particular, the authors found that PEGylated formulations exhibited a reduced binding efficacy compared to plain and chitosan-coated ones, whereas chitosan-coated liposomes were binding mucin significantly more compared to plain ones (Jøraholmen et al., 2017).

As previously stated, the interaction of liposomal formulations with the mucus layer can be affected by numerous factors, such as the pH of the physiological environment, pH of the specific formulation and pKa and related degree of ionization of the associated drug (Groo and Lagarce, 2014; Jøraholmen et al., 2017; Lieleg et al., 2010; Shaw et al., 2005). In this study, chitosan-coated formulations were prepared at acidic pH and were then adjusted to pH 7.40. This process was carried out to ensure the same pH environment of the liposome-associated drug for all formulations (plain, chitosan-coated, PEGylated liposomes). This pH was selected as a model pH, however the next step would be to adjust it to the targeted mucosal site (e.g. around pH 6 depending on which part of the intestine or 4.5 for the vaginal site).

Table 2

The permeability of metronidazole, indomethacin and naproxen from different liposome formulations (Fig.6) indicated decreased permeability for liposomally-associated

drugs compared to drugs in solution, confirming that liposomes assured a sustained release of the associated drugs. This is a very important feature considering prolonged release of drugs at the administration site, e.g. vaginal site (Jøraholmen et al., 2014).

For metronidazole-containing liposomes, the drug permeability did not vary between the different formulations in the absence of mucus, suggesting that the chitosan coating and PEGylation processes had a negligible effect on drug release from the liposomes compared to the plain ones, evidence supported by the results obtained by Chen et al. (2013). However, in the presence of the mucus layer, metronidazole's permeability changed according to the type of liposome formulation. In fact, chitosan-coated liposomes displayed a lower permeability of the drug compared to the plain ones, suggesting that the potential interaction between mucus and the chitosan-coating could slow down the permeation process of metronidazole, whereas PEGylated liposomes could easily penetrate through the mucus layer, contributing to a higher permeability. These results can be also explained by the different zeta potentials of the three formulations. Chitosan-coated liposomes, bearing a slightly positive zeta potential, could interact with the negatively charged mucus leading to a mucoadhesive effect, whereas the PEGylated liposomes, having a slightly negative zeta potential, could freely diffuse through the mucus layer. These results are supported by the findings of Chen and colleagues (2013), who clearly depicted the different mucus penetration potentials of plain phosphatidylcholine, chitosan-coated and Pluronic®-modified liposomes in *ex vivo* penetration studies. Their *in vivo* pharmacokinetic study further demonstrated that the Pluronic®-modified formulation (bearing a zeta potential of -4 mV) could provide the best oral absorption profile for the chosen drug, indicating that the *ex vivo* data correlate well with the *in vivo* one.

The indomethacin- and naproxen-containing liposomes, exhibited a different penetration behaviour; indomethacin-containing plain, chitosan-coated and PEGylated liposomes were all found to be negatively charged (-25, -19 and -10 mV, respectively), a

feature that could lead to a lack of significant differences in the diffusion potential of the formulation and permeability of the drug. On the other hand, for the naproxen-containing liposomes, the PEGylation lead to an increase in permeability in the absence of mucus, suggesting an intrinsic penetration behaviour of the formulation. These deviations from the trends described above for the metronidazole-containing liposomes can be ascribed to the complexity of the physicochemical characteristics of the specific liposomal formulation, highlighting the problem/challenge of generalization when studying mucus diffusion properties and permeability potentials of different types of formulations (Fabiano et al., 2017; Netsomboon and Bernkop-Schnürch, 2016). Moreover, we found that the surface potential of the liposomes prepared varied according to the drug incorporated. Therefore, the mucopenetrating or mucoadhesive behaviour could mainly be linked to the specific zeta potential of the formulation. The permeability of the drugs depends on numerous factors including the penetration potential of the liposome formulation through the mucus layer and the interaction with it, the vesicle surface properties and size, but also the release of the drug from the delivery system, the chemical and structural properties of the specific compound and the drug equilibrium between the different layers. This confirms the high importance and need to have reliable *in vitro* permeability models able to predict the effect of mucus on the permeability of both drugs in solutions and in more complicated formulations.

Fig. 6

Conclusions

The novel mucus-PVPA model was developed and exploited to better mimic the *in vivo* environment of mucosal tissues by adding a mucus-simulating layer on top of the PVPA

barriers. The reliability of this upgraded version of the original PVPA model was proven in terms of the barrier tightness and functionality, and the barriers demonstrated maintained integrity under the chosen conditions. As expected, the mucus layer proved to be an additional barrier to the permeation of the selected drugs. The permeability varied depending on the different chemical structures and properties of the tested drugs. Moreover, the mucus-PVPA barriers were able to discriminate between different types of nanodelivery systems. The mucus-PVPA model was proven as a reliable tool in drug/active compound screening and can serve in the development and optimization of formulations destined for transmucosal delivery.

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Conflict of interest

No conflict of interest are declared by the authors.

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Figure and table captions

Table 1: Overview of the model drugs/marker included in this study.

Compound	Abbreviation	pKa	Log P	Log $D_{7.4}$[d]	Charge at pH 7.4[b]	Detection method	Wavelength (nm)	St.curve (nmol/mL)
Calcein	CAL	1.8/9.2[a]	-1.71[b]		-	Fluorimeter	Ex.: 485 Em.: 520	0.10-2.25
Atenolol	ATN	9.54[c]	0.16[d]	-1.03	+	UV	274	0.20-80.45
Ibuprofen	IBP	4.45[c]	3.97[d]	0.81	-	UV	220	10-150
Indomethacin	IND	4.42[c]	4.27[d]	0.77	-	HPLC-UV	254	0.016-320
Metronidazole	MTR	2.62[e]	-0.02[d]	0.14	0	UV	320	30-200
Naproxen	NPR	4.18[c]	3.18[d]	1.70	-	UV	270	0.8-84

[a]: Flaten et al. 2006b
[b]: Naderkhani et al. 2014b
[c]: Avdeef 2003
[d]: Benet et al. 2011
[e]: Rediguieri et al. 2011

Fig. 1: P_{app} values for calcein and electrical resistance of the PVPA barriers in the presence and absence (control) of different concentrations of mucin (10, 20, 40 mg/mL). The results are indicated as mean ± SD (n=3).

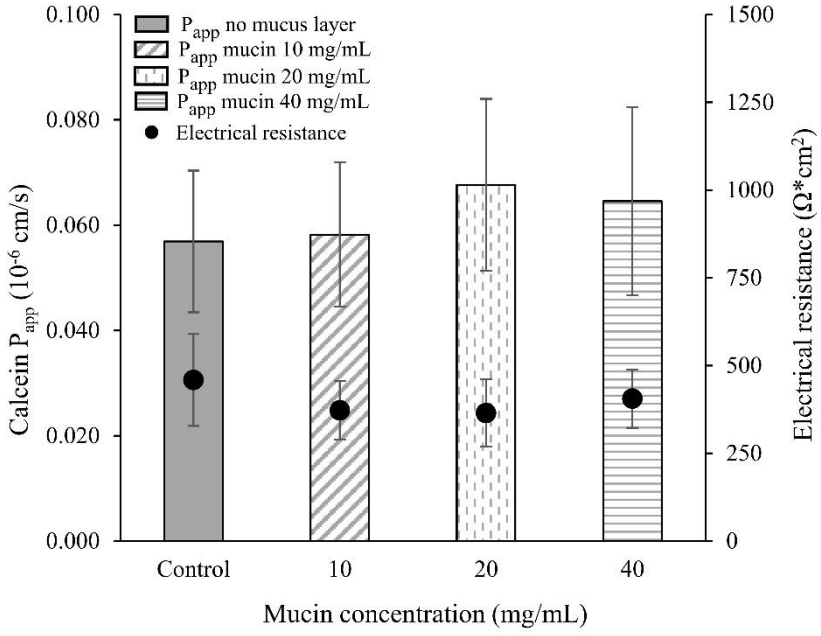


Fig. 2: Mucin viscosity of three mucin concentrations (10, 20 and 40 mg/mL).

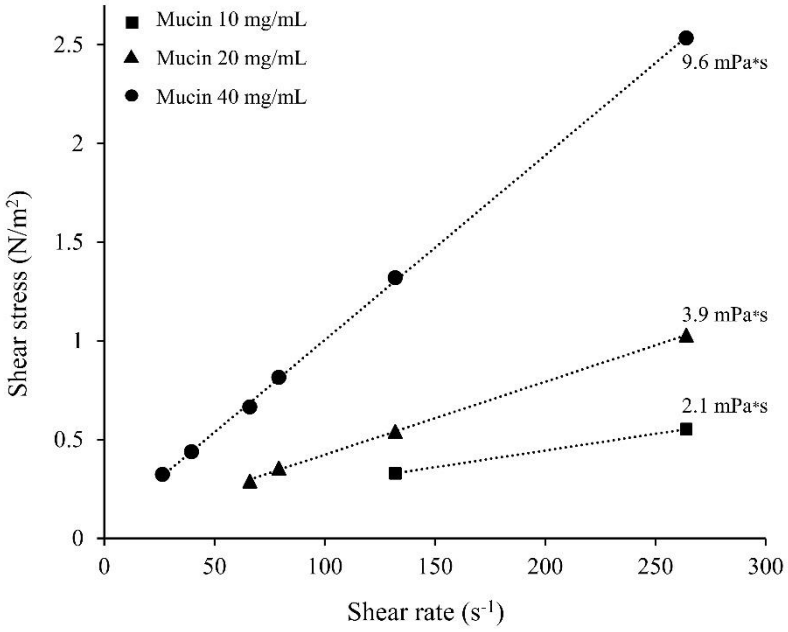


Fig. 3: Naproxen P_{app} (left axis) in the presence of a mucus layer with varying thicknesses (right axis, shaded area), and compared to the physiological mucus layer thickness (◄►), dependent on the volume of mucus (mucin 10 mg/mL) added to the PVPA. The results are indicated as mean ± SD (n=3). Statistical significance ($p < 0.05$) was investigated with one-way ANOVA using the Bonferroni *post hoc* test.

* Statistically significant difference in drugs' P_{app} in the presence of different mucus volumes compared to its absence.

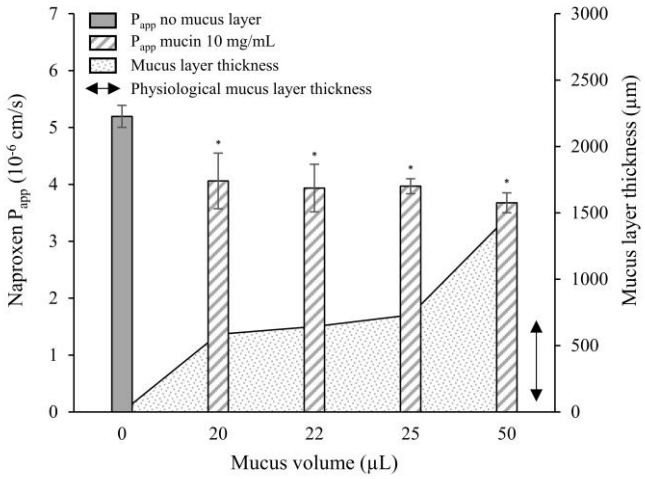


Fig. 4: Drug permeability in the presence and absence of different mucin concentrations (0, 10, 20, 40 mg/mL). The results are indicated as mean ± SD (n=3). Statistical significance ($p < 0.05$) was investigated with one-way ANOVA using the Bonferroni *post hoc* test.

* Statistically significant difference in drugs' P_{app} in the presence of mucus with different mucin concentrations compared to its absence.

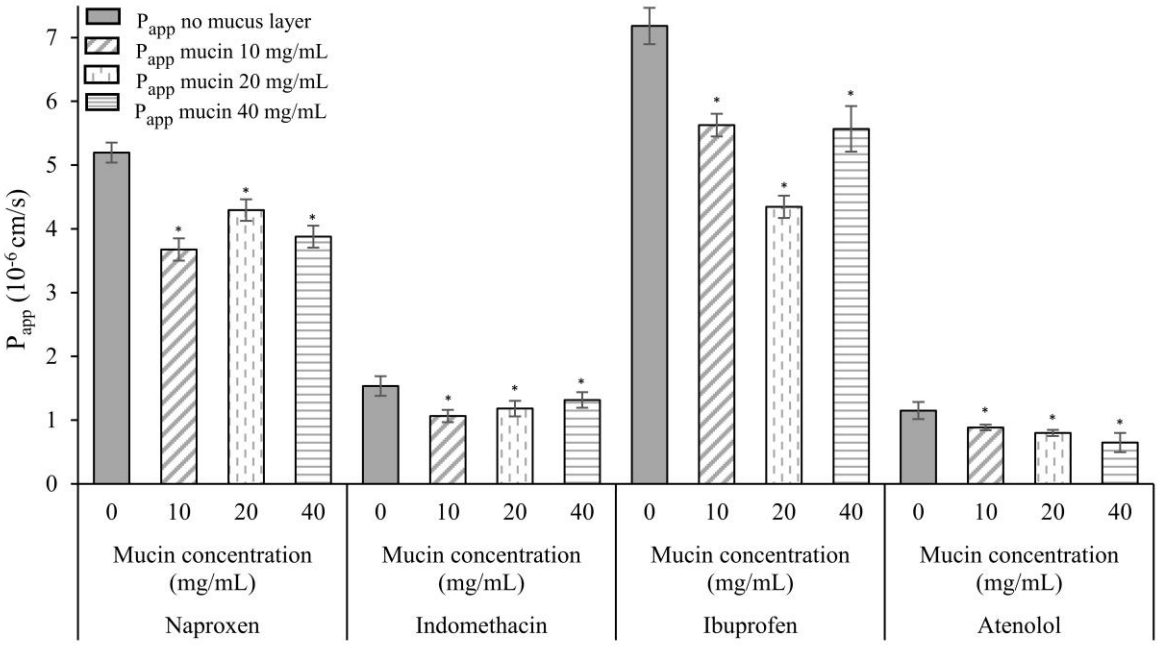


Fig 5. Permeability of different compounds in the presence and absence of mucin (10 mg/mL) at room temperature (23-25°C) and at 37°C. The results are indicated as mean ± SD (n=3). Statistical significance ($p < 0.05$) was investigated with one-way ANOVA using the Bonferroni *post hoc* test.

* Statistically significant difference in drugs' P_{app} in the presence of mucus compared to its absence.

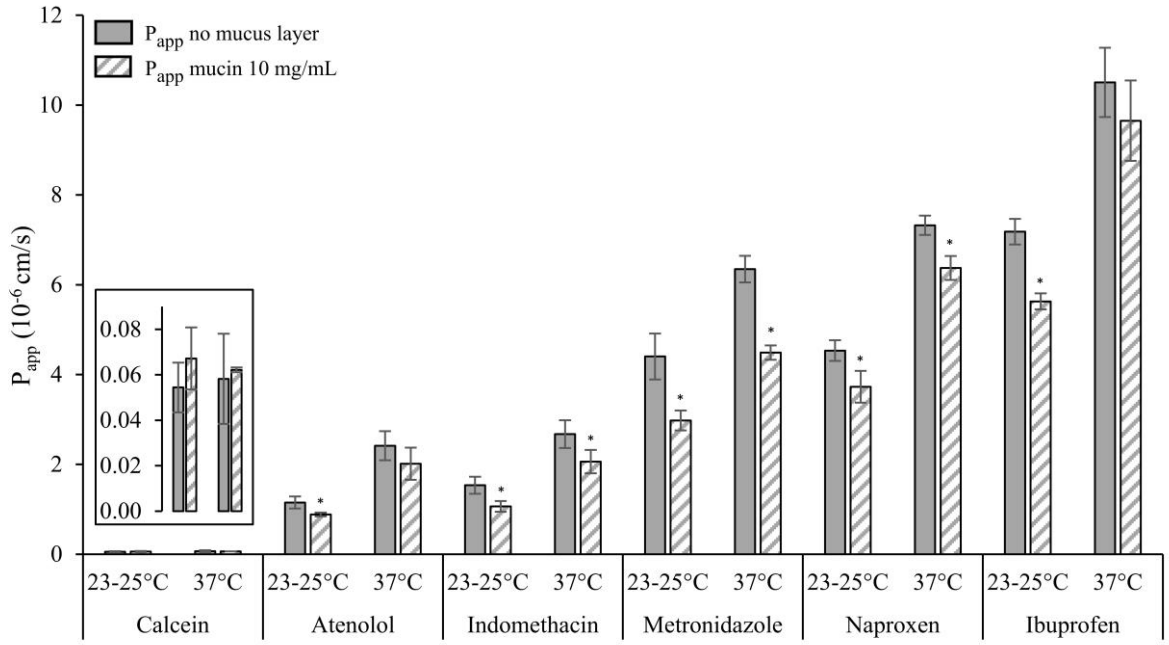


Table 2. Liposomal characteristics. The results are expressed as mean ± SD (n=3).

Formulation*	Vesicle size (nm)	PI	Zeta potential (mV)	Entrapment (%)
Plain liposomes containing NPR	146.30 ± 13.15 (100%)	0.28	-2.32 ± 1.20	26.15 ± 2.19
Coated liposomes containing NPR	138.10 ± 4.38 (95.2%)	0.38	0.19 ± 0.50	37.43 ± 5.79
PEGylated liposomes containing NPR	128.00 ± 6.36 (99.8%)	0.18	-10.89 ± 2.13	23.58 ± 0.31
Plain liposomes containing IND	140.85 ± 5.87 (97.6%)	0.27	-24.75 ± 0.35	83.30 ± 3.88
Coated liposomes containing IND	134.15 ± 18.74 (96.5%)	0.30	-18.68 ± 1.53	73.87 ± 4.03
PEGylated liposomes containing IND	96.22 ± 5.11 (98.3%)	0.23	-10.60 ± 0.34	77.81**
Plain liposomes containing MTR	202.52 ± 2.24 (70.1%)	0.52	-2.13 ± 1.34	2.82 ± 0.14
Coated liposomes containing MTR	162.27 ± 8.44 (68.9%)	0.63	1.91 ± 0.24	2.78 ± 0.01
PEGylated liposomes containing MTR	105.40 ± 5.11 (98.6%)	0.20	-4.38 ± 0.519	2.58 ± 0.20

* Naproxen (NPR), indomethacin (IND) and metronidazole (MTR)
** Only one batch was prepared

Fig. 6: Permeability of metronidazole, indomethacin and naproxen from different liposomal formulations in the presence and absence of mucin (10 mg/mL). The results are indicated as mean ± SD (n=3). Statistical significance ($p < 0.05$) was investigated with one-way ANOVA using the Bonferroni *post hoc* test.

* Statistically significant difference in drugs' P_{app} between the highlighted bar and all the others or between 2 different bars.

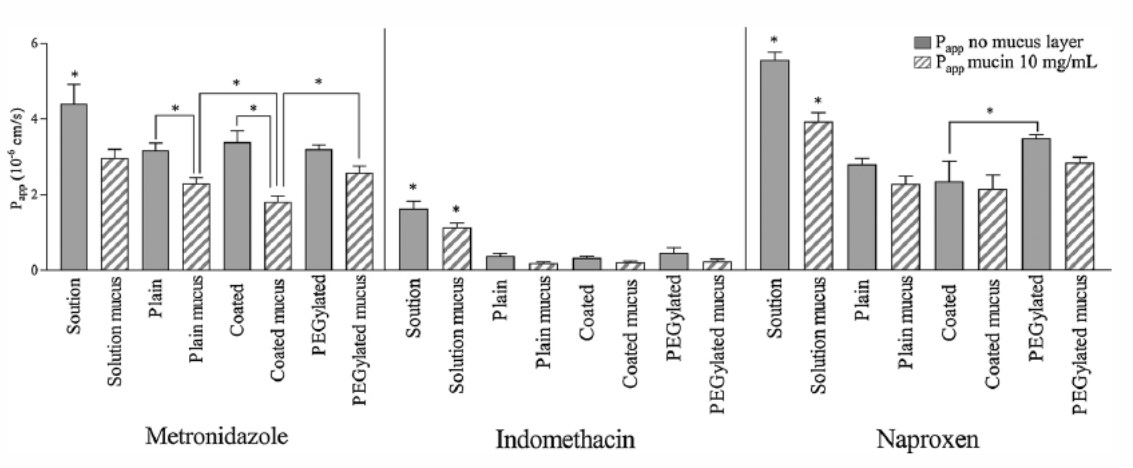


Fig. S1. Confocal laser scanning microscopy of the PVPA barrier labelled with rhodamine (red) after soaking for 3 hours in calcein solution (green). The two micrographs were taken from two different positions in the barrier. The white lines mark the placement of the cross-sections *a* and *b* shown at the top and bottom, respectively.

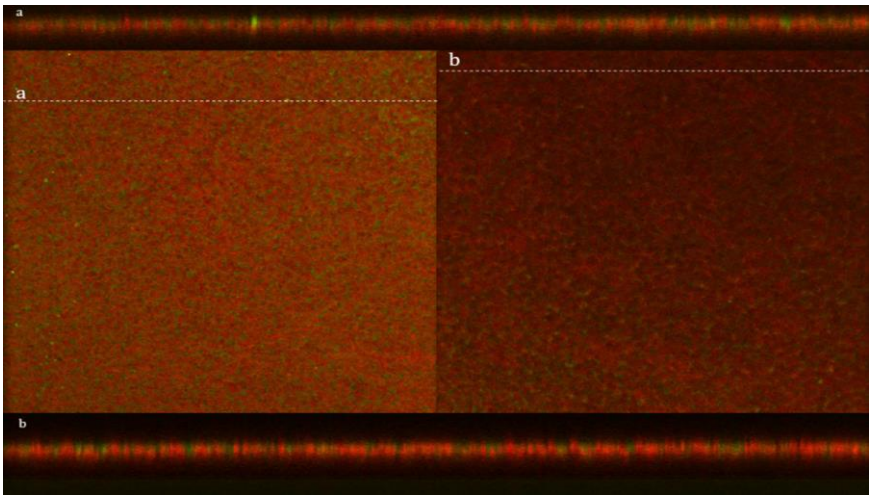


Fig. S2. Confocal laser scanning microscopy of the PVPA barrier labelled with rhodamine (red) after soaking for 3 hours in mucin 10 mg/mL marked with calcein (green). The two micrographs were taken from two different positions in the barrier. The white lines mark the placement of the cross-sections *a* and *b* shown at the top and bottom, respectively.

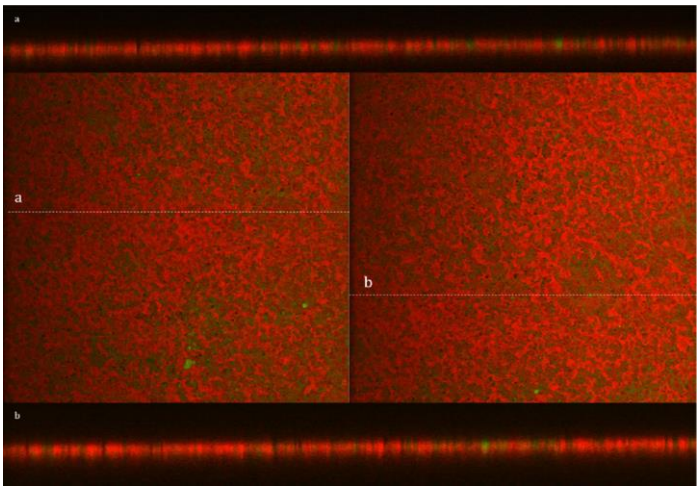
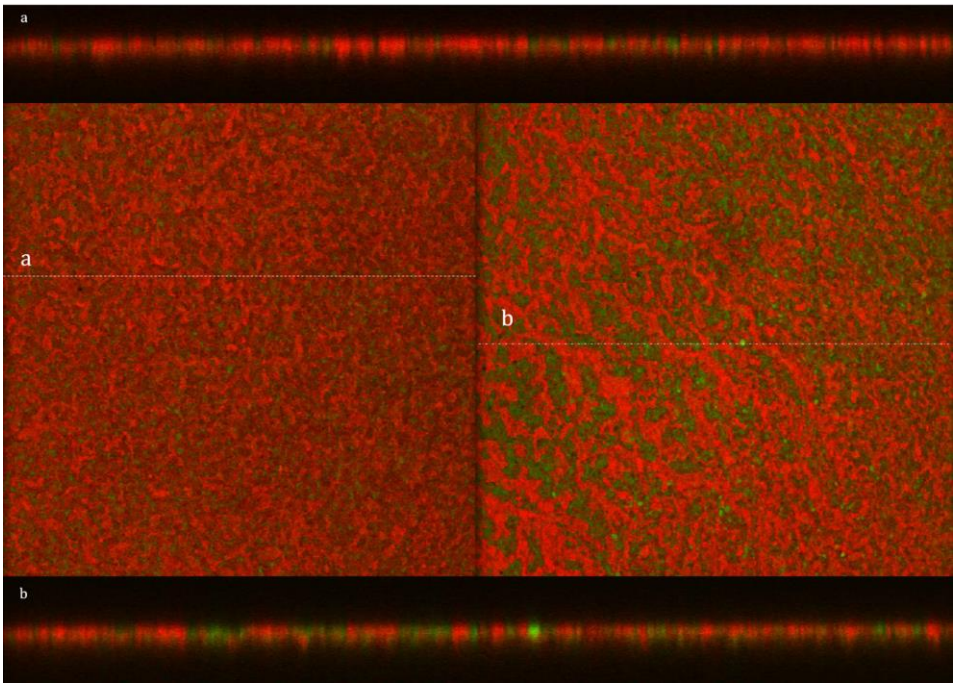


Fig. S3. Confocal laser scanning microscopy of the PVPA barrier labelled with rhodamine (red) after soaking for 3 hours in mucin 40 mg/mL marked with calcein (green). The two micrographs were taken from two different positions in the barrier. The white lines mark the placement of the cross-sections *a* and *b* shown at the top and bottom, respectively.



Supplementary

Confocal laser scanning microscopy

Methods

Confocal laser scanning microscopy (CLSM) was used to assess possible interactions between the mucin suspensions and the PVPA barriers. The barriers were prepared as described in section 2.2 with the only exception that 0.2 mol% of the Lipoid E80 was replaced by 1,2-dioleoyl-*sn*-glycero-3-phophoethanolamine-N-(Lissamine rhodamine B sulfonyl) (ammonium salt) rhodamine (purchased from Avanti Polar Lipids, Inc., Alabama, USA) to visualise the lipids composing the barriers. Calcein solution (1.65 mg/mL) was used to produce two suspensions with different mucin concentrations (10 mg/mL and 40 mg/mL). Before the experiment, 50 µL of either calcein solution or mucin suspensions (10 mg/mL and 40 mg/mL) were added to the donor compartment and the system was left to soak for three hours in the acceptor wells containing 600 µL of PBS pH 7.4 to visualise possible aqueous channels throughout the barriers' thickness caused by the mucus layer . After soaking, the donor fluids were removed and the filters carefully detached from the inserts. The CLSM analysis was performed on a Leica TCS SP5 microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) equipped with an Argon laser for calcein and a DPSS 561 laser for rhodamine. Laser lines of 488 and 568 nm were used to excite calcein and rhodamine, respectively. For calcein, fluorescence was detected in the spectral range of 500-550 nm, while rhodamine was detected at 570-610 nm (Ternullo et al., 2017). Images were acquired with a 10x0.4 objective taking z-section micrographs (z-step size of 0.25 µm). To make sure the defects were not present throughout the whole thickness of the barriers, 420 z-sections were analysed for each barrier. The gain, off-set and zoom were kept as constant as possible to maintain the same setup for all

the micrographs. The micrographs were superimposed using Volocity® v.6.3 software (PerkinElmer, MA, USA).

Results

In a previous study, Flaten and colleagues have analysed via confocal electron scanning microscopy the filters composing the PVPA barriers without the addition of the liposomes (Flaten et al., 2006a) and used it as a control in order to visualise how aqueous channels look like in the absence of the lipid component. By comparing this control to confocal images of the PVPA barriers, they were able to see that no significant aqueous channels were present throughout the thickness of the PVPA barriers. In our study, we wanted to visualise if the addition of mucus would cause the formation of aqueous channels in the PVPA barriers, especially since the high permeability of molecules (in or case the highly hydrophilic marker calcein) can be traced back to a significant number or defects and aqueous channels in the barriers (Richter et al., 2016).

Confocal images are shown in Fig. S1 (calcein solution in the donor), Fig. S2 (10 mg/mL mucin suspended in calcein solution the donor) and Fig. S3 (40 mg/mL mucin suspended in calcein solution in the donor).

Fig. S1

Fig. S1 displays the PVPA barrier after exposure to the calcein solution, showing a dominant red fluorescence representing the rhodamine-associated PVPA barrier and a green fluorescence of the hydrophilic calcein solution. The cross-sections *a* and *b* taken in different positions confirm lack of aqueous channels through the barrier, suggesting the maintenance of

the barrier's integrity in the given condition. This is in agreement with previous CLSM studies of the original PVPA barriers (Flaten et al. 2006a).

Fig. S2

Fig. S2 indicates that no aqueous channels were present after the exposure to the lowest concentration of mucus suspension.

Fig. S3

As it can be observed from Fig. S3, calcein was more abundant as compared to previous results (Fig. S1 and S2). The first cross-section (*a*) indicates a barrier similar to the one when PVPA barrier was exposed to calcein solution (Fig. S1 and S2). In the second micrograph and cross-section (*b*) calcein was visible in a higher concentration in the donor side of the barrier. However, no significant breaches in the barrier were observed, suggesting that the barrier's integrity was maintained also in the presence of the highest concentration of mucus suspension.