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Barrier properties of ex vivo porcine intestinal mucus are highly independent of isolation and storage conditions

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ABSTRACT
Porcine intestinal mucus (PIM) is often utilized as an ex vivo mucus model in mucus interaction studies. However, numerous isolation procedures and storage conditions for PIM are reported, yet their potential impact on preserving the critical properties of PIM remains unknown. This study investigated the effect of isolation procedures (rinsing and anatomical site of mucus isolation) and storage conditions (–20 ºC, –80 ºC, snap frozen in liquid nitrogen prior to storage at –80 ºC, or freeze-dried followed by storage at room temperature and reconstitution) of PIM in regard to the permeation of fluorescein-isothiocyanate-labelled dextran (FD) macromolecules of 4, 40 and 150 kDa, rheological properties as well as pH, osmolality, protein and water content. Rinsing intestines with tap water or phosphate-buffered saline as well as isolating PIM from different regions of the first five meters of the proximal jejunum did not affect the pH or osmolality of isolated PIM. The permeation of FD4, FD40 and FD150 through stored PIM was similar to permeation through fresh PIM. The rheological properties of stored PIM were similar to properties of fresh PIM. Osmolality, protein and water content were similar in stored and fresh PIM whereas pH decreased with 0.3 unit for all stored PIM. Overall, PIM samples stored at –20 ºC, –80 ºC, snap frozen or freeze-dried were found to have similar properties to freshly isolated PIM and can all be consi-dered good alternatives to fresh PIM for mucus studies.

1. Introduction
Intestinal mucus constitutes a steric and interactive barrier for diffusion of macromolecules such as biopharmaceuticals, small hydrophobic drugs and drug delivery systems (DDS) intended to facilitate absorption of drugs [1]. Because of limited access to human intestinal mucus, porcine intestinal mucus (PIM) is often used for ex vivo studies due its high structural and viscoelastic similarities to that of human intestinal mucus [1–3].

PIM can be collected from intestines by gently scraping the mucosal surfaces of the excised tissue [1,3–5]. The small intestine consists of the duodenum, jejunum and ileum and as each part has specific physiological properties, mucus properties may vary depending on the anatomical site of isolation [1,3]. Prior to PIM isolation, mucosal surfaces have been rinsed with e.g. water [6], isotonic and isohydric buffers [4,5], and ice-cold simulated intestinal fluid [3] to remove food debris. As the viscoelastic properties of mucus is strongly dependent on pH and ionic strength, pooling mucus from larger sections of the small intestine or rinsing mucus can potentially be problematic due to altered barrier properties of PIM [2,4,5,7]. This was reported by Boegh et al., who found that rinsing with an isotonic and isohydric buffer decreased osmolality and viscosity of ex vivo PIM [4,5].

Isolated PIM has been used for numerous drug delivery and inter- action studies [2–4]. Both freshly isolated PIM [6,8] and stored PIM (–20 ºC [4,5,9] or snap frozen in liquid nitrogen before storage at –80 ºC [1,5,6]) have been employed in different experiments [2–5]. As a biological material, PIM is susceptible to degradation over time, thus appropriate conditions are necessary to maintain integrity and general properties of PIM during storage. Mucus properties are generally found to be independent of storage conditions though these data are often based on other types of mucus than PIM or is limited to one storage condition or one experimental method [2–5,7,9,10]. Thus, there is a gap in understanding the effect of storage on PIM properties. Rheology and permeability studies are often employed to evaluate properties of mucus [2,4,5,8,9]. Thus, rheological properties and permeability of macro- molecular marker molecules across PIM stored at different conditions

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are essential to evaluate to ensure that results from mucus studies are not affected by the storage conditions.

This study aimed to investigate if isolation procedures and storage conditions would affect the physicochemical characteristics and barrier properties of isolated ex vivo PIM.

2. Materials and methods

2.1. Materials

Fluorescein-isothiocyanate-labelled dextrans of average 4 kDa (FD4), 40 kDa (FD40) and 150 kDa (FD150), Hank Balanced Salt Solution (HBSS), bovine serum albumin (> 98%) (BSA) and phosphate-buffered saline (PBS); Sigma-Aldrich (St. Louis, MO, USA). 2-(N-morpholino)-ethanesulfonic acid anhydrous BioChemica (MES); PanReac AppliChem (Darmstadt, Germany). Pierce™ BCA protein assay kit; Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Isolation

Intestines from healthy fasted (18–24 hrs) gilts (40–60 kg, 3–4 months, Danish Landrace) were obtained after experimental surgery. Immediately after euthanization, up to 5 min jejunum was isolated distal to the ligament of Treitz. Sections were opened by a latitudinal cut and mucus was isolated by gently scraping the mucosal surface. Intestines and mucus were kept on ice at all times. Rinsing procedure: The jejunum was either unrinsed or rinsed with tap water or PBS, mucus was isolated and either used fresh or stored at −20 °C, or at −80 °C directly or after snap freezing in liquid nitrogen, or freeze-drying followed by storage at room temperature (RT) until use. Freeze-dried PIM was reconstituted in demineralized water volumes corresponding to the weight loss after freeze-drying (stirring 4–6 hrs at RT, stirring at 4 °C for 12–15 hrs, and storage at 4 °C without stirring). Procedures were according to the authorization by Danish Veterinary and Food Administra-trion (license number DK-13-oth-931833).

2.3. Permeability

Permeability of fresh or stored (4–26 months, average 8 months for each condition) PIM was assessed using permeable filter inserts (Corning, Costar Transwell®, Thermo Fisher Scientific; tissue culture treated polycarbonate membrane, 1.12 cm², 0.4 µm pore size). PIM was hydrated to ensure complete mucus coverage of the insert without physical intervention that might create holes and compromise mucus integrity. Hydration: 3.5 parts PIM to 1 part 10 mM MES in HBSS pH 6.5 or 6.9 (mHBSS) the day prior to the experiment and storage at 4 °C. 250 µL hydrated PIM was applied to the filter inserts and equilibrated for 10 min on a shaking table (37 °C, 50 rpm). 1000 µL mHBSS was added to the receiver compartments and the equilibration continued for another 10 min. At 0 min, 100 µL of donor solution (125 µg/mL FD4 kDa, 40 kDa, or 150 kDa) was added gently and dropwise to the donor compartment. At 10, 20, 30, 50, 60, 90, 120, 150, 180 and 240 min, 200 µL samples were collected from the receiver compartments, which were replenished with 200 µL mHBSS (37 °C). The samples’ fluorescence was quantified at λEM 485 nm and λEX 520 nm using 96-well black microplates in a plate reader (FLUOstar Omega, BMG LABTECH, Ortenberg, Germany) including standard curves based on serial dilutions in the range of 0.03125–2.0000 µg/mL. Maximum ± 15% deviation (±20% for the lower limit of quantification) of the calculated fluorescence intensity from the measured value was allowed for data points to be accepted and included in the standard curve of minimum four data points. Lower and upper limit of quantification (LLOQ and ULOQ) was determined from the standard curve lowest and highest acceptable concentration. The accumulated amount (Q, mol/cm²) of permeated FD was plotted as a function of time (t) and the steady state flux (JSS, mol · min⁻¹ · cm⁻²) was calculated as the linear slope of minimum three data points and only accepted if sink condition. The apparent permeability coefficient (PAPP, cm/s) was calculated using equation (1). Co, donor was the concentration of FD in the donor compartment at 0 min.

\[
P_{\text{APP}} = \frac{J_{\text{SS}}}{C_{0,\text{donor}}} \tag{1}
\]

The lag time was calculated as the linear regression slope intercept with the x-axis. The permeated amount (%) was calculated based on the accumulated amount in the receiver compartment after 240 min relative to initial added FD.

2.4. Rheology

Rheological measurements of PIM stored for 5 months at different conditions were conducted with an AR-G2 rheometer equipped with a 40 mm cone with a cone angle of 1° and a Peltier plate (TA Instruments, New Castle, DE, USA). The plate temperature was 37 °C and a solvent trap (TA Instruments) was used to prevent sample dehydration. First, a 10 s pre-shear step with 100 s⁻¹ was conducted followed by 5 min equilibration. Measurements were conducted in five consecutive steps: 1) frequency sweep; range of 0.1–10 rad/s, oscillation stress of 0.5 Pa ensuring measurements were conducted within the linear viscoelastic range (LVE), 2) continuous flow ramp; increasing shear rates from 0.01 to 3000 s⁻¹, 3) stress sweep; constant frequency of 1 rad/s, oscillation range 0.1–500 Pa. Equilibration of 5 min was used between each step. LVE end was determined by maximum 5% deviation of storage modulus G’ from the mean of five data points of G’, which were stress independent. Only biological replicates were conducted rather than technical replicates to capture the higher biological variation [4].

2.5. Protein content

The protein content in mucus was determined by the commercial Pierce™ BCA protein assay kit. Briefly, a 500-fold dilution of PIM in demineralized water was analyzed and quantified using a BSA standard curve ranged from 0.1 to 0.8 mg/mL. Technical triplicates of 25 µL demineralized water, BSA standard or diluted PIM was added to 200 µL kit reaction solution in 96-well clear plates. Incubation for 30 min at 37 °C and subsequent cooling to RT (10 min) was done prior to measu-ring absorbance at λ 562 nm using a plate reader (FLUOstar Omega).

2.6. Water content

Thermogravimetric analysis (TA Instruments, New Castle, DE, USA) was used to determine the water content in mucus stored at different conditions. Briefly, approximately 50 µL PIM was added to platinum pans in duplicate and the weight measured with increasing temperature (ramps of 10 °C/min from 40 to 300 °C). Water content was calculated based on the weight loss at 300 °C.

2.7. Statistics

All data are presented as means and standard deviations (SD). One-way analysis of variance (ANOVA) followed by Dunnett multiple comparisons test assuming equal variance and normal distribution of data was used to determine significant differences between three or more means. In case of unequal variance, a Welch test was used. GraphPad Prism 8.3 from GraphPad Software (La Jolla, CA, USA) was used.

3. Results and discussion

3.1. Rinsing and anatomical site do not affect pH and osmolality of PIM

Prior to isolation of PIM, the mucosal surface was either unrinsed, rinsed with tap water or PBS. It was found that the pH and osmolality of
isolated PIM were 6.89 ± 0.11, 6.82 ± 0.06, 6.78 ± 0.07 and 385 ± 37 mOsm, 365 ± 69 mOsm, 399 ± 64 mOsm for unrinsed, tap water rinsed and PBS rinsed PIM, respectively (n = 4–5 pigs). Thus, in the present study, the rinsing procedure had no effect on the slightly acidic pH and the hyperosmolality of isolated PIM. The pigs were fasted prior to PIM isolation and barely any visual food debris was present so little rinsing was needed. The differences between our findings and those by Boegh et al. [4,5] could be caused by different extents of rinsing. Rinse with tap water, which is hypotonic and has no buffer capacity, did not affect pH or osmolality compared to unrinsed PIM in this study. Extensively rinsing mucosal surfaces prior to PIM collection can lead to significant changes such as hydration, change in pH and osmolality of PIM, which would lead to conformation changes of mucins and thus altered PIM properties [2,4,5]. If possible, extensive rinsing should be avoided by e.g. fasting the animals prior to PIM isolation or by using limited amounts of rinsing media with pH and osmolality similar to that of native PIM.

The pH and osmolality of PIM are reported to vary throughout the small intestine [1,3]. As PIM normally is isolated and pooled from the proximal part of the jejunum, it was investigated if PIM isolated from the first 5 m of jejunum varied in pH and osmolality (Table 1). Neither pH nor osmolality were found to differ significantly amongst the isolated segments, thus mucus from the proximal 5 m of this anatomical site may be pooled without risking alterations in pH and osmolality.

### Table 1

<table>
<thead>
<tr>
<th>Distance from ligament of Treitz (m)</th>
<th>pH</th>
<th>Osmolality (mOsm/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>6.89 ± 0.11</td>
<td>376 ± 35</td>
</tr>
<tr>
<td>1.0</td>
<td>6.83 ± 0.10</td>
<td>402 ± 26</td>
</tr>
<tr>
<td>1.5</td>
<td>6.77 ± 0.08</td>
<td>401 ± 41</td>
</tr>
<tr>
<td>2.0</td>
<td>6.80 ± 0.10</td>
<td>426 ± 73</td>
</tr>
<tr>
<td>2.5</td>
<td>6.82 ± 0.05</td>
<td>423 ± 66</td>
</tr>
<tr>
<td>3.0</td>
<td>6.86 ± 0.09</td>
<td>413 ± 52</td>
</tr>
<tr>
<td>3.5</td>
<td>6.84 ± 0.03</td>
<td>421 ± 88</td>
</tr>
<tr>
<td>4.0</td>
<td>6.84 ± 0.03</td>
<td>381 ± 38</td>
</tr>
<tr>
<td>4.5</td>
<td>6.84 ± 0.03</td>
<td>465 ± 57</td>
</tr>
<tr>
<td>5.0</td>
<td>6.88 ± 0.09</td>
<td>399 ± 80</td>
</tr>
<tr>
<td>Pooled 0-5</td>
<td>6.78 ± 0.12</td>
<td>441 ± 75</td>
</tr>
</tbody>
</table>

Fig. 1. A-C) Apparent permeability coefficients (P_{app}) D-F) lag times and G-I) permeated amounts (%) after 240 min of the initially added of fluorescein-isothiocyanate dextrans 4 kDa (A, D, G), 40 kDa (B, E, H) and 150 kDa (C, F, I) through ex vivo porcine intestinal mucus (PIM) fresh or stored at either 20 °C, 80 °C, snap frozen in liquid nitrogen and stored at −80 °C (snap frozen) or freeze-dried followed by storage at room temperature and reconstitution (freeze-dried). Data are given as mean ± standard deviation of n = 1–5, N = 2–12. For FD150 most replicates in the −20 °C, −80 °C and snap frozen condition were below the limit of quantification, thus total number of replicates < 3 and no SD is included. Data were not statistically different from the fresh condition unless indicated by * (P < 0.05).
3.2. Storage of PIM did not affect permeability and physio-chemical properties of PIM except for pH

Due to practicalities, storage of PIM is often necessary. In literature, PIM is reported to be stored at \(-20^\circ C\) or \(-80^\circ C\), snap frozen in liquid nitrogen before storage at \(-80^\circ C\). Snap freezing is often done due to a concern that slow freezing may lead to the formation of ice crystals, which can cause mechanical damage to the physical structure of PIM. To investigate this, both PIM stored at \(-80^\circ C\) and freeze-dried and reconstituted PIM were also included. To assess the effect of freezing and storage of PIM at either \(-20^\circ C\), \(-80^\circ C\), snap frozen in liquid nitrogen and storage at \(-80^\circ C\) or freeze-dried followed by storage at room temperature and reconstitution, permeation of different molecular weight FDs (Fig. 1), rheological properties (Fig. 2), pH, osmolality, protein and water content (Table 2) were investigated.

The permeation of different molecular weight FD was found similar with regards to permeability coefficient (\(P_{\text{APP}}\)), lag time and permeated amount. Only, the lag time for FD4 permeation through PIM that had been frozen and stored was longer compared to that found for fresh PIM. Storage of PIM seemed to reduce the \(P_{\text{APP}}\) values and the permeated amount of the pathogen marker, FD150, whereas the lag time seemed similar to that of fresh PIM. Significant differences could not be demonstrated due to high biological variation in fresh PIM samples. Additionally, at \(-20^\circ C\), \(-80^\circ C\) or snap frozen conditions, FD150 was detectable, but often below LLOQ and no statistical difference was demonstrated due to too few replicates higher than LLOQ. Further, comparison of \(P_{\text{APP}}\) values, lag times and permeated amounts of FD4, FD40 and FD150 at short storage (4 months) versus longer storage.
(12–24 months) of PIM at −20 °C or freeze-dried did not display any clear tendency of effects as a function of storage time.

Overall, the PIM permeability after storage at −20 °C, −80 °C directly or after snap freezing or freeze-dried and reconstituted was found to be similar and all good alternatives to fresh PIM for permeability studies. Similarly, the diffusion of 500 nm latex particles in PIM were found independent of the storage condition [3].

Rheological measurements were conducted to evaluate the physical properties of PIM. To investigate if the different storage conditions affected the stability of the gel network, comparisons of G’ at 1 rad/s, slopes of the linear fit of log (G’) versus log (frequency), apparent viscosities at 0.4 s⁻¹, slopes of the linear fit of log (viscosity) versus log (shear rate), LVE end and G’<G” transitions were made (Fig. 2).

PIM from all storage conditions displayed viscoelastic behaviour (G’>G”). The storage modulus G’ and slopes of the linear fit of log (G’) versus log (frequency) remained similar between the different storage conditions (Fig. 2A-C). Thus, the stiffness and frequency dependency of PIM were independent of the storage condition. Viscosity was also found to be independent of the storage condition (Fig. 2D-F) both when comparing apparent viscosities at 0.4 s⁻¹ and viscosity shear rates dependency (0.1–3000 s⁻¹). Freeze-dried reconstituted PIM displayed a lower apparent viscosity at 0.4 s⁻¹ compared to PIM stored at different storage conditions. From the stress sweep, it was evaluated if storage of PIM would affect the stress needed to disrupt the gel-network. Both the LVE end and G’<G” transition for PIM stored at −80 °C, snap frozen or freeze-dried and reconstituted seemed to be reduced compared to that of fresh PIM and PIM stored at −20 °C. Viscoelastic properties of human cystic fibrosis mucus has previous been found independent of storage condition [10], whereas, freezing and storage of PIM at −20 °C as well as reconstituted freeze-dried mucus displayed compromised viscoelastic properties [4,5] as seen with human cervical mucus [7]. Our study found rheological properties to be independent of storage condition.

Lastly the chemical properties of PIM upon storage were investigated (Table 2).

The pH in stored PIM was significantly and similarly decreased compared to that of fresh PIM, irrespective of the storage condition. Changes in mucus pH is generally correlated with altered rheological properties [2], yet not observed in this study probably due to only minimal pH change. Osmolality and protein and water content of PIM was retained independently of storage condition.

In conclusion, we demonstrated that PIM samples stored at −20 °C, −80 °C, snap frozen or freeze-dried were found to have similar properties compared to freshly isolated PIM and can all be considered good alternatives to fresh PIM for mucus studies. This expands current reports that are limited to investigating one storage condition and one experimental method at a time.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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