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Understanding the gastrointestinal mucus and its impact on drug absorption

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Abstract
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The gastrointestinal mucus is a hydrogel lining the luminal side of the gastrointestinal epithelium. Mucus is vital for gut homeostasis because it protects the epithelium from the noxious external environment. However, from a drug delivery perspective, drugs have to permeate through the mucus to reach the epithelium; therefore, mucus might pose a barrier to drug absorption. Most of the information about mucus derives from fundamental studies performed on rodents. However, information from larger preclinical animal species is highly warranted for improving study designs and guiding better interpretation of data from preclinical assessments. Furthermore, improved understanding of the nature of the gastrointestinal mucus would enable the development of *in vitro* mucus models with increased biorelevance. These could then be implemented in drug absorption assays to improve the (bio)predictability. Well-informed *in vitro* mucus models would enable quick and less variable screening of drug candidates in the early drug development stages. Finally, these models would contribute to reduction, refinement, and replacement (the 3Rs) of animal usage in the drug development process.

This thesis aims to improve our understanding of the nature of gastrointestinal mucus and its impact on drug absorption. For this purpose, mucus from the complete gastrointestinal tract of pigs and dogs was characterized and the diffusion of physicochemically diverse FITC-dextrans through colonic mucus was studied, both *ex vivo* and *in vitro*. The characterization of the gastrointestinal mucus focused on properties relevant for drug absorption and revealed the physiological characteristics, composition, and structural profiles from the various gastrointestinal regions. The findings pointed towards substantial differences between small intestinal and colonic mucus in both species and served as the basis for developing artificial colonic mucus models for drug permeation assessments. Porcine and canine artificial mucus models were developed and validated against the respective native secretions in terms of structural properties and demonstrated their potential to capture the key diffusion patterns of FITC-dextrans observed in native colonic mucus. Overall, this work provided insights into key properties of mucus from large preclinical species and validated tools for the assessment of the impact of mucus on drug absorption.

Keywords: mucus, gastrointestinal, pigs, dogs, physiology, colonic, in vitro assay, drug diffusion, macromolecules

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“Life shrinks or expands in proportion to one's courage.” ***Anais Nin***

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Barmpatsalou**, **V.**, Dubbelboer, I.R., Rodler, A., Jacobson, M., Karlsson, E., Pedersen, B.L. and Bergström, C. A. S. (2021) Physiological properties, composition and structural profiling of porcine gastrointestinal mucus. *Eur J Pharm Biopharm* 169: 156-167

II. Dubbelboer, I. R., **Barmpatsalou**, **V.**, Rodler, A., Karlsson, E., Nunes S. F., Holmberg, J., Häggström, J. and Bergström, C. A. S. (2022) Gastrointestinal mucus in dog: Physiological characteristics, composition, and structural properties. *Eur J Pharm Biopharm* 173: 92-102

III. **Barmpatsalou**, **V.**, Rodler, A., Jacobson, M., Karlsson, E., Pedersen, B. L. and Bergström, C. A. S. (2023) Development and validation of a porcine artificial colonic mucus model reflecting the properties of native colonic mucus in pigs. *Eur J Pharm Sci* 181:106361

IV. **Barmpatsalou**, **V.**, Tjakra, M., Li, L., Dubbelboer, I. R., Karlsson, E., Pedersen, B. L. and Bergström, C. A. S. Development of a canine artificial colonic mucus model for drug diffusion studies. (In manuscript)

Other contributions:

Kourentas, A., Vertzoni, M., **Barmpatsalou**, **V**., Augustijns, P., Beato, S., Butler, J., Holm, R., Ouwerkerk, N., Rosenberg, J., Tajiri, T., Tannergren, C., Symillides, M., and Reppas, C. (2018) The BioGIT System: a Valuable In Vitro Tool to Assess the Impact of Dose and Formulation on Early Exposure to Low Solubility Drugs After Oral Administration. *AAPS J* 20(4):71

Contents

Abbreviations

3D	Three-dimensional
BSA	Bovine serum albumin
$CACM_{II}$	Canine Artificial Colonic Mucus containing mucin Type II
$CACM_{III}$	Canine Artificial Colonic Mucus containing mucin Type III
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CryoSEM	Cryo scanning electron microscopy
DIOS	Distal intestinal obstruction syndrome
EMA	European Medicines Agency
FITC	Fluorescein isothiocyanate
FRAP	Fluorescence recovery after photobleaching
GI	Gastrointestinal
GIT	Gastrointestinal tract
H-CACM	High viscosity canine artificial colonic mucus
HBSS	Hanks' balanced salt solution
L-CACM	Low viscosity canine artificial colonic mucus
LVR	Linear viscoelastic region
MES	2-(N-morpholino)ethanesulfonic acid
MMC	Migrating motor complex
MPT	Multiple particle tracking
MW	Molecular weight
PAA	Polyacrylic acid
$PACM_{II}$	Porcine Artificial Colonic Mucus containing mucin Type II
$PACM_{III}$	Porcine Artificial Colonic Mucus containing mucin Type III
PTS	Proline, threonine, serine
ROI	Region of interest
SPT	Single particle tracking
TMT	Targeted mass tandem
UC	Ulcerative colitis
UV	Ultraviolet
Zg16	Zymogen granulae protein 16

Introduction

The gastrointestinal mucus

The gastrointestinal (GI) mucus is a viscoelastic hydrogel lining the luminal side of the epithelium along the gastrointestinal tract (GIT) (1). The physiological role of the mucus is to protect the sensitive epithelium from the external environment while allowing passage and subsequent absorption of nutrients. The GI mucus plays a key role in keeping bacteria and particulate matter at distance from the epithelium, which it does by means of a highly sophisticated mechanism involving steric hindrance and antibacterial mediators. Due to its viscoelastic characteristics, the mucus tolerates mechanical stress generated by the intraluminal contents, especially solid-semisolid intracolonic material. The hydrogel nature of the mucus lining prevents dehydration of the underlying epithelium. Finally, the mucus lubricates the intraluminal contents and facilitates their propulsion to lower parts of the GIT until they are excreted from the body.

Mucins

Although the properties of the GI mucus layer vary along the GIT in order to address the physiological needs of the separate regions (1-7), the key building blocks of mucus from all gastrointestinal segments are the mucins.

Mucins can be secreted to form a gel layer over the epithelium (gel-forming mucins) or they can remain anchored to the epithelium where they cover the apical surface of the enterocytes (transmembrane mucins) (8). The production and secretion of mucins is highly regulated spatially and temporally. For instance, MUC5AC is expressed by surface and glandular mucous cells in the stomach (9), while MUC2 is expressed by surface goblet cells along the small and large intestine (10). Mucins are continuously secreted at a baseline level. Their release is strictly controlled and can be triggered by a plethora of signals, such as variations in Ca^{2+} (11), bicarbonate (12), histamine (13), and acetylcholine (14). A typical example is the release of mucin from the gastric mucous cells in response to cellular injury. The release of Ca^{2+} that occurs upon disruption of the plasma membrane triggers mucus secretion and the released mucus acts as a protective layer over the affected tissue to minimize cellular damage (15).

The mucins belong to a family of divergent glycoproteins and have a "worm-like" structure. The mucin monomers present a hydrophobic protein core with oligosaccharide branches, resulting in the characteristic brush-like structure (Fig. 1) (8). Mucin monomers are 0.2–0.6 μm in length (16) and contain disulfide-rich cysteine domains at the amino- and carboxy-terminal regions. The sulfhydryl groups (−SH) at the cysteine domains facilitate the formation of disulfide bridges between monomers to form dimers (17) and trimers (post glycosylation) (18) at the carboxy- and amino- terminals, respectively. The polymerization of mucins by C-terminal dimerization and N-terminal trimerization results in the formation of an extensive three-dimensional (3D) network organized in polymeric sheets (11).

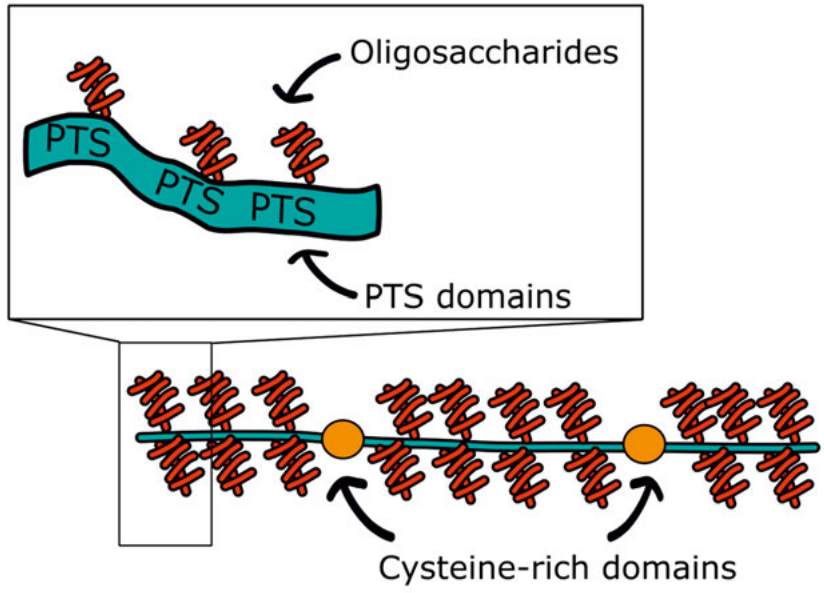


Fig. 1: Schematic representation of intestinal gel-forming MUC2. The protein core with repeated regions of proline, threonine and serine (PTS) domains is shown in blue, oligosaccharides in red, and cysteine-rich mucin domains in orange

The protein domains of the mucins show high sequence similarity, containing a high proportion of proline, threonine, and serine (PTS) amino acids which are often arranged in tandem repeats (19). The exposed hydroxyl groups of threonine and serine provide attachment sites for O-glycosylation which occurs in the Golgi apparatus (20). Depending on the physiological needs of the mucosal surface, the mucins undergo different glycosylation processes (7). Mucin monomers can harbor more than 200 unique glycan structures (21) and the resulting glycosylation fingerprint determines the nature of interactions and the interplay between mucins and other components, such as bacteria or endogenous substances. The high degree of glycosylation (approximately 50–

80% (w/w) carbohydrates (22)) comes at a huge expenditure of energy and resources for the cellular production machinery, but it is necessary for shielding the protein backbone from acidic environments and preventing proteolytic degradation by the pancreatic digestive enzymes (23). Most glycans attached to the mucin core are tipped with a negative charge due to carboxyl or sulfate groups; this feature provides hydrophilic domains interspersed in the non-glycosylated hydrophobic regions of the protein core (16). A gradient in sialylation of glycans exists from the upper to the lower GI tract (24) and is associated with enhanced resistance of the mucins against bacterial degradation (25).

The presence of hydrophilic glycans enables the binding of copious amounts of water, which constitutes most of the mucus (26). The mucus network is rather complex and is also composed of lipids, proteins, sloughed epithelial cells, DNA and inorganic salts. All these components contribute to the viscoelasticity of the mucus (27), a property essential for the mucus to exert its protective, hydrating and lubricating functions (16). For instance, lipids bind to mucin monomers both covalently and non-covalently (28) and contribute to the wettability (charged lipids) and hydrophobicity (neutral lipids) (29) and markedly increase the viscoelasticity of the gel (30). Removal of lipids from the mucus network substantially decreases the viscoelasticity (30). The rheological properties of the mucus can also be affected by external stimuli (22). Increased concentration of divalent cations, Mg or Ca, may lead to the collapse of the mucus gel (31, 32), while increased concentrations of monovalent cations, Na or K, are associated with a reduction in mucus viscosity (33). Overall, the viscoelastic behavior of mucus can be considered the product of the interplay of multiple endogenous and exogenous factors and is of great importance for the transport of particles through mucus (34).

Mucus organization

The organization of the mucus reflects the physiological needs and environmental challenges of the underlying epithelium.

Gastric mucus plays a pivotal role in protecting the sensitive epithelium from the harsh environment of the stomach. The high acidity of the intragastric contents, in combination with the presence of enzymes such as pepsin and various proteases, necessitate enhanced protection of the epithelium. This protection is mediated by bicarbonate and mucus secretion, which results in the creation of a pH gradient from the acidic lumen (pH 1–2) to neutral pH at the cell surface (35). Gastric secretions have the potential to cross through the gastric mucus through temporary channels (36); however, the mucus can efficiently block back-diffusion of hydrochloric acid and pepsin. Co-secretion of trefoil peptides stabilizes the mucus network (37) which is organized as a firmly

adhered layer over the gastric mucosa (1), providing a physical barrier between the luminal enzymes and the epithelium (Fig. 2).

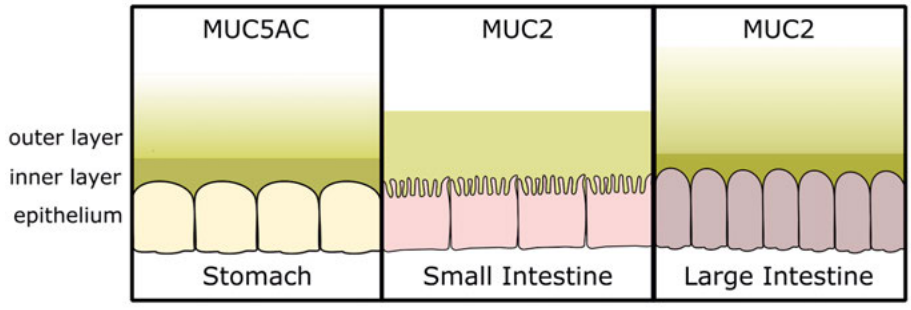


Fig. 2: Regional organization of the mucus. In the stomach and the large intestine, the mucus is organized in two layers—an outer one that is "worm-like" and mixes with the intraluminal contents and an inner one that adheres tightly to the epithelium. In the small intestine, the mucus forms a loose layer. MUC5AC is the major component in gastric mucus, while MUC2 is most prevalent in small and large intestinal mucus. The thickness of mucus in the segments shown here has not been adjusted to physiologically relevant values

The main site for absorption of orally ingested xenobiotics is the small intestine. The presence of nutrients in a form that can be readily absorbed could attract large bacterial populations. Antibacterial peptides and proteins secreted from crypt Paneth cells and enterocytes remain concentrated in the mucus network. These play a key role in keeping bacteria at a distance from the epithelium (38, 39). Small intestinal mucus forms a loose layer (Fig. 2) that can be easily removed (1-3, 6), and the migrating motor complex (MMC) facilitates the migration of the mucus distally, ensuring removal of pathogens and other entrapped matter (40). Post secretion, mucus expansion is controlled by release of bicarbonate ions (HCO_3^-) which is regulated by the cystic fibrosis transmembrane conductance regulator (CFTR) (41). The inherited disease cystic fibrosis (CF) is associated with a dysfunction of the CFTR channel leading to mucus accumulation and pathologically strong adherence to the epithelium (12) and often resulting in distal intestinal obstruction syndrome (DIOS).

The human large intestine harbors approximately 100 trillion bacteria (42) which are in a symbiotic relationship with the host. Colonic mucus acts as a physical barrier by separating this huge bacterial load from the sensitive epithelium. In the colon, the mucus is organized in a two-layer configuration (Fig. 2)—an outer, loose, and "wormlike" layer that mixes with the intracolonic contents, and an inner layer that is tightly adhered to the epithelium and is devoid of bacteria (43). The outer layer forms ~50 µm (mouse) (43) or 100 µm (rat) (1) from the epithelium, where endogenous protease activity (44) converts the inner mucus into the outer mucus layer. It has been demonstrated that

the proteolytic activity results in a lower MUC2 concentration (43) in the outer mucus layer that expands 2–3-fold in volume and results in pore size increase, allowing bacteria to penetrate the outer layer. The colonic mucus acts not only as a physical barrier (due to its mesh-like architecture that limits bacterial penetration), but also as an immunological barrier. For example, immunoglobulin A hinders epithelial attachment of antigens capable of triggering an immune response (45). Another protein, Zg16 (zymogen granulae protein 16), found abundantly in mucus, binds Gram-positive bacteria via its carbohydrate binding domain. The binding triggers aggregation and subsequent limited bacterial mobility, which slows penetration (46). Furthermore, findings suggest that transmembrane mucins could have an active role in cell signaling (47). Although colonic mucus restricts penetration of pathogens through various mechanisms, it also promotes the proliferation of a diverse microbial ecosystem by providing glycans as source of energy. It is therefore essential for the homeostasis of beneficial bacterial communities in the large intestine (25). The thickness of the colonic mucus has been reported remarkably constant (43) and is the outcome of a balance between mucus production and bacterial degradation and shedding due to the propulsion of luminal contents. An imbalance in these processes can result in an absent or compromised mucus barrier, in which case access of antigens to the epithelium can lead to disease. One example is ulcerative colitis (UC), in which there is a reduction of core mucus structural components that is believed to contribute to the disease's pathogenesis (48). Thinning of the mucus layer, where bacterial populations come in contact with the epithelium, triggering a severe immune response and subsequent inflammation has also been reported as a result of a low-fiber diet (49).

Impact of mucus on drug absorption

Although mucus is essential for gut homeostasis, it complicates drug delivery because the drug molecules have to permeate it to reach the epithelium and eventually be absorbed. The mucus is a complex hydrogel presenting several barrier levels to diffusing entities (50). Firstly, the mucins present a plethora of functional groups, such as hydrophilic, negatively-charged glycans. These are interspersed with hydrophobic protein domains and cysteine-rich subdomains, offering a wide range of interactions with varying degrees of affinity for the diffusing drugs. Other components, such as lipids and proteins, provide additional potential for physicochemical interactions and contribute to the high selectivity of the mucus network (26) (Fig. 3).

Secondly, the mucus acts as a size exclusion filter, restricting passage of entities larger than the mucus mesh space (Fig. 3). There have been several attempts to determine the "cut off" of the mucus mesh network, with values suggested between 20 and 200 nm (51) (52). The variability in the data might

be related to methodological and sample preparation differences between studies, aspects that can lead to over- or underestimation of the mesh space (53). It should be stressed, however, that diffusion through the mucus network of particles up to 2 μm has been also reported (2). The determination of an absolute "cut off" value is further complicated by the genuine heterogeneity of the mucus network and the static conditions under which measurements are performed, which might not sufficiently capture the dynamic characteristics of the barrier *in vivo*. Thirdly, the constant turnover of the mucus layer is a necessary clearance mechanism to transfer entrapped pathogens to lower parts of the GIT where they are eventually excreted from the body (Fig. 3). This protective mechanism dictates the "time window" for diffusing drugs to permeate the mucus layer to reach the epithelium before they are flushed away. In humans, the mucus turnover is in the range of a few hours (54) (55) and is believed to be a shear dependent process.

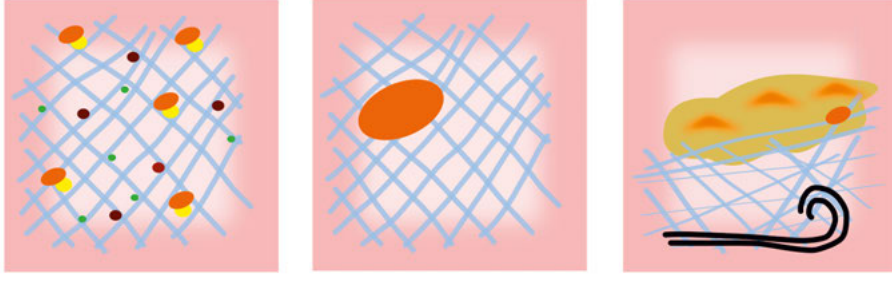


Fig. 3: Schematic representation of the barrier properties of the mucus towards drug absorption. Physicochemical interactions (left), steric hindrance (middle) and mucus renewal (right). Drug candidates represented in orange

Several studies have investigated the impact of the barrier properties of mucus on drug absorption, using mucus from various sources. To examine small-molecule drug space, Larhed et al. studied the diffusion of mannitol, metoprolol, propranolol, hydrocortisone and testosterone through porcine jejunal mucus. They identified lipids as the main component interacting with lipophilic drugs of low molecular weight (MW) (26). Boegh et al. used porcine small intestinal mucus to study the diffusion of testosterone and mannitol, which was reduced 6.8 and 1.6 times, respectively, suggesting greater hindrance for lipophilic compounds compared to hydrophilic ones (56). Shaw et al. highlighted the impact of drug ionization on mucus diffusion by comparing the diffusion of paracetamol and ibuprofen in porcine gastric mucus at a range of pH values. They found increased diffusion for ibuprofen when in ionized form compared to its non-ionized one. The authors related the findings with decreased lipophilicity of the ionized form of ibuprofen, which thereby limited its interactions with the mucus (57). Overall, the findings of these studies corroborate the concept that compounds with a sufficiently low MW, limited

hydrophobicity, and resistance to enzymatic degradation can permeate through mucus with limited hindrance.

For larger macromolecular drugs, diffusion through mucus is more challenging. Boegh et al. observed a non-proportional decrease in apparent permeability values of FITC-dextrans in porcine jejunal mucus (56). Another study related the structural properties of peptides with their permeation profiles through porcine jejunal mucus and identified strong binding of cationic peptides with high hydrogen-bonding potential (58). Desai et al. studied the diffusion of radiolabeled Na and macromolecules (MW between 14,400 and 186,000) through porcine gastric mucus. They reported slower diffusion with increasing MW, but no absolute "cut off" (59). A study by Larhed et al. revealed comparable diffusion rates for two peptides of similar MW through porcine intestinal mucus (60). Bernkop-Schnürch et al. studied the permeation of polypeptides through 1-mm thick porcine intestinal mucus over a period of 5 h and reported a decrease in permeated amount with increasing MW for peptides up to 12.4 kDa (61). For polypeptides with MW from 12.4 to 66 kDa, the permeation was constant at 1±0.5 %, suggesting limited mobility for peptides larger than 12.4 kDa.

Net charge is also a key factor governing diffusion of macromolecules through the mucus, as shown in a study investigating the mucus diffusion of two peptides of equal MW but opposite net charges. This study revealed very limited interactions between the anionic peptide and reconstituted gastric mucin (62), but strong attractive interactions with the cationic peptide. Interestingly, the same study showed that a peptide carrying both negative and positive charge domains exhibited enhanced diffusion that was higher than the combined effect of the separate charge profiles. It also showed that spatial charge distribution affected peptide transport through mucus, proving that mucus is a highly sophisticated barrier. This observation might be related to advanced mucus-penetrating mechanisms of pathogens which present a combination of surface features.

Preclinical animal models to study mucus and its impact on drug absorption

Early studies on mucus characterization involved mainly rats and mice (1, 2, 63). Although these studies established early knowledge regarding the mucus, there is currently a need to shift towards larger animal species more relevant to humans and with mucus yields of sufficient amounts for experimentation. In light of this, some studies on GI mucus from larger species, such as pigs and dogs, have been conducted (5, 26, 30, 64, 65).

The pig model has gained increasing attention from the pharmaceutical industry (66, 67) due to its similarities to the human GIT (68). Pigs are omnivorous species, like humans (69). Porcine gastric mucus has a similar thickness to the human one; the porcine small intestine is also covered with finger shaped villi (70) and the colon in both species is sacculated (71). Pigs and humans have comparable pH values in the small and large intestine and a comparable GI water content when normalized for body weight and gut length (70). The latter two parameters play a key role in drug stability/ionization and dissolution, respectively. The half-lives of 13 peptide drugs in pig and human gastric fluids are well correlated (R^2: 0.72) (70). The potential of the porcine model to adequately predict most absorption, distribution, metabolism, excretion and toxicity (ADMET) endpoints has also been demonstrated (72).

Dogs are an established large-animal species in preclinical testing for evaluation of controlled release formulations. They can be administered the large dosage forms intended for ingestion by humans and the gastric capacity of dogs is comparable to that of humans. Dogs and humans have relatively similar pH values in the small and large intestine (70) as well as similar anatomical division of the colon (ascending, transverse, descending) (73). The bioavailability values of 22 acidic drugs in dogs and humans have been correlated with some success (R^2: 0.64) (70).

In vitro assays to study drug diffusion through mucus

Mucus sources

Given the complexity and inherent sophistication of GI mucus—both in terms of composition and structural properties—the use of native mucus in transport studies is highly warranted to aim for conclusions of high biorelevance value. However, access to GI mucus from healthy volunteers can be ethically challenging and might not yield sufficient amounts for routine experimentation. An alternative is the use of mucus sourced from the stomach or the small intestine of abattoir pigs, a process that typically yields mucus in appreciative amounts and does not rely on availability of research animals. Porcine jejunal mucus can be stored under common laboratory conditions, without compromising its barrier properties (74). Several research groups have used mucus from porcine gastric or small intestinal origin, to assess the permeation of drugs and nanoparticulate formulations (52, 56, 58-61, 74-79). Although the use of native mucus in permeation studies undoubtedly provides data of high *in vivo* relevance (Fig. 4), from a practical perspective, the collection of native mucus is a rather labor-intensive (especially in the large intestine) and time-sensitive process. Additionally, the biological origin of native mucus requires proper disinfection of any contaminated experimental equipment and has been associated with high variability in the acquired experimental data (58).

The European Medicines Agency (EMA) supports the implementation of the 3R principle regarding the replacement, refinement, and reduction of animal usage in the drug development process (80). To this end, several mucus-producing, cell-based approaches that aim to replicate the key properties of mucus without dependence upon animal sources, have been reported (81-84) (Fig. 4). A typical example of mucus-producing cell lines is the MTX29-Caco2 coculture. The MTX29-Caco2 coculture combines the mucus-secreting feature of methotrexate-treated HT29 cells with the tight junction characteristics of Caco2 cells. Under specific conditions, this cell coculture can present a mucus layer; however, its homogeneity and thickness may vary substantially, requiring further optimization of culturing conditions to achieve replication of the mucus barrier *in vivo* (85). Despite the potential of these models, it should be stressed that the cell-based models necessitate cell-dedicated facilities and typically require long cultivation times, parameters that restrict their use for early high-throughput screening.

Alternatives for faster and more inexpensive screening of drug candidates in the early stages of drug development are artificial intestinal mucus models that can be prepared *in vitro* with commercially available chemicals (26, 56, 86). The complexity of these models ranges from mucin solutions to mixtures that incorporate multiple mucus components (Fig. 4).

Mucin solutions of commercially available sources are widely used, mainly due to easy access and a simple preparation protocol. Although the use of mucin solutions in permeation studies can provide insights into drug/formulation binding to mucins, this simplified approach excludes the representation of other mucus components that contribute to the mucus barrier properties. It should also be highlighted that commercially available mucins undergo an intense purification treatment, which results in loss of functional domains and consequently binding sites. This could explain the compromised gel-forming potential of commercially available mucins reported in the literature, even at high, non-physiologically relevant concentrations (87-89).

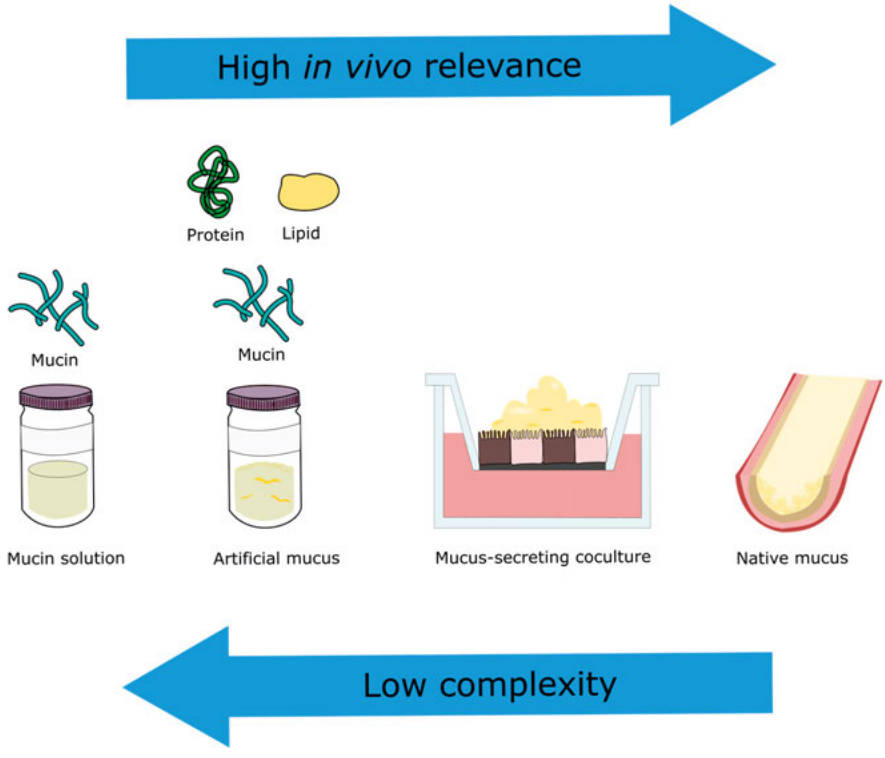


Fig. 4: Relationship of biorelevance and complexity of mucus models

The characterization of mucus by Larhed et al. provided fundamental insights into the composition of porcine jejunal mucus and the authors attempted to replicate the native secretion by mixing commercially available components identified from their component analysis (26). Drug diffusion in their artificial mucus was comparable to that in the native one. However, due to the limited gel-forming properties of the mucins, the authors recommended further studies to assess the applicability of artificial mucus models. In light of their work, several strategies have been reported which incorporate mechanical adjuvants to compensate for the loss of the gel-forming properties of commercially available mucins.

Further rheological profiling of porcine jejunal mucus by Boegh et al. provided the potential to develop an *in vitro* mucus model. They called this "biosimilar mucus", and it mimics the barrier properties of native mucus of small intestinal origin (56). The rheological profile of biosimilar mucus is enhanced by the presence of polyacrylic acid (PAA). The biosimilar mucus is a highly versatile model and has showcased its applicability in various set ups, including cell-based assays to monitor mucus diffusion and cellular uptake processes

simultaneously (56). It has also been used to enhance the potential of lipolysis assays for predicting *in vivo* performance (90).

Improved network rigidity could also be achieved by chemical cross-linking, as presented by Hamed et al. (91). In this study, they used glutaraldehyde as a bifunctional cross-linking agent to strengthen the rigidity of the mucus mixture, which they developed to reflect the properties of tracheal mucus. Another approach to enhance the cross-linking of mucins involves UV-assisted cross-linking. Duffy et al. designed a mucus model to mimic the sputum of healthy individuals (92). Their mucus model was prepared by introducing a photo-initiator to a mixture of methacrylated mucins; subsequent gelation was induced via photo-induced cross-linking with a 365-nm UV light.

These models were designed to mimic the mucus barrier of either the upper GI tract or other epithelia. However, as the impact of GI mucus on drug absorption can be region-dependent (93), the need to develop models reflecting the barrier properties of colonic mucus has emerged and has not been addressed to date.

Methods to study drug diffusion

Given the need for precise predictions *in vitro* before proceeding to costly *in vivo* measurements, many techniques to study drug diffusion through native or artificial mucus have been developed over the years.

One widely-used experimental setup to study drug permeation through a cellular monolayer are Transwell inserts (Fig. 5). This setup consists of an apical and a basolateral side, separated by a semipermeable filter membrane that serves as a support for cell cultivation. The Transwell setup has been adjusted to accommodate mucus and enable drug diffusion studies (56, 90, 94-96). The mucus is deposited on top of the filter membrane and the drug is introduced to the system on the apical side, where it diffuses through the mucus to reach the basolateral side. Thereafter, the permeated fraction can be quantified by liquid chromatography, radioactivity, or fluorescence measurements. The Transwell setup is commercially available and can incorporate a cell monolayer or artificial cellular barrier to monitor mucus diffusion and cellular uptake simultaneously. However, the mucus thickness achievable in the Transwell setup does not reflect the *in vivo* one. Additionally, the formation of an uneven mucus layer upon introduction of the drug solution to the system, along with the static nature of the assay, might cause it to not sufficiently capture the dynamic GIT environment.

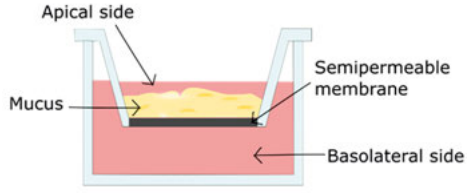


Fig. 5: Transwell permeation assay. A semipermeable membrane separates the apical side (corresponding to the lumen) from the basolateral side (corresponding to the systemic circulation). The mucus can be deposited on top of the semipermeable membrane and the drug is introduced to the system on the apical side

Another option to study drug diffusion is the rotating tube proposed by Dünnhaupt et al. (76). This method involves small tubes filled with mucus, to which the drug solution is added on top and left to diffuse through. To quantify the permeated amount, the tubes are frozen, cut in slices, and the permeated amount is quantified by fluorescence. The rotating tube technique can provide quick assessments and be used for rank ordering of formulations. However as the diffusion is studied macroscopically, drugs need to permeate sufficiently to allow quantification of permeated amount (85).

Microscopy-based techniques have been developed to enable monitoring the diffusion of minute amounts of compounds through very small volumes of mucus. This is particularly attractive in the early stages of the drug discovery process, when drug candidates are typically available only in low amounts. Single particle tracking (SPT) and multiple particle tracking (MPT) are techniques to visualize the diffusion path of individual particles through mucus; the mobility is then expressed as the mean squared displacement. MPT has been used to examine diffusion through native gastrointestinal mucus (77, 97, 98), but it has size limitations for successful detection and quantification.

Fluorescence Recovery After Photobleaching (FRAP) is another technique for tracking mucus diffusion (99). In principle, it involves mixing the native or artificial mucus sample with a fluorophore, and subsequent rapid and irreversible bleaching of a small area by a high intensity laser beam (Fig. 6). The diffusion of unbleached fluorophore into the bleached area is measured to extract the diffusivity value. FRAP allows acquisition of quick measurements and has the potential to capture time sensitive events; however, its applicability is limited to fluorescent species.

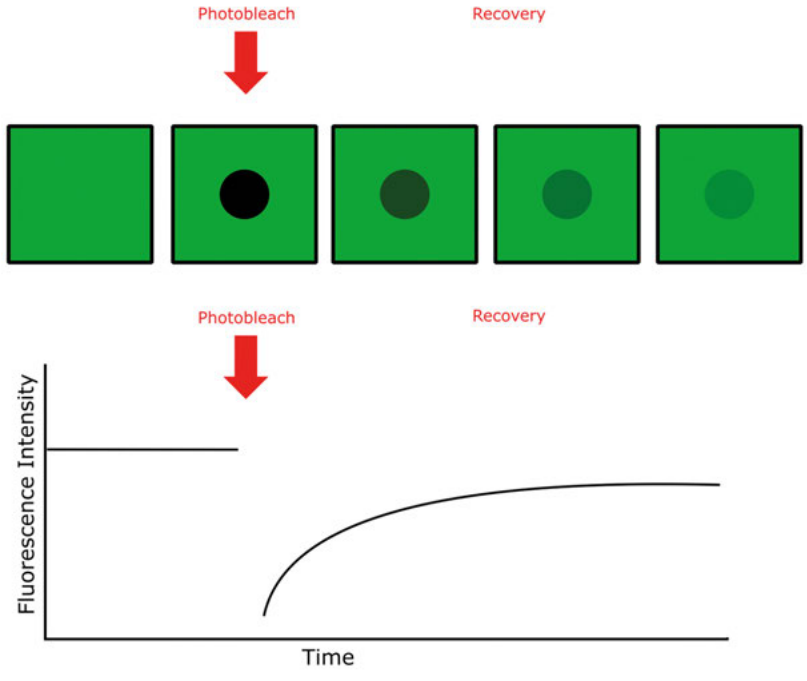


Fig 6: The FRAP assay: Briefly, the mucus is mixed with a fluorophore and irreversible bleaching of a small region is induced with a laser, resulting in a decrease in the intensity of the fluorescence. The recovery of fluorescence in the bleached region by neighboring non-bleached fluorophores is used to calculate the diffusivity

Finally, microfluidic devices have emerged as a technology with potential to replicate physiologically relevant shear conditions. These dynamic models allow experimentation under physiologically relevant flow rates with reagent volumes on a microliter scale. They have been used to develop a cell model with a 2-layer mucus configuration (100) that allows mucus diffusion studies of high *in vivo* relevance. Mucin solutions and reconstituted mucus from porcine stomach scrapings have also been implemented in microfluidic devices, enabling the assessment of the impact of particle surface functionalization (101) and spatial charge arrangement in macromolecules (102), respectively. In summary, microfluidic devices can replicate the dynamic GI (micro)environment in a physiologically relevant way and capture time-sensitive phenomena in real time with microscopy-based detection.

Aims of the thesis

The overall aim of the thesis was to improve our understanding of the gastrointestinal mucus in preclinical, large animal species and assess its impact on drug absorption in *ex vivo* native and *in vitro* artificial colonic mucus.

The specific aims of the thesis were the following:

- To characterize the GI mucus of pig (Paper I) and dog (Paper II) and elucidate key mucus properties that are relevant for drug permeation.
- To study the diffusion of macromolecules with various charge and size characteristics through porcine and canine colonic mucus (Papers III and IV).
- To develop an *in vitro* porcine colonic mucus model and assess its usefulness in predicting the permeation of macromolecules through colonic mucus (Paper III).
- To develop an *in vitro* canine colonic mucus model and to implement the newly proposed model in various experimental setups assessing the permeation of macromolecules through colonic mucus (Paper IV).

Methods

Animals

Mucus was collected from pigs and dogs. Collection of the porcine samples and from the canine private veterinary patient was done at the Swedish Agricultural University (SLU), while the collection of samples from dogs originating from a research colony in Astra Zeneca (Mölndal, Sweden) was performed at the Department of Pharmacy, Uppsala University.

Pigs

The porcine mucus (Papers I and III) was sourced from a local abattoir in Uppsala. The crossbreed pigs were 20–22 weeks of age and 100–110 kg. The animals were farmed for commercial meat production and thus, no ethical approval was needed as the sample collection used waste tissue. As per the abattoir's standard routines, the animals were fasted for at least 12 h prior to slaughter and water was allowed *ad libitum*.

Dogs

The canine mucus (Papers II and IV) was obtained from five dogs. Four of them originated from a research colony in AstraZeneca (Mölndal, Sweden) and will be herein referred to as laboratory dogs, D_{lab} (Paper II), or DZ (Paper IV). The laboratory dogs were Labradors, 5-8 years of age, and 35.9-38.7 kg. Permanent nipple-valve stomas were surgically inserted into the duodenal, jejunal or proximal colonic abdominal wall. The fifth dog was a canine private veterinary patient, recruited from the Animal Hospital at the Swedish Agricultural University (SLU) and will be herein referred to as domestic dog, D_{do}. This domestic dog was a Siberian Husky, 3 years old, and 20 kg. All animals were euthanized for reasons other than this study and therefore no ethical permit was needed. Exclusion criteria included: i) GIT-related diseases, ii) invasive GIT treatment/surgery/endoscopy/colonoscopies, or iii) vomiting or diarrhea episodes within the final month before euthanasia.

Mucus collection

Pigs

Sample collection occurred within one hour of slaughter. Mucus samples were collected from all GI segments, as illustrated in Fig. 7.

The stomach pouch was dissected and gastric mucus was scraped off the tissue. The remaining intestinal tube was cut longitudinally and duodenal mucus was collected 1 cm distal to the pyloric sphincter. The jejunal mucus was collected from the middle of the small intestine, while the ileal mucus samples were collected within 8 cm from the ileocecal valve. The cecum pouch was dissected, and the tissue was submerged into an ice-cold isotonic buffer (10 mM MES buffer containing 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 137 mM NaCl, pH 6.5) to remove digesta. The first part of the large intestine (distal to the cecum orifice) was cut open to collect proximal colonic mucus. Accordingly, distal colonic mucus samples were collected from the beginning of the descending colon. The proximal and distal colonic tissues were quickly submerged into ice-cold MES buffer to remove any remaining digesta if necessary, prior to mucus collection.

Mucus was collected with a metal spatula. Upon collection, the samples were placed in ice to minimize bacterial degradation and the collection process was completed within 1 h.

pH values of all mucus samples were recorded with a micro-electrode (Orion Sure-Flow, Thermo Fisher Scientific) within 1 h of sample collection. Thereafter, samples were aliquoted, snap-frozen in liquid nitrogen, and stored at -80 °C until further analyses.

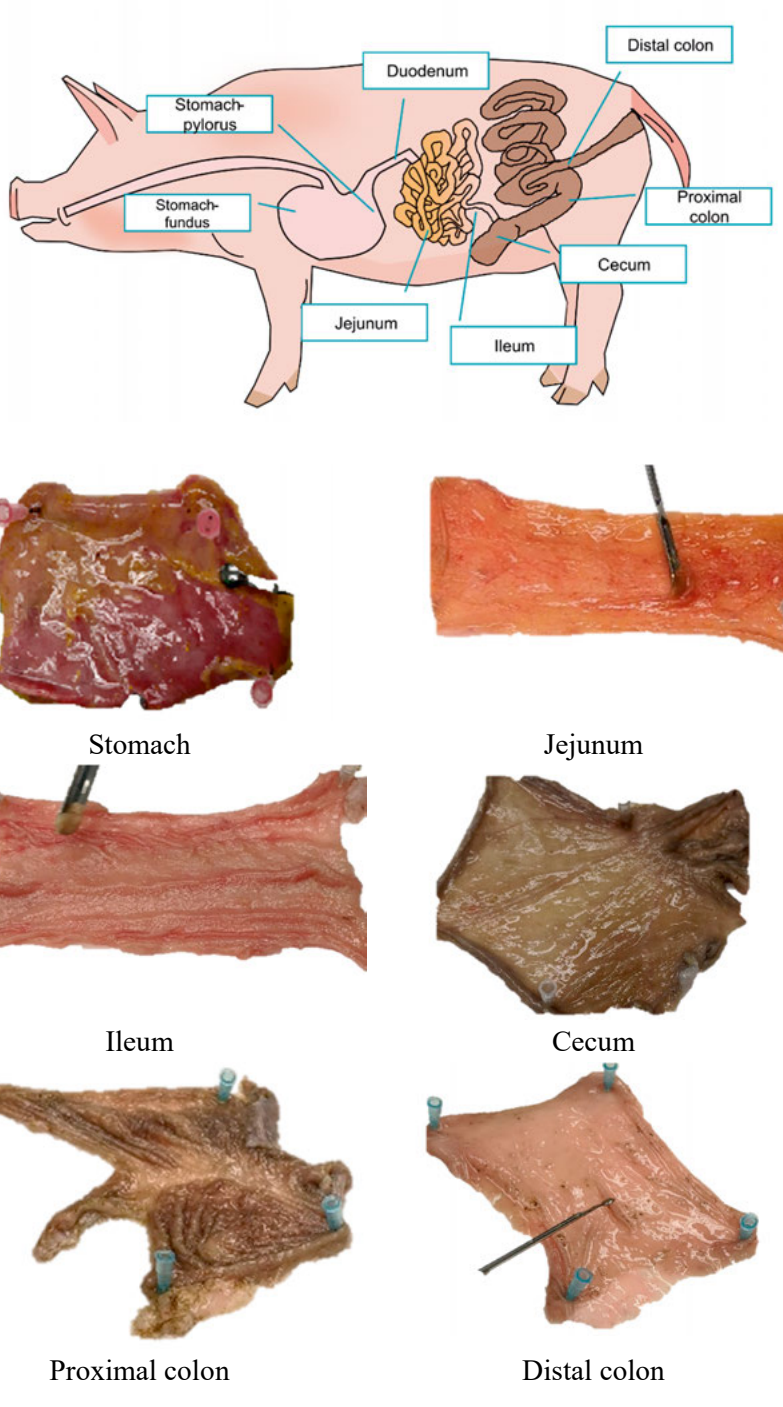


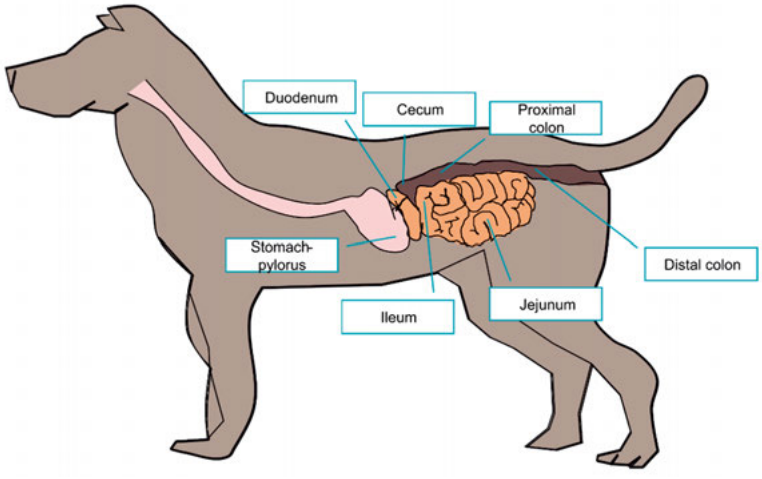
Fig. 7: Porcine GI segments from which mucus was collected, along with representative photos of tissue segments taken during sample collection

Dogs

Dissection of the canine tissue was initiated within one hour after euthanasia. Mucus was collected from all GI segments, as illustrated in Fig. 8. Gastric tissue (10 x 15 cm) was obtained from the fundus of the stomach pouch. The duodenal and jejunal tissues were sampled distal to the pylorus and from the middle of the small intestine, respectively. The ileal tissue sample was collected 8 cm above the ileocolic valve. The cecal sac was harvested in its entirety and the proximal and distal colonic samples were collected 5 cm distal to the ileocolic valve/cecum and 10 cm from the sigmoidal colon, respectively.

Upon excision, samples from the laboratory dogs were stored for 6 h on ice in ice-cold buffer [Ringer lactate (D_{lab}01, pH 5–6) or Krebs Ringers (D_{lab}02-04, pH 7.3)] before collecting the mucus. For the domestic dog, mucus collection was done immediately after excision.

The mucus collection was performed as described above for the pigs. Briefly, intestinal tube segments were cut longitudinally and the cecal pouch was dissected. If needed, the tissue was rinsed quickly with cold buffer (10 mM MES isotonic buffer containing 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 137 mM NaCl, pH 6.5) prior to mucus collection. Thereafter mucus samples were collected with a metal spatula. Immediately after collection, samples were stored on ice, pH values were measured, then aliquoted, snap-frozen in liquid nitrogen, and stored at -80 °C until further analyses.



Stomach Duodenum Jejunum Ileum

Cecum Proximal colon Distal colon

Fig. 8: Canine GI segments from which mucus was collected, along with representative photos of the respective tissue segments taken during sample collection

Mucus characterization

Physiological properties

Appearance

Samples were inspected under ambient light against a white background to report the color and the consistency/texture.

Water content

Aliquots of mucus were weighed and transferred to a laboratory-scale freeze dryer (VirTis Sentry 2.0, SP Scientific, or a Flexi-Dry MP, FTS systems, both from CiAB, Sweden) with a condenser temperature of -80 °C. After at least 48 h, the samples were taken out, weighed, and the water content was calculated from the weight difference.

Zeta potential

Mucus was dispersed in distilled water and dissolved under magnetic stirring. A portion of the mixture was transferred to an Omega cuvette and the zeta potential was measured with a Litesizer 500 (Anton Paar, Austria) dynamic light scattering instrument. All measurements (3-5 per sample) were conducted after equilibration for 1 min, using the Smoluchowski approximation and Henry Factor of 1.5, at 25 °C. The data were processed with the Kalliope software (Anton Paar, Austria).

Composition (Papers I & II)

Proteomics analysis

Label-free and targeted mass tandem (TMT)-labeled global proteomic analyses were performed at the Clinical Proteomics Mass Spectrometry facility (Science for Life Laboratory at Karolinska Institutet/University Hospital). The data were processed with Perseus (version 1.6.14.0) (103). Proteins unique to or shared among the GI segments were identified using the Venn diagram option in Perseus on data obtained from the label-free analysis. To determine significant differences in protein abundance in jejunal, proximal, and distal colonic mucus, the Volcano plot option in Perseus was used on data obtained from the TMT-labelled proteomics analysis.

Lipidomics/metabolomics analyses

The lipidomics and metabolomics analyses were performed at the Swedish Metabolomics Center in Umeå, Sweden. The analyses were performed on jejunal mucus samples, where most drug absorption occurs, and on proximal and distal colonic mucus samples, as these segments are relevant from a sustained release perspective. To identify significant differences in lipid and

metabolite abundance in the mucus of jejunal, proximal, and distal colonic origins, Volcano plots were generated in Perseus.

Structural properties (Papers I-IV)

Rheological profiling

The apparent viscosity of the mucus samples was measured under continuous flow conditions, with increasing shear rate, to compare the resistance to flow in the mucus samples. This information could provide insights into the amount of shear needed to turn the mucus into a low-viscosity fluid that is "flushable" to lower parts of the GIT. The viscoelastic properties of mucus samples were measured from frequency sweeps by monitoring the storage and loss moduli under oscillatory shear conditions. This yielded information about the rigidity and the cross-linking of the mucus network. The rheological measurements were performed using an ARES-G2 strain-controlled rheometer (TA Instruments, Sollentuna, Sweden) with the Advanced Peltier System (APS) accessory for the lower plate. As a first step, the linear viscoelastic region (LVR) was determined by performing an amplitude sweep. The oscillation strain was increased from 0.1 to 100% at a frequency of 1 Hz oscillation and an oscillation strain value within the LVR was selected to ensure that the network structure would remain intact during the measurements. All measurements were performed at 37 ˚C.

Microstructure

The microarchitecture of the mucus network was visualized by cryo scanning electron microscopy (CryoSEM) at the Umeå Centre for Electron Microscopy (UCEM), using a Carl Zeiss Merlin field-emission cryogenic scanning electron microscope, fitted with a Quorum Technologies PP3000T cryo preparation system. Images were acquired at -140 °C using an in-chamber secondary electron detector at an accelerating voltage of 2 kV and a probe current of 50 pA.

Preparation of artificial colonic mucus models (Papers III & IV)

During the mucus characterization studies (Papers I & II), it became evident that mucus collection was time-sensitive (to prevent degradation of the mucus), tedious, and labor-intensive. Especially in the porcine large intestine—where the mucus adheres tightly to the epithelium, —the collection did not yield adequate amounts for routine experimentation. This led to the development of bio-mimicking artificial mucus models based on the composition and structural characteristics of native colonic mucus. The development used data

obtained from native colonic mucus (Papers I & II), as no artificial colonic mucus model was available in the literature. The characteristics of proximal colonic mucus were selected for the development, as this colonic region is relevant from a controlled release point of view. Further, there are higher chances of drug absorption here compared to the distal colonic segment, where intracolonic contents have solidified. The models were designed to reflect the colonic mucus in pigs and dogs, as these animals are regarded as promising and well-established preclinical species in the drug development process, respectively.

Design of artificial colonic mucus

The design of the artificial colonic mucus models was based on protocols for artificial jejunal mucus preparation from Boegh et al. (56), mainly due to easy commercial accessibility of the components, limited cost, use of commonly available laboratory equipment, and the absence of high-toxicity materials. As the original protocol described the preparation of artificial porcine jejunal mucus, the amounts of each component were adjusted to reflect the composition of porcine and canine colonic mucus. Calculation of the exact amounts for the porcine artificial colonic mucus (PACM) **(Paper III)** used the abundance ratios (proteomics, lipidomics, and metabolomics analyses) of mucus components found in porcine jejunal and colonic mucus **(Paper I)**. The same approach was applied for the canine artificial colonic mucus (CACM) **(Paper IV)**, using the abundance ratios from **Paper II**. The percentage values of porcine jejunal mucus components reported by Larhed et al. (26) were used as references for the absolute quantity values for porcine jejunal mucus.

Polyacrylic acid (PAA) was used as a mechanical adjuvant to the mucus mixtures to increase the viscosity in the mixtures (104). PAA belongs to the family of carbomer-based materials commercially known as carbopols. Carbopols can form hydrogen bonds between carboxyl groups in the carbomer subunits and the sialic acid and sulphate residues in mucins and via electrostatic interactions between functional groups (105). Accordingly, appropriate amounts of PAA were added to the artificial colonic mucus models to mimic the structural properties of native porcine and canine colonic mucus. Purified mucin from porcine stomach is commercially available in two forms, namely Types II and III. As the two forms undergo different purification processes that result in different functional group residuals (106), both types were investigated, to choose the functionally best suited one for the artificial models.

PACM samples contained 0.91% (w/v) mucin Type II or mucin Type III, 7.02% (w/v) bovine serum albumin (BSA), 1% (v/v) lipid mixture (0.21% cholesterol and 0.12% phosphatidylcholine, mixed with Tween 80 at a 3:1 ratio). CACM samples contained 2.67% (w/v) mucin Type II or mucin Type

III, 12.08% (w/v) BSA, 1% (v/v) lipid mixture (0.12% cholesterol and 0.14% phosphatidylcholine, mixed with Tween 80 at a 3:1 ratio). In both sample types, the lipid mixture was prepared by dissolving the lipids in isotonic buffer (10 mM MES with 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 137 mM NaCl, pH 6.5) in the presence of Tween 80. PAA 0-1.8% (w/v) was dissolved in a non-isotonic buffer (10 mM MES with 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, pH 6.5) under intense stirring. Mucin Type II or III was added to the PAA-containing solutions and the resulting mixtures ($PACM_{II}$ and $CACM_{II}$ contained Mucin Type II and $PACM_{III}$ and $CACM_{III}$ contained Mucin Type III) were vortexed until all solids were dissolved. Subsequently, NaOH (5M) and BSA were added and the resulting mixtures were vigorously vortexed again until all visible solids were dissolved. Finally, the lipid mixture was added and the pH adjusted to 7.0 ($PACM_{II}$ and $PACM_{III}$) and 6.5 ($CACM_{II}$ and $CACM_{III}$) by dropwise addition of NaOH (5M). All mixtures were stored overnight at 4 ˚C, then at -80 ˚C until further analyses.

Assessment of the diffusion of macromolecules through mucus (Papers III & IV)

Model compounds

The diffusion of seven macromolecules through native and artificial hydrogels was assessed in the present thesis. FITC-dextrans are model compounds, used extensively in *in vitro* assays, due to their commercial availability, and their well-defined structural and fluorescent properties. Dextrans are hydrophilic polysaccharides comprised of varying lengths of branched glucose molecules and are commercially available in a wide range of MW. Fluorescein isothiocyanate (FITC) is a fluorescent marker that can be attached to the dextran for easy fluorescence-based quantification. The attachment of FITC can occur randomly to any free hydroxyl group of the dextran with degrees of substitution ranging from 0.001 to 0.020 for 4K, 40K and 70K FITC-dextrans (107). Dextrans are neutral but the presence of FITC is associated with a slightly negative charge (108). However at these low levels of substitution, the charge contribution is minimal (109) (107). Indeed, values reported for 70K dextran labelled with FITC suggest zeta potential values around -2.7 (109). Dextrans on the lower end of the MW scale exhibit less branching (110), while dextrans with MW higher than 10K are highly branched (107). The approximate Stokes' radii for 4K, 40K and 70K FITC-dextrans have been reported to be 14, 45 and 60 Ångström, respectively (107).

FITC-dextrans can be further modified to exhibit more distinct charge properties. For instance, FlTC–carboxymethyl–dextran (FITC-CM-Dextran), is an

anionic fluorescent probe synthesized by introducing O-carboxymethyl groups along the dextran chain. The carboxymethyl groups ensure the presence of anionic properties, as reflected by zeta potential values of approximately −11.6 for 70K FITC-CM dextran (109). FITC-diethylaminoethyl-dextran (FITC-DEAE-Dextran) is synthesized by introducing two types of DEAE-substituents into the dextran chain, which provide cationic properties, with zeta potential values of 8.9, for 70K FITC-DEAE dextran (109). Charged FITC-dextrans enable the study of the impact of charge on mucus diffusion and there is no significant effect on the diffusion of 70K FITC-CM or FITC-DEAE dextrans in solution (109). The permeation of FITC-dextrans through native or artificial mucus samples has been previously studied, allowing for comparisons between mucus of various sources and properties (74, 79, 111, 112).

Mucus models

The diffusion of macromolecules was assessed in colonic mucus of both native and artificial origin. Porcine and canine native colonic mucus samples were harvested from three crossbreed pigs and two Labrador dogs originating from a research colony in AstraZeneca (Mölndal, Sweden), respectively. PACM and CACM samples, containing either mucin Type II ($PACM_{II}$ and $CACM_{II}$) or mucin Type III ($PACM_{III}$), were prepared according to the protocols described above, to evaluate the impact of each mucin Type on the barrier properties of the artificial models and thereafter to select the one functionally best suited to be incorporated in the mucus models. As PAA was the main structural adjuvant of the colonic mucus models, diffusion of macromolecules through an aqueous mixture containing PAA in equal quantities to PACM and CACM was monitored, with the aim to investigate whether PAA alone could capture key mucus properties. Additionally, this assessment would provide information regarding the contribution of the other mucus components (mucin, protein, and lipids) in the barrier properties of the artificial mucus models. Furthermore, as various disease states might be associated with altered rheological properties of mucus, high- and low-viscosity CACM samples with either higher or lower viscosity and storage modulus values were compared to reference CACM samples. Finally, the impact of mucus components (mucin, BSA, lipids) on the permeation of macromolecules was evaluated in CACM samples in which either the mucin, BSA or lipids were excluded. These measurements provided information regarding the degree of interaction between each component and diffusing macromolecule.

Methods to study drug diffusion through mucus

Fluorescence recovery after photobleaching (FRAP) assay (Paper III & IV)

FRAP is a microscopy method that was used to assess the impact of charge and MW on the diffusion of macromolecules through native and artificial hydrogels and to evaluate the potential of the artificial hydrogels to capture key diffusion trends observed in the native colonic mucus secretions of porcine and canine origin. Solutions of FITC-dextrans covering a range of charge and MW characteristics (4 and 40 K for both cationic and anionic FITC–dextran molecules, and 4, 40 and 70K for the non-ionic ones) in Hanks' Balanced Salt Solution (HBSS) buffer with pH 7.4 were prepared. Each solution was mixed with a hydrogel sample, the mixture was vortexed, and mechanical mixing using a pipette tip followed, in order to ensure homogenous distribution of the fluorophore in the hydrogel.

A small volume of the hydrogel-fluorophore mixture was placed between two glass coverslips on a microscope slide, as previously illustrated (111). A consistent sample thickness of 100 μm was used for all measurements. To avoid hydrogel drying, the FRAP measurements were initiated promptly, using a Zeiss CLSM 780, and the data were collected with the ZEN Black software (Carl Zeiss GmbH, Jena, Germany). The sample was imaged with a 20× objective lens (CFI Plan Apochromat), at a numerical aperture of 0.8, and the gain of the detector was adjusted to ensure no pixels were saturated. Two circular regions of interest (ROI) were selected and included in a rectangular frame. After initial scanning, one ROI was bleached with the 488-nm line of an argon laser at 100% power, while the other ROI was not bleached and served as a reference to detect potential photofading. A series of images was immediately taken at low laser transmission, to monitor fluorescence recovery in the bleached ROI. All measurements were performed at least in triplicates and at room temperature.

The diffusivity values were calculated as previously described (113). Briefly, the fluorescence intensity values of the bleached and the reference ROI were normalized to the pre-bleach intensity values (fluorescence intensity inside the ROI as recorded in the last caption prior to the bleaching event). A least-squares fit was performed on the recovery curve (Eq. 1) in R to determine the characteristic diffusion time τD.

$$F(t) = k \cdot e^{-\frac{\tau D}{2t}} \left[Io\left(\frac{\tau D}{2t}\right) + \mathrm{I}_1\left(\frac{\tau D}{2t}\right) \right] \qquad \text{(Eq. 1)}$$

where I_0 and I_1 are the zero and first-order modified Bessel functions of the first kind, respectively, k corresponds to the mobile fraction, and t is time (s). The diffusivity values were calculated by solving $D= w^2/\tau D$, where w is the radius of the bleached ROI (16 μm).

Snapwell assay (Paper IV)

The Snapwell assay is a variation of the Transwell setup commonly used for permeability assessments. It is commercially available and allows quantification of drug permeation in a variety of ways. Therefore, its potential to incorporate CACM was assessed. In preliminary experiments, "holes" in the apical side formed upon introduction of the donor solution to the artificial mucus. Such "holes" could create a path for the diffusing molecules to bypass the mucus barrier, leading to substantial diffusion overestimation. A nylon filter was therefore carefully applied on top of the artificial mucus, ensuring the formation of a homogenous mucus layer, while allowing free diffusion due to the large pore size (11 μm) (114). The impact of mucus with altered rheological properties, and the impact of mucus components on the diffusion of macromolecules in CACM were then explored. The permeation experiments were performed in 6-well Snapwell plates with 12-mm insert filters. CACM was transferred to the filters and left to equilibrate for 10 minutes under continuous shaking to obtain a homogenous mucus layer. Thereafter, circular, 12-mm nylon filters were carefully positioned on top of the mucus, to ensure the layer remained intact after the addition of the donor solution to the insert. HBSS, pH 7.4, supplemented with 0.05% BSA was introduced to the basolateral side. The experiment was initiated with the gentle transfer of FITC-dextran donor solution on top of the nylon filter inserts to avoid the creation of holes in the hydrogel. Samples were collected from the basolateral side of each filter at predefined time intervals, and the aspired volume was replenished with an equal volume of HBSS buffer (at 37 °C). The amount of diffused FITC-dextran was quantified by measuring the fluorescent signal at λ_{ex} 485 nm and λ_{em} 520 nm in a TECAN SPARK Microplate Reader (TECAN Austria).

The amount (%) of FITC-dextran that diffused through the mucus layer was determined based on the total amount of FITC-dextran that permeated to the basolateral side in relation to the initial amount of FITC-dextran introduced at the apical side of the filters.

Results and discussion

Characterization of native gastrointestinal mucus (Papers I & II)

Physiological properties of porcine and canine gastrointestinal mucus

Upon collection, the gastrointestinal mucus samples were visually inspected in both species. For both pigs and dogs, the mucus samples differed substantially, depending on where in the GI they originated (Fig. 9A and 9B). This observation has been reported for murine mucus (2) and can be explained by the differences in intraluminal conditions in the various GI segments.

The porcine gastric mucus was yellow and stringy (Fig. 9A), and easily visible on the gastric tissue. Canine gastric mucus was transparent and rubbery, with a slight pink tone (Fig. 9B). The mucus of small intestinal origin had an opaque, light-orange and glossy texture, except for ileal mucus, which had a "paste-like" consistency in both species (Fig. 9A and 9B). The characteristic orange color of small intestinal mucus is probably due to bilirubin, a compound that is secreted in the bile in the small intestine. Large intestinal mucus samples were translucent gels in pigs (Fig. 9A), similar to what has previously been reported for mice (1), and gray in dogs (Fig. 9B). Both gastric and small intestinal mucus were available in large quantities, in contrast to large intestinal mucus, where only small volumes could be collected in pigs. Canine mucus was available in large amounts all along the GIT.

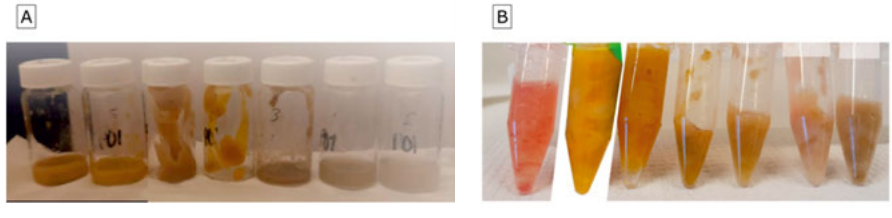


Fig. 9: (A) Porcine and (B) canine mucus samples. Left to right: gastric, duodenal, jejunal, ileal, cecal, proximal, and distal colonic mucus

The pH values of porcine and canine gastrointestinal mucus are shown in Fig. 10A and 10B. In the porcine stomach, the mean pH of the mucus was 5.6. In

the porcine small intestine, the mean duodenal, jejunal and ileal mucus pH values were 6.7, 7.1 and 7.1, respectively, and in the large intestine, the mean cecal, proximal and distal colonic mucus pH values ranged between 7.3 and 7.5 (Fig. 10A). In the canine gastric mucus, pH values were ~6.5 and 4.8 for the laboratory dogs and domestic dog, respectively. The high gastric mucus pH of the laboratory dogs could be due to the long storage time in buffer and not necessarily representative of *in vivo* values. The median canine intestinal mucus pH ranged between 6.5 and 6.8 (Fig. 10B); however, there were no literature canine mucus pH values available for comparison. For pigs, the pH values were in line with literature data (115-118), suggesting that sample collection procedures developed in other laboratories were similar. Overall, it can be concluded that the intestinal mucus for both species maintains near-neutral pH values to protect the sensitive epithelium from intraluminal pH variations.

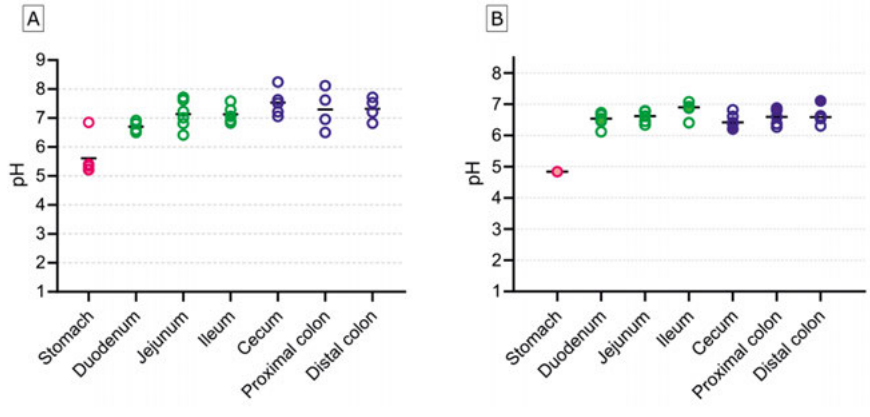


Fig. 10: pH values of (A) porcine and (B) canine mucus from various segments of the GIT. Circles depict individual values and lines depict mean porcine and median canine values. In (B), circles representing data from D_{do} are closed and D_{lab} open. Data from gastric (pink), small intestinal (green), large intestinal (blue) segments

In both species, the water content was less variable, and slightly higher, for gastric and large intestinal mucus, compared to small intestinal mucus (Fig. 11A and 11B). The values for the porcine gastric, jejunal and ileal mucus were in agreement with other reports in pigs (77, 96), as were the values of canine gastric and jejunal mucus, albeit the measurements were performed in other species (26, 119).

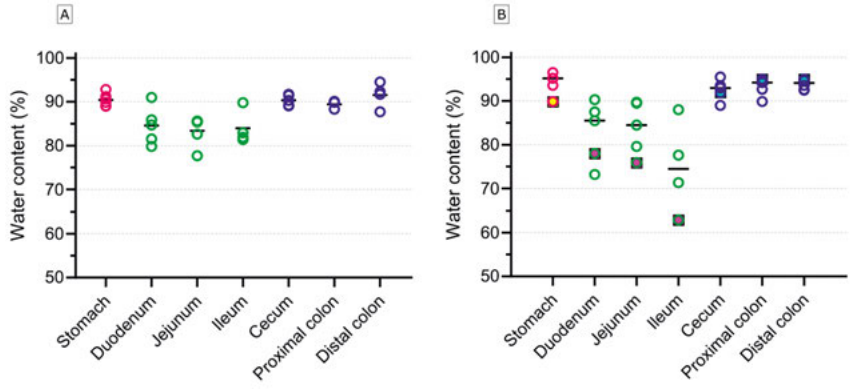


Fig. 11: Water content (%) of (A) porcine and (B) canine mucus from various GIT segments. Circles depict individual values and lines depict mean porcine and median canine values. In (B), circles representing data from D_{do} are closed and D_{lab} open. Data from gastric (pink), small intestinal (green), large intestinal (blue) segments

Composition of porcine and canine gastrointestinal mucus

The proteomics analysis in pigs (Fig. 12Ai) revealed that 66% of the proteins identified were common to all GI segments. Excluding the stomach, 69% were shared among the intestinal segments and less than 12% were unique to the various segments. Significant differences in protein abundance between jejunal and colonic mucus were identified. Only seven proteins were significantly different in abundance between proximal and distal colonic mucus (Fig. 12Aii). However, more than 700 proteins had significantly different abundance in the jejunal mucus, compared to either colonic mucus samples (Fig. 12Aiii-iv). Jejunal mucus had an overrepresentation of proteins from the digestion and absorption pathways, while colonic mucus had an overrepresentation of proteins from the O-linked glycosylation of mucins, the immune system, and antimicrobial peptides. These findings might be associated with the physiological function of the small intestine, where most digestion and metabolism occur, and with the presence of a huge bacterial load in the colon.

In dogs, 43% of the identified proteins were detected in all GIT segments (Fig. 12Bi). More than 75% of the proteins detected in mucus from the small intestinal region were present in all small intestinal segments (duodenum, jejunum, ileum) as were 64% of those in large intestinal mucus common to all large intestinal segments (cecum, proximal and distal colon). No significant differences in protein abundance between jejunal and colonic mucus were identified (Fig. 12Bii-iv).

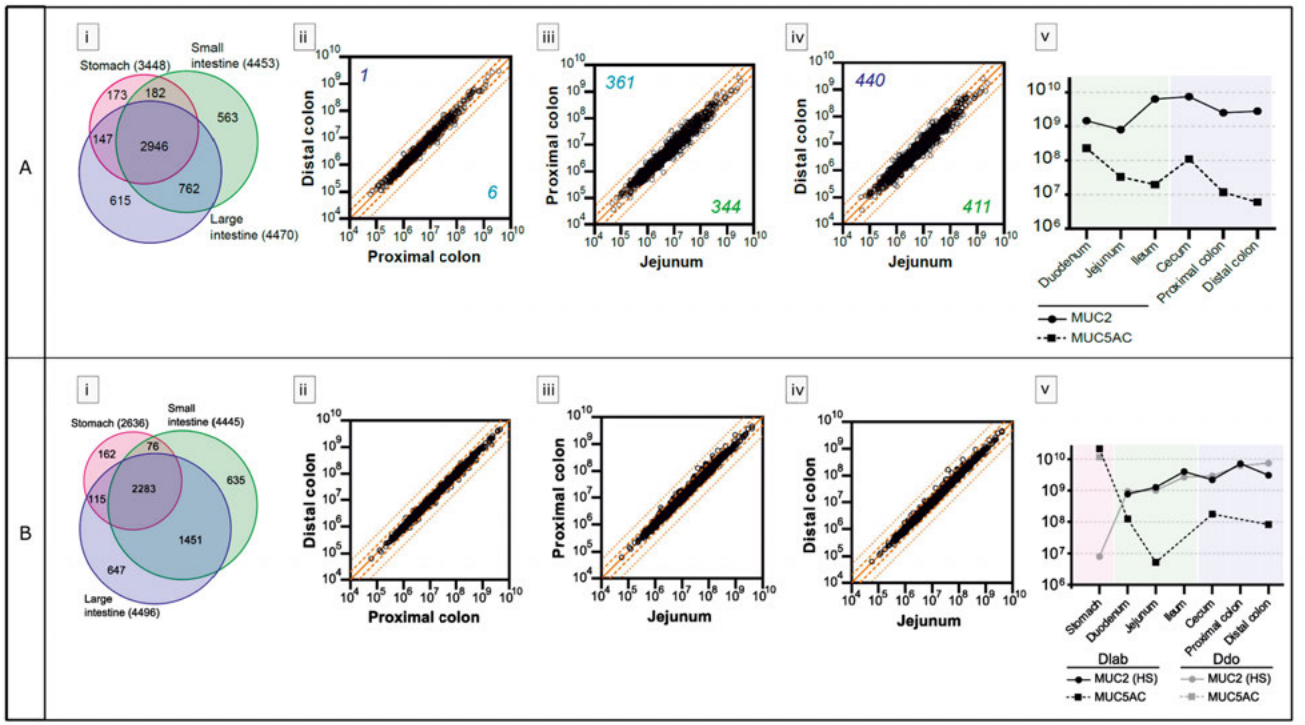


Fig. 12: Composition of (A) porcine and (B) canine GI mucus. (i) Venn diagrams showing the number of shared and unique proteins in GIT regions; total number identified for that segment is given in parentheses. (ii), (iii), and (iv): Comparisons of protein abundance in the different segments. The orange solid line represents the line of unity, the dashed lines the 2-fold difference and the dotted lines the 5-fold one. Numbers indicate proteins with significantly higher abundance in jejunal (green) or colonic (blue) mucus. (v) Abundance of gel-forming mucins

In both pigs and dogs, the MUC5AC (gel-forming mucin) abundance decreased throughout the intestinal tract, while the abundance of MUC2 (gel-forming mucin) remained constant and was dominant in the small and large intestine (Fig. 12Av and 12Bv). The protein abundance was similar between the laboratory dogs and the private veterinary patient, (Fig. 12Bv). The predominance of MUC2 in the small and large intestinal mucus has been previously reported in humans (48), pigs (120), mice (121), and rats (122).

No significant differences in compound abundance between proximal and distal colonic mucus were detected in the lipidomics and metabolomics analyses, for either pigs or dogs. Most of the total identified lipids and metabolites had significantly higher abundance in the jejunal mucus than in the colonic mucus, where only 4% (pigs) and 7% (dogs) of the total compounds detected were significantly more abundant. Lipids and metabolites which were significantly more abundant, were all digestive products. This might be again related to the physiological function of the small intestine, i.e., to digest food and absorb nutrients.

Structural profiling of porcine and canine gastrointestinal mucus

Rheological profiling

All porcine and canine mucus samples showed non-Newtonian fluid characteristics and displayed viscoelastic properties. The apparent viscosity profiles of porcine small intestinal mucus samples are presented in Fig. 13Ai. Ileal mucus exhibited the highest mean apparent viscosity values throughout the examined shear range. These samples were also homogenous, with limited inter-animal variability (average 2-fold difference between maximum and minimum values). Jejunal mucus showed the lowest mean apparent viscosity values and the highest variability (18-fold), while the variability for duodenal mucus was 12-fold. These data are supported by visual observations of the mucus, as the ileal samples had a “paste-like” texture, while the jejunal samples ranged from gel-like to liquid-like. Data from another investigation have previously highlighted the high inter-animal variability in porcine jejunal mucus (56). The median apparent viscosity values of canine small intestinal mucus samples are presented in Fig. 13Bi, and were similar for all segments. The inter-animal variability ranged from 2-fold to 9-fold, while ileal mucus had a “paste-like” consistency.

In the large intestinal segments of both species, mucus from the distal colon and the cecum exhibited the highest and lowest apparent viscosity values, respectively (Fig. 13Aii and 13Bii). Histological work in pigs has shown substantial differences in the mucosal architecture between cecum and colon (123), and the differences in apparent viscosity values between cecal and colonic mucus were more pronounced in pigs than in dogs. All large intestinal

samples were homogenous, with the inter-animal variability ranging from 2- to 8-fold for the pigs and 3- to 4-fold for the dogs. The low variability in colonic mucus might be related to its protective function; the rheological properties must be within certain margins to exert its crucial functional role. The rheological properties of mucus collected from the domestic dog were within the range observed for the laboratory ones, suggesting that differences in size, breed, and the presence of stomas, had no significant effect.

For both species, apparent viscosity values were higher in the colonic mucus than in the small intestinal mucus and this can be interpreted as higher resistance to flow for the former. Indeed, colonic mucus forms a tight barrier that resists the generated shear stress without being flushed away, in order to protect the epithelium. Additionally, excessive amounts of mucosal cells in the jejunal compared to colonic mucus have been related to the weaker rheological behavior of jejunal mucus (124). Porcine mucus exhibited higher apparent viscosity values compared to canine mucus, both in the small and large intestinal segments. This suggests that the porcine mucus has a higher resistance to flow.

All porcine and canine mucus samples behaved like true gels, with the storage modulus being higher than the loss modulus (data not shown for reasons of clarity). In both species, no statistically significant differences were identified between the storage modulus values (at 1 rad/sec) of mucus from the various GI segments. In pigs, a trend towards higher storage modulus values was observed in proximal colonic, distal colonic, ileal, and duodenal compared to gastric, jejunal and cecal mucus samples, suggesting a higher degree of cross-linking for the former (Fig. 13Aiii). The storage modulus values were in accordance with available literature values for the same species (87, 89, 124, 125). In dogs, the extent of rigidity of the mucus network increased progressively towards the distal parts of the small and large intestine, with a trend towards higher storage modulus values in the gastric, ileal, and distal colonic mucus samples compared to other segments (Fig. 13Biii). There was a high degree of variability in the canine gastric mucus. Variability in the viscoelastic properties of canine gastric mucus has been reported previously and it is hypothesized that gastric mucus is organized into two rheologically different layers (65). The storage modulus profiles did not correlate with any physiological, compositional, or structural parameters, indicating that the rheological behavior is a product of the contribution of various components, as discussed elsewhere (120, 126).

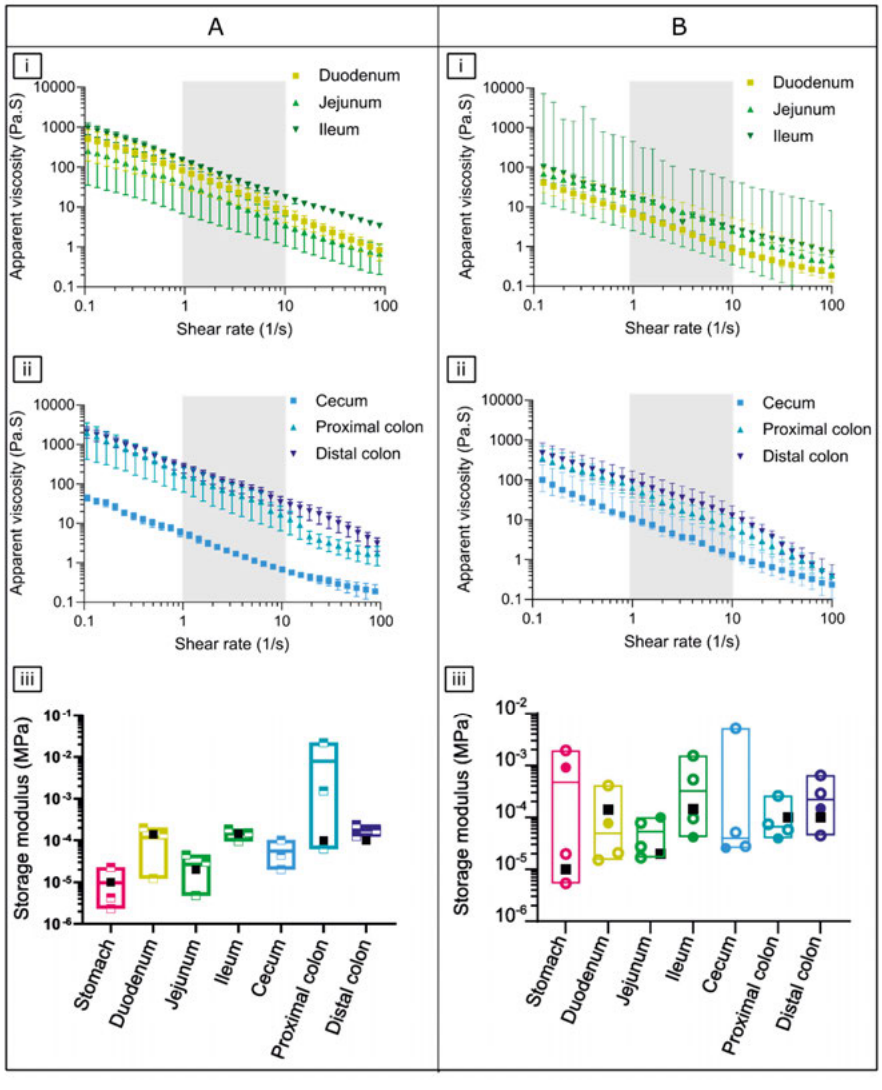


Fig 13: Structural profiling of (A) porcine and (B) canine gastrointestinal mucus. Apparent viscosity curves of mucus samples from various segments of the (i) small and (ii) large intestine as function of shear rate. Porcine data are depicted as mean ± SEM and canine data as median ± range; shaded area illustrates the physiologically relevant shear-rate range. (iii) Floating bars of storage modulus of mucus samples from the porcine GIT (porcine mean and canine median storage modulus values at 1 rad/sec are depicted with line, n = 3). Half-filled squares represent the porcine individual values, open circles represent the laboratory dogs, closed circles represent the domestic dog and black squares the literature values

Network microarchitecture

As seen in the CryoSEM images (Fig. 14A and 14B) all GI mucus samples from both species present an extensive network that cannot be adequately captured with conventional microscopy. Due to their highly sophisticated structure, mucins can form multiple types of bonds, such as disulfide and non-covalent bonds. This results in a highly entangled network (127) which is responsible for the sieving function of the mucus. Several studies have attempted to determine the pore size of the resulting network, with reported values ranging from 20 nm to 200 nm (51). However, particles up to 2 μm can diffuse through the mucus network (2, 128). These observations suggest that, due to the dynamic nature of the GI mucus *in vivo*, the determination of an absolute cut-off value for pore size is complicated, especially if physicochemical interactions or the effects of antimicrobial agents are also to be considered. The aim of the analysis in this thesis was to compare the microarchitecture of mucus network characteristics from various porcine and canine GIT segments.

In both species, the microarchitecture of mucus from jejunal and ileal origin was similar, containing small, near-circular pores with a high degree of homogeneity. The similarity of jejunal and ileal mucus has also been reported by a study using microscopy and multiple particle tracking analysis (77). In both species, cecal mucus consisted of large, elongated pores, while mucus of proximal colonic origin presented large, elongated pores but also “islets” of small, near-circular pores. These "islets" were also visible in the porcine distal colonic mucus, but could not be detected in canine distal colonic mucus. The latter contained mainly larger pores. The presence of two pore populations could be due to the two-layer configuration of the colonic mucus, where the outer layer is formed by proteolytic activity in the inner layer. The heterogeneity of the GI mucus samples suggests that the microarchitecture of the network depends on a combination of factors, even though MUC2 and MUC5AC are the main mucins responsible for the network formation of intestinal and gastric mucus, respectively.

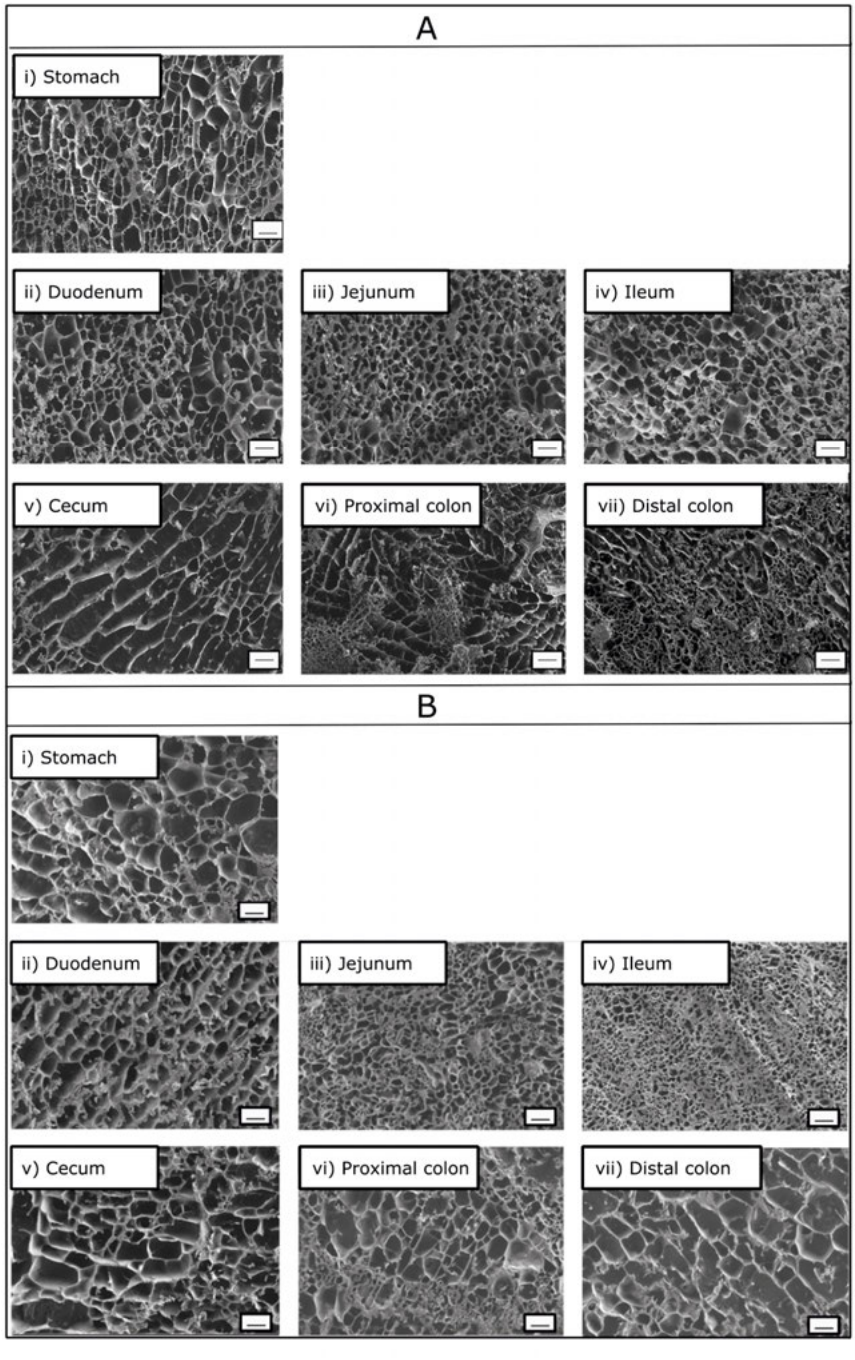


Fig 14: Structural profiling of (A) porcine and (B) canine gastrointestinal mucus: Representative cryo-scanning electron micrographs of mucus samples collected from (i) stomach, (ii) duodenum, (iii) jejunum, (iv) ileum, (v) cecum, (vi) proximal, and (vii) distal colon at 2000× magnification. Scale bar: 10 μm

Barrier properties of porcine and canine colonic mucus (Papers III and IV)

The barrier properties of porcine **(Paper III)** and canine **(Paper IV)** native colonic mucus were examined to understand the impact of charge and size on the diffusion of macromolecules in mucus. The diffusivity values of FITC-dextrans with various size and charge properties are presented in Fig. 15 for colonic mucus from three pigs and for two dogs in Fig. 16.

In pigs P01 and P05 and dog DZ02, the diffusivity values of neutral and anionic FITC-dextrans were similar and significantly higher compared to the values obtained for the cationic 4K FITC-dextran, in each animal. For pig P04 and dog DZ04, the diffusivity values of charged FITC-dextrans were different from each other, and both were significantly lower compared to the neutral one (Fig. 15A-D and 16A and 16B).

Overall, diffusivity values tended to decrease more in porcine and canine colonic mucus for cationic FITC-dextrans compared to the neutral and anionic ones. However, the decrease in diffusivity of the positively charged FITC-dextran was more pronounced in dogs than pigs. This could be related to the lower zeta potential in canine native colonic mucus (-31.6 and -32.6 for DZ02 and DZ04, respectively) compared to the respective porcine secretion (ranging from -27.1 to -25.8). This suggests stronger interactions and binding of the cation to the negatively charged mucin domains. Other studies have demonstrated that diffusion is limited for positively charged molecules in porcine gastric mucus (129) and nanoparticles in porcine jejunal mucus (98). These observations could be explained by electrostatic interactions between the cation and the negatively charged mucin domains (130, 131).

The diffusivity values of FITC-dextrans with higher MW (40K) are illustrated in Fig. 15E and 16A and 16B. No significant differences were reported in any of the animals, and this could be because the higher MW of the dextrans dominates the diffusion behavior. Although the diffusivity of cationic 40K FITC-dextran was also included in the investigations, the fluorescence recovery after the photobleaching event was too slow for calculation of the diffusion values (Fig. 15Fi-iii.) As seen in the representative confocal image in 15F, the 40K FITC-dextran was not homogenously distributed in the porcine mucus sample despite vigorous mixing. Instead, the dextran bound to specific sites on the mucus network. The combination of strong electrostatic interactions (due to the positive charge) and steric hindrance (due to high MW) resulted in complete immobilization and electrostatic entrapment within the porcine colonic mucus network. Substantial differences have been reported in the diffusion of anionic and cationic synthetic peptides of equal MW in porcine gastric mucus (62) and between anionic and cationic 40K FITC-dextran in a mucin solution

(111). These data suggest that the permeation of high MW cations through porcine colonic mucus is highly restricted.

The diffusivity values of higher MW (40K and 70K) FITC-dextrans were significantly lower compared to the corresponding values for the 4K FITC-dextran, in both pigs and dogs (Fig.15G-I and 16A and 16B). High MW can be associated with limitations in mobility and a high likelihood of physicochemical interactions. It is worth mentioning that the diffusion of 40K and 70K FITC-dextrans was delayed, but they were not completely immobilized. Similarly, two other studies have reported retardation of solute flux in porcine gastric and intestinal mucus with increasing MW (59) (61). Another recent study reports decreasing apparent permeability values in porcine jejunal mucus with increasing MW (4K to 150K) for FITC-dextrans (74). The decrease in diffusivity values in this thesis was non-proportional to the increase in MW, as also reported for porcine gastric (59), jejunal mucus (74), and for cystic fibrosis sputum (112).

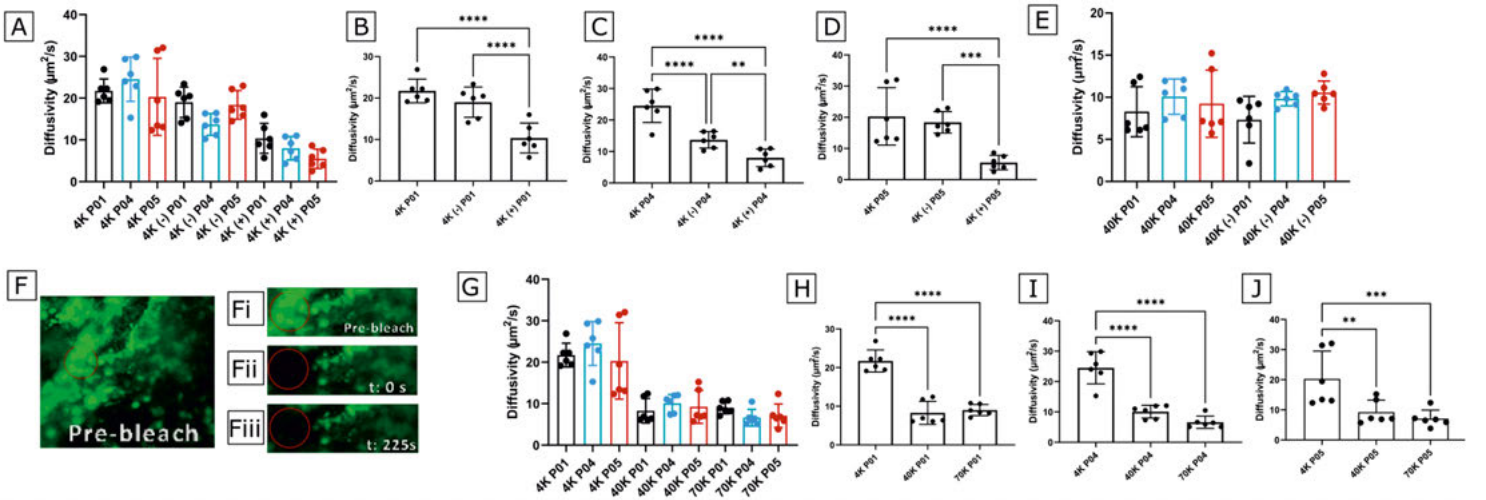


Fig 15: Comparison of FITC-dextran diffusivity values in native porcine colonic mucus. (A) Diffusivity values of neutral, anionic (-) and cationic (+) 4K FITC-dextrans in native colonic mucus from three pigs (P01, P04 and P05). Individual profiles and statistical analysis of diffusivity values from (B) P01; (C) P04; and (D) P05. (E) Diffusivity values for the neutral and anionic (-) 40K dextrans in native porcine colonic mucus from the same three pigs. (F) Confocal images of native colonic mucus of pig P04 during a FRAP experiment with the cationic (+) 40K FITC-dextran, including (Fi) pre-bleach caption. (Fii) Captions were taken immediately after the photobleaching event and (Fiii) at t: 225 s. (G) Diffusivity values of the 4K, 40K, and 70K FITC-dextrans from three pigs (P01, P04, and P05) and respective individual profiles and statistical analysis for (H) P01; (I) P04; and (J) P05; n=3-6. Filled circles represent individual measurements. Bars: Mean±SD (** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)

Although the same diffusion trends were identified in DZ02 and DZ04, the diffusivity values were higher for DZ04 than DZ02. The trend towards higher diffusivity values in DZ04 might be because of the higher water content in this dog's colonic mucus (94.5%), compared to DZ02 (89.9%), as previously reported (3). Smith at al. have showcased a strong correlation between water content and the diffusivity of butyrate in porcine colonic mucus (132).

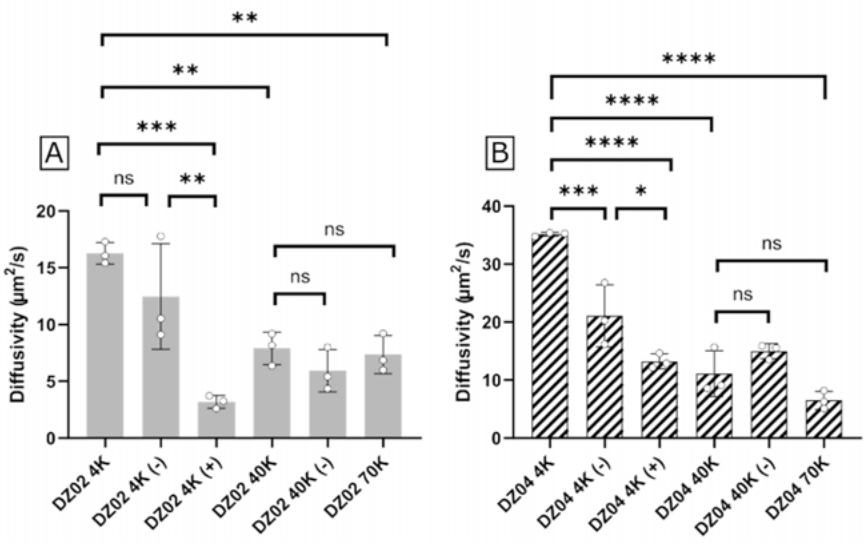


Fig. 16: Individual profiles and statistical analysis of diffusivity values of FITC-dextrans in colonic mucus from (A) DZ02 and (B) DZ04; n=3. Open circles represent individual measurements. Bars:Means±SD (* $p< 0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns: no significance)

Development of artificial colonic mucus models (Papers III & IV)

Initial preparations of porcine artificial colonic mucus models with either mucin Type II or III did not present gel-like characteristics. The limited gel-forming ability of commercially available mucins has been extensively reported in the literature (87-89) and it could be related to the purification process that the mucins undergo. Harsh conditions deprive the mucins of binding sites that can be instrumental in the formation of the characteristic 3D network (106). PAA is a thickening agent used as a mechanical adjuvant to compensate for the limited gel-forming potential of purified mucins (56). PAA associates with mucins through mucoadhesion, by physical entanglement, and functional group interactions (105).

Gradual addition of PAA increased both the apparent viscosity and storage modulus values, suggesting enhanced cross-linking (Fig. 17A and 17B). The values were higher in the samples containing mucin Type III than Type II (Fig. 17C and 17D). The two mucin Types undergo different purification processes that may result in the preservation of different functional groups, allowing for different types of interactions between the mucins and the PAA (106). Additionally, ions play a key role in the rheological synergism between PAA and mucins (133) and therefore, different levels of ions due to different purification processes could further contribute to this phenomenon.

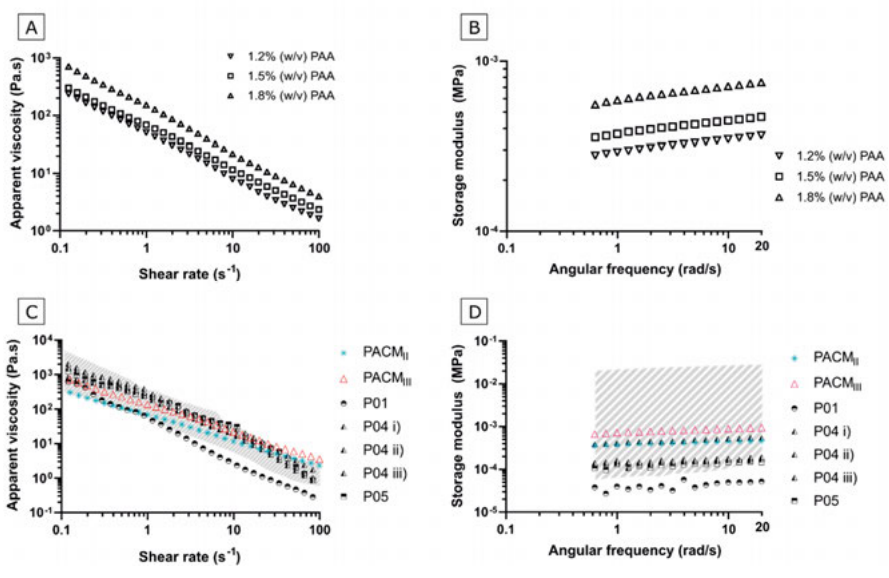


Fig. 17: Rheological profiling of PACM. Effect of increasing amounts of PAA on (A) the apparent viscosity and (B) storage modulus of $PACM_{II}$ samples. Reverse triangles, squares and triangles represent 1.2%, 1.5% and 1.8% (w/v) PAA additions to the samples. Comparison of PACM and porcine native colonic mucus in terms of (C) apparent viscosity, and (D) storage modulus values. The shaded area represents the range of data from **Paper I**. $PACM_{II}$, blue stars; $PACM_{III}$, red triangles. Native colonic mucus values are from three pigs (P01, P04, and P05), represented in black half circles, triangles, and squares, respectively. i), ii) and iii) represent multiple independent measurements of colonic mucus samples from pig P04

Porcine artificial colonic mucus models containing mucin Type II and mucin Type III with 1.5% (w/v) PAA had rheological properties similar to the native colonic mucus from six pigs (three pigs from Project III and three pigs from Project I, shown as shaded area) (Fig. 17C and 17D). Similarly, the rheological properties of canine artificial colonic mucus containing mucin Type II and mucin Type III with 1.5% and 1.3% (w/v), respectively, were within the range of the rheological properties measured in native colonic mucus from five dogs.

CryoSEM imaging showed that porcine artificial colonic mucus models containing either mucin Type II or Type III, in the absence of PAA, had "loose" network (Fig. 18A and 18B). The gradual addition of PAA resulted in the adaptation of a "honeycomb" structure for both sample types (Fig. 18C and 18D). Upon addition of appropriate amounts of PAA, both mucus mimics presented a microarchitecture with features comparable to the respective native colonic mucus secretion (Fig. 18E). This highlights the ability of PAA to associate with mucins and other components to present a native-like mucus (134).

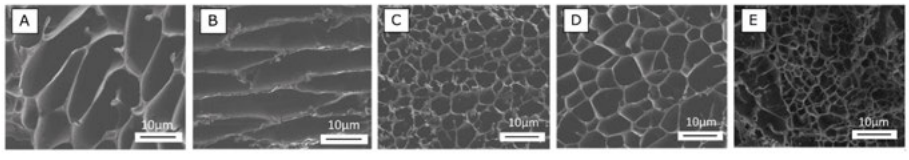


Fig. 18: Structural validation of $PACM_{II}$ and $PACM_{III}$: Representative cryo-scanning electron micrographs of: (A) $PACM_{II}$ samples without PAA; (B) $PACM_{III}$ samples without PAA; (C) $PACM_{II}$ samples with 1.5% (w/v); (D) PAA $PACM_{III}$ samples with 1.5% (w/v) PAA; and (E) porcine native colonic mucus. All at 5000 × magnification. Scale bars (A-E): 10 µm

Barrier properties (Papers III & IV)

Diffusion of model macromolecules through artificial colonic mucus models was compared to values obtained with the corresponding native mucus to assess how well they could replicate the barrier properties of the latter. The $PACM_{II}$ and $PACM_{III}$ samples containing 1.5% (w/v) PAA showed comparable rheological properties to porcine native colonic mucus and were selected for validation against the native secretion in terms of macromolecular diffusion. A pure PAA aqueous gel was also included in the investigations to examine whether PAA alone could capture diffusion trends, as it played a critical role in the artificial mucus structure.

Diffusion of macromolecules in both $PACM_{II}$ and $PACM_{III}$ was not significantly different to the profiles obtained from porcine native colonic mucus, with the exception of the cationic 4K FITC-dextran (Fig. 19A and 19B). $PACM_{II}$ and $PACM_{III}$ discriminated between charges and sizes, thereby adequately capturing the key diffusion trends observed in porcine native mucus. The diffusion of cationic 4K FITC-dextran was significantly more restricted in $PACM_{II}$, $PACM_{III}$, and the PAA mixture compared to the native mucus. This could be related to the more negative charge of the artificial models (range -48.7 to -35.1) compared to the native mucus (range -27.1 to -25.8). The greater negative charge results in stronger electrostatic interactions between the mucus and the diffusing cation. Thus, the stronger interactions can

result in significant retardation of the diffusing cation and translate into lower diffusivity values. The diffusion of anionic and neutral macromolecules with low MW (4K) in the PAA mixture was significantly higher compared to the values obtained from porcine native colonic mucus. This observation emphasizes the key role of mucus components such as mucins, proteins, and lipids as they offer a plethora of potential interaction sites. This is particularly important for the diffusion of small molecules and low MW macromolecules. As their diffusion is not sterically hindered, physicochemical interactions govern the diffusion pattern. For FITC-dextrans of higher MW, the difference between native mucus and artificial variants was not significant. The trend towards slightly lower diffusivity values in the PAA mixture, $PACM_{II,}$ and $PACM_{III}$, was not significant and could be attributed to the more rigid network of PAA.

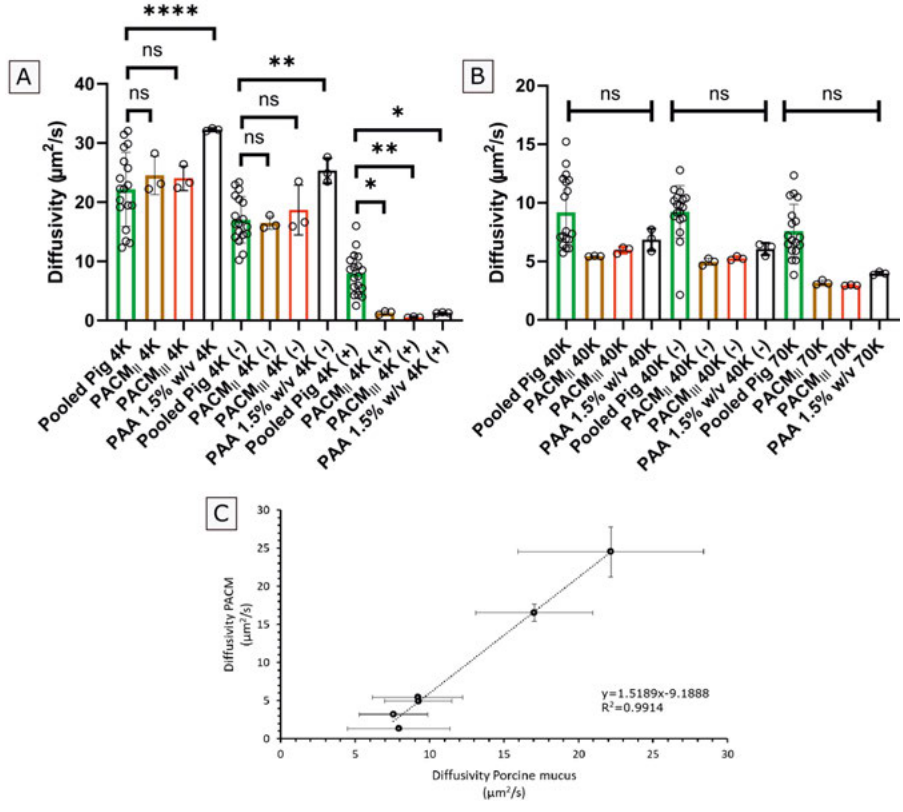


Fig. 19: Comparison of diffusivity values of FITC-dextrans in native porcine colonic mucus from three pigs (P01, P04, and P05), in $PACM_{II}$, $PACM_{III}$, and PAA 1.5% (w/v) gel. (A) 4K; (B) 40K and 70K molecular weights. Open circles represent individual measurements. n: 3-6, Bars: Mean±SD. (C) Correlation between average diffusivity values of various FITC-dextrans in $PACM_{II}$ and porcine native colonic mucus (Mean±SD) (* $p< 0.05$, ** $p<0.01$, **** $p<0.0001$, ns: no significance). Data from porcine native colonic (green), $PACM_{II}$ (brown), $PACM_{III}$ (red) and the 1.5% (w/v) PAA mixture (black)

Both the $PACM_{II}$ and $PACM_{III}$ models captured key trends observed in native mucus. However, preparation of $PACM_{III}$ was more cumbersome, as it resulted in high viscosity mixtures where mixing of the components was rather difficult. Insufficient mixing could result in undissolved components in the mixture, an aspect that would hinder production reproducibility. Therefore, $PACM_{II}$ was selected to be the PACM model. A good correlation between the diffusivity values of FITC-dextrans in PACM and porcine native colonic mucus was established (R^2: 0.99); Fig. 19C.

As $CACM_{III}$ was also difficult to prepare, $CACM_{II}$ was selected to represent CACM and undergo the validation against native colonic mucus from two laboratory dogs. The diffusivity profiles of FITC-dextrans of various charges and sizes from DZ02, DZ04, and CACM are shown in Fig. 20A and 20B. The values obtained for CACM were not significantly different from the DZ02 native colonic mucus. However, they were different compared to the DZ04 native mucus, with the exception of 70K FITC-dextran. As also seen with PACM, diffusion of high MW FITC-dextrans was more restricted in CACM compared to the canine native colonic mucus, probably because of the higher rigidity of the network created by PAA. Thus, CACM captured key diffusion trends in macromolecular diffusion related to charge and size, as observed in both dogs.

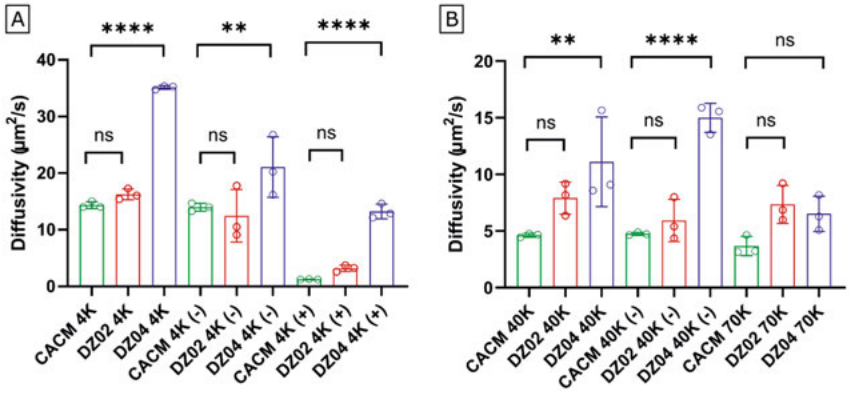


Fig. 20: Diffusivity of FITC-dextrans in native and artificial canine mucus. (A) Comparison of diffusivity values in canine native colonic mucus from two laboratory dogs and CACM of 4K molecular weight FITC-dextrans of different charges. (B) Similar profiles for 40K and 70K FITC-dextrans. Open circles represent individual measurements; n= 3, Bars: Mean±SD (** $p<0.01$, **** $p<0.0001$, ns: no significance). Data from CACM (green), DZ02 (red) and DZ04 (blue)

Impact of altered viscosity on the diffusion of macromolecules in CACM (Paper IV)

Several disease conditions, such as ulcerative colitis and cystic fibrosis, are associated with altered mucus rheology. CACM samples containing lower and higher amounts of PAA (L-CACM and H-CACM, respectively) were prepared to assess the permeability of macromolecules through mucus with altered rheological properties. As shown in Fig. 21, there was a trend towards higher diffusion of FITC-dextrans in L-CACM, compared to reference CACM. Similar findings have been reported for the diffusion of 4K FITC-dextrans in rat mucus treated with N-acetyl-cysteine to exhibit lower viscosity and cross-linking (135). The diffusion of FITC-dextrans in H-CACM was similar to that for reference CACM and this might have been related to the limited additional amount of PAA added in H-CACM. Further PAA addition was deemed impossible as it was difficult to dissolve the solids and obtain a homogenous mixture.

Overall, the findings suggest that CACM can be modified to reflect the rheological properties of low-viscosity mucus that are reported in some disease states, and it was possible to replicate diffusion patterns described in the literature. Further optimization of the low-viscosity CACM model would involve adjustment of the mucus components to amounts reported in the literature for disease-state mucus, in order to enhance the biorelevance of the model.

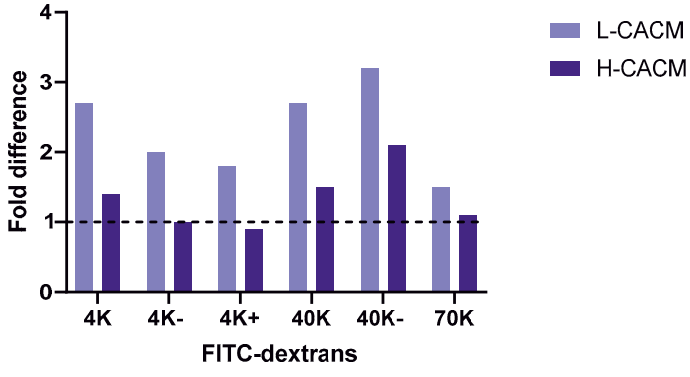


Fig. 21: Comparison of diffusion in low- and high-viscosity CACMs with reference CACM. Fold difference is given for the mean permeated amount (%) of six FITC-dextrans. The dotted line represents the reference CACM baseline

Impact of key mucus components on the diffusion of macromolecules in CACM (Paper IV)

CACM was also used to assess the impact of key mucus components on the diffusion of macromolecules. This study was performed by monitoring the FITC-dextran permeation through CACM samples in which either the mucin, the BSA, or the lipids were omitted in the sample preparation.

In the absence of mucin and BSA, the permeated amount of all FITC-dextrans increased considerably (Fig. 22A and 22B) due to the loss of the binding potential that these additives provide for the macromolecules. As expected, the absence of lipids had limited impact on the diffusion of the hydrophilic FITC-dextrans. Indeed, it has been previously demonstrated that lipids substantially impact the permeation of lipophilic compounds but have limited effect on the hydrophilic ones (26). For the charged FITC-dextrans, the diffusion of the cationic one was more affected by the absence of mucin than of albumin, while the opposite was observed for the anionic ones. The presence of mucin is known to greatly decrease the diffusivity of cationic FITC-dextrans compared to anionic ones (demonstrated for 4K, 40K, and 150K FITC-dextrans) (111).

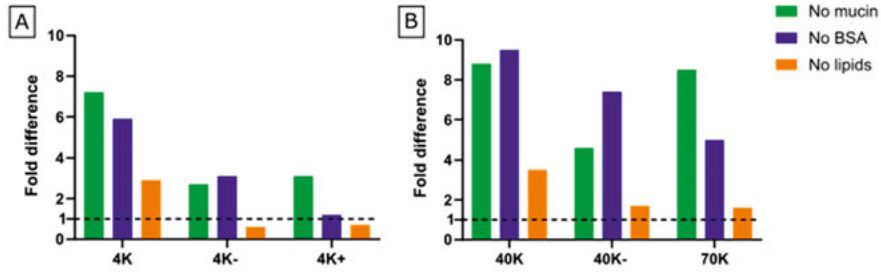


Fig. 22: Impact of components included in the CACM artificial mucus on diffusivity. Comparison of fold difference in mean permeated amount of FITC-dextrans in CACM without mucin (green), CACM without BSA (blue) and CACM without lipids (orange) for: (A) 4K molecular weights, and (B) 40K and 70K; n: 3-6. The dotted line represents the CACM baseline containing all three components (mucin, BSA, lipids)

Conclusions

This thesis focused on the characterization of gastrointestinal mucus from two large animal species (pigs and dogs) and studied the impact of mucus on drug absorption using *ex vivo* and *in vitro* approaches. The findings improve our understanding of the key mucus properties relevant for drug absorption and demonstrate the potential of artificial mucus models in capturing the barrier properties of native mucus.

The key conclusions of this thesis are summarized as follows:

- Small intestinal mucus forms a "loose" layer that can be easily detached from the tissue in both pigs and dogs and is typically available in large quantities. In contrast, gastric and large intestinal mucus adheres tightly to the tissue for both species **(Papers I & II)**

- Proximal and distal colonic mucus was available in lower amounts in pigs compared to dogs **(Papers I & II)**

- There are significant differences in the composition of small and large intestinal mucus, and these are more pronounced in pigs than in dogs. These differences could be a result of the different physiological functions of the gastrointestinal regions **(Papers I & II)**

- Mucus of small intestinal origin exhibits lower apparent viscosity compared to mucus of large intestinal origin, in both pigs and dogs. This may be related to the necessity of the colonic mucus to withstand the higher mechanical stress generated in the large intestine so that it adheres to the epithelium **(Papers I & II)**

- Diffusion of cationic 4K FITC-dextran through porcine and canine colonic mucus is substantially delayed, compared to its neutral and anionic counterparts. This observation is likely related to electrostatic binding of the cation to the anionic domains of mucins. The delay was more pronounced in artificial mucus models, due to the more anionic nature of the polymer used as mechanical adjuvant in these models compared to native mucins **(Papers III & IV)**

- In both pigs and dogs, FITC-dextrans of higher MW (40K and 70K) had similar diffusion profiles in colonic mucus, and were significantly lower than the 4K ones. Although diffusion of 40K and 70K FITC-dextrans was substantially delayed, the dextrans were not completely immobilized **(Papers III & IV)**

- The positively charged 40K FITC-dextran was immobilized, suggesting that the combination of steric hindrance and electrostatic interactions can be detrimental to diffusion through mucus. As a consequence, the diffusion of cationic macromolecules with high MW was highly restricted through the colonic mucus **(Paper III)**

- Artificial colonic mucus models, prepared using common laboratory ingredients, could capture the diffusion trends observed in native porcine and canine colonic mucus. Although the artificial colonic mucus models cannot completely replace the use of native mucus due to differences in network rigidity and charge between the native mucins and the mechanical adjuvant, they could enable quick screening of drug candidates and provide useful insights about mucus diffusion in the early stages of the drug development process **(Papers III & IV)**

- The inclusion of mucin, protein, and lipid components in the canine artificial colonic mucus model was necessary to replicate the mucus barrier *in vitro.* The canine artificial colonic mucus model was successfully implemented in microscopic and macroscopic permeation assays, enabling the assessment of the diffusion of both fluorescent compounds available in small quantities and nonfluorescent compounds **(Paper IV)**

Popular scientific summary

Absorption of orally administered drugs is a complicated process. There are many barriers in the human body that the drug needs to overcome in order to exert its effect. One such barrier is a hydrogel that lines the gut and its purpose is to protect it from bacteria and other noxious matter. This hydrogel, which is called “mucus”, can also restrict the absorption of drugs. In order to understand what is the role of this hydrogel in drug absorption, information about the characteristics of this barrier is needed. Until today, most of the available information comes from mucus from mice and rats, because these are commonly used animals in research. There are differences however between the gut characteristics of rodents and humans. This is why information about the mucus in large animals, whose gut characteristics are closer to humans, is needed. Then we have a better idea of how mucus behaves in humans. In the first part of this thesis, waste tissue with mucus from pigs (from a local abattoir) and dogs (euthanized for other reasons than these studies) was analyzed to report key properties, such as the mucus components and the mucus structure. The mucus components might bind to drugs and the mucus structure forms a net that could restrict the passage of drugs through the mucus and affect drug absorption.

In order to understand how mucus impacts drug absorption, it is common to study how drugs permeate through mucus harvested from animals in preclinical experiments. However, animal (and animal products) usage in the drug development process should be reduced, in order to minimize animal distress, a goal that is also emphasized by the European Medicines Agency. To replace/reduce the use of animals or animal products in experiments, artificial models that are mimicking animal characteristics can be used. If the artificial models have a well-informed design, then they can predict trends that are observed in animals, without the need to include animals in the experiments. This means that using these models to screen many drugs will result in only the most promising drug candidates proceeding to the costly clinical stages, an aspect that can cut the unnecessary costs of late-stage drug failures. In the second part of this thesis, information about mucus in pigs and dogs was used to design artificial mucus models. These models were validated and it was shown that they can be useful in predicting how drugs pass through the mucus. These models can be used to test what drug characteristics are favorable to

permeate through the mucus. The models can be easily prepared with common laboratory ingredients, at low cost and can be used to screen drug formulations and select the best-performing candidate.

Εκλαϊκευμένη επιστημονική περίληψη

Η απορρόφηση των φαρμάκων που χορηγούνται από το στόμα είναι μια περίπλοκη διαδικασία. Υπάρχουν πολλοί φραγμοί στον ανθρώπινο οργανισμό οι οποίοι περιορίζουν την απορρόφηση ξενοβιωτικών, με σκοπό την προστασία από το εξωγενές περιβάλλον. Έναν τέτοιο μηχανισμό προστασίας αποτελεί μια υδρογέλη που καλύπτει το έντερο και σκοπός της είναι να το προστατεύει από βακτήρια και άλλες επιβλαβείς ουσίες. Τα φάρμακα θα πρέπει να διαπεράσουν αυτή την υδρογέλη, η οποία ονομάζεται «βλέννα», ώστε να απορροφηθούν. Για να γίνει κατανοητός ο ρόλος αυτής της υδρογέλης στην απορρόφηση φαρμάκων, πρέπει πρώτα να υπάρχουν πληροφορίες σχετικά με τα χαρακτηριστικά αυτού του φραγμού. Μέχρι σήμερα, οι περισσότερες πληροφορίες που υπάρχουν στην βιβλιογραφία προέρχονται από μελέτες σε βλέννα που έχει συλλεχθεί από ποντίκια και αρουραίους, επειδή αυτά είναι ζώα που χρησιμοποιούνται συνήθως στην έρευνα. Ωστόσο, υπάρχουν διαφορές στα χαρακτηριστικά του εντέρου μεταξύ τρωκτικών και ανθρώπων. Αυτός είναι ο λόγος για τον οποίο χρειάζονται πληροφορίες για τη βλέννα σε μεγάλα ζώα, των οποίων τα χαρακτηριστικά του εντέρου είναι περισσότερο όμοια με αυτά του ανθρώπου. Τότε έχουμε μια καλύτερη ιδέα για τα χαρακτηριστικά της βλέννας στους ανθρώπους. Στο πρώτο μέρος αυτής της διατριβής, ιστοί από χοίρους (από τοπικό σφαγείο) και σκύλους (που υποβλήθηκαν σε ευθανασία για λόγους άλλους από αυτές τις μελέτες) αναλύθηκαν για να καταγραφούν βασικές ιδιότητες, όπως τα συστατικά και η δομή της βλέννας. Τα συστατικά της βλέννας ενδέχεται να αλληλεπιδράσουν φυσικοχημικά με φάρμακα και η δομή της βλέννας σχηματίζει ένα δίχτυ που θα μπορούσε να περιορίσει τη διέλευση των φαρμάκων μέσω της βλέννας και να επηρεάσει την απορρόφηση τους.

Προκειμένου να γίνει κατανοητός ο τρόπος με τον οποίο η βλέννα επηρεάζει την απορρόφηση φαρμάκων, είναι σύνηθες να μελετάται η διαπέραση των φαρμάκων σε βλέννα που συλλέγεται από ζώα σε προκλινικά πειράματα. Ωστόσο, η χρήση ζώων (και ζωικών προϊόντων) στη διαδικασία ανάπτυξης φαρμάκων θα πρέπει να μειωθεί, προκειμένου να ελαχιστοποιηθεί η δυσφορία των ζώων, στόχος που τονίζεται επίσης από τον Ευρωπαϊκό Οργανισμό Φαρμάκων. Για την αντικατάσταση/μείωση της χρήσης ζώων ή ζωικών προϊόντων σε πειράματα, μπορούν να χρησιμοποιηθούν τεχνητά μοντέλα που μιμούνται τα χαρακτηριστικά των ζώων. Εάν τα τεχνητά μοντέλα έχουν καλά

ενημερωμένο σχεδιασμό, τότε μπορούν να προβλέψουν τις τάσεις που παρατηρούνται στα ζώα, χωρίς να χρειάζεται να συμπεριληφθούν ζώα στα πειράματα. Αυτό σημαίνει ότι η χρήση αυτών των μοντέλων για τον έλεγχο πολλών φαρμάκων θα έχει ως αποτέλεσμα μόνο τα υποψήφια φάρμακα με τις περισσότερες πιθανότητες να γίνουν επιτυχή φαρμακευτικά σκευάσματα να προχωρήσουν στα δαπανηρά κλινικά στάδια, μια πτυχή που μπορεί να μειώσει το περιττό κόστος των αποτυχιών φαρμάκων στα τελευταία στάδια της ανάπτυξης. Στο δεύτερο μέρος αυτής της διατριβής, χρησιμοποιήθηκαν πληροφορίες σχετικά με τη βλέννα σε χοίρους και σκύλους για το σχεδιασμό μοντέλων τεχνητής βλέννας. Αυτά τα μοντέλα επικυρώθηκαν και αποδείχθηκε ότι μπορούν να είναι χρήσιμα στην πρόβλεψη του τρόπου με τον οποίο τα φάρμακα διαπερνούν τη βλέννα. Τα μοντέλα μπορούν να παρασκευαστούν εύκολα με κοινά εργαστηριακά συστατικά, με χαμηλό κόστος και μπορούν να χρησιμοποιηθούν για τον έλεγχο πολλών φαρμάκων και την επιλογή του υποψηφίου με τις καλύτερες επιδόσεις.

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