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Artificial colonic mucus for studies of the mucus absorption barrier

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Abstract
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Colonic diseases affect more than 10 million people in Europe, and by 2045, more than 1% of the global population is expected to be affected by inflammatory bowel diseases. Drug development targeting colonic diseases is urgently needed. However, translating in vitro research into in vivo clinical relevance remains a challenge, with significant time and effort required in drug discovery and pre-clinical stages. Therefore, the development of efficient drug study platforms is necessary, in accordance with the ethical 3Rs principles and the UN SDGs 2030. In addition, the FDA Modernization Act 2.0 encourages the improvement of in vitro models with in vivo relevance.

Colonic mucus is the first interface in contact with drugs targeting colonic diseases. Mucus is a hydrogel composed of complex macromolecular crosslinks and acts as a structural barrier. In healthy conditions, colonic mucus consists of a stratified layer, with the outer layer hosting bacteria. Previous studies have reported changes in mucus in 85% of patients with various colonic diseases. However, characterization of the viscoelastic and barrier properties of colonic mucus in diseased states is still underexplored.

This PhD project focuses on studying the characteristics of the mucus barrier that may influence drug diffusion. Properties of native colonic mucus from human patients are characterized. Combined with studies of particle diffusion and viscoelastic properties in colonic mucus, general parameters influencing drug diffusion in the mucus are identified. Characteristics dependent on pH, surface charge, viscosity, and macromolecular composition of mucus have been investigated. Studies of drug interaction with mucus models from pig, dog, and artificial colonic mucus were performed to observe drug diffusion, drug binding, and drug permeability. Various methods to improve experimental (3D printing) and analytical (machine learning classifiers and physiology-based pharmacokinetic models) approaches were incorporated to enhance reproducibility and provide in-depth data analysis.

From this study, the macrorheology and microrheology profiles of the mucus were compared, and artificial colonic mucus was shown to capture the properties of native colonic mucus. By formulating a biosimilar artificial colonic mucus based on the native form, computational studies connecting to in vivo settings allow for better prediction and improved clinical relevance.

Keywords: hydrogel; mucus; drug diffusion; drug absorption; interspecies; colon drug delivery

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Somewhere between mind and matter, deep in the Sea of Souls,
“It takes a village to raise a child”
– unknown origin –
(perhaps it is a shared consciousness?! Collective unconscious?!)

"It takes a whole world to raise a scientist!"

-Marco Tjakra-

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., Tjakra, M., Li, L., Dubbelboer, I.R., Karlsson, E., Pedersen Lomstein, B., Bergström, C.A.S. (2024) Development of a canine artificial colonic mucus model for drug diffusion studies. *European Journal of Pharmaceutical Sciences*, 194:106702

II. Tjakra, M., Chakrapeesirisuk, N., Jacobson, M., Sellin, M.E., Eriksson, J., Teleki, A., Bergström, C.A.S. (2025) Optimized artificial colonic mucus enabling physiologically relevant diffusion studies of drugs, particles and delivery systems. *Molecular Pharmaceutics*, 22:4032−4045

III. Tjakra, M., Lidayová, K., Avenel, C., Bergström, C.A.S., Hossain, S. (2025) Machine learning framework for investigating nano- and micro-scale particle diffusion in colonic mucus. *Journal of Nanobiotechnology*, 23:583.

IV. Tjakra, M., Teleki, A., Mickols, E., Nyström, N., Ideström, M., Bergström, C.A.S. (2025) Development of patient-centric human artificial colonic mucus based on adult and pediatric samples under healthy and inflamed conditions. *In manuscript*.

V. Tjakra, M., Alkakaei, M., Karlgren, M., Kesisoglou, F., Bergström, C.A.S. (2025) Improved prediction of orally administered budesonide by including the mucus as an absorption barrier. *In manuscript*.

List of additional papers and projects not included in the thesis

I. Naranjani B, Hossain S, Tjakra M, Azhand P, Bergström C, Sinko P, Larsson P. Uncovering optimum motor activity of the small intestine for oral delivery of macromolecules with enhancers. (submitted)

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Abbreviations

MES	2-morpholinoethanesulfonic acid
3D	Three dimensions
3R	Replacement, Refinement and Reduction
ACAT	Advanced Compartmental and Transit
ACM	Artificial colonic mucus
ADME	Absorption, distribution, metabolism, and excretion
API	Active pharmaceutical ingredient
BES	N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
CACM	Canine Artificial Colonic Mucus
CD	Crohn’s Disease
CNCM	Canine Native Colonic Mucus
CRC	Colorectal cancer
Cryo-SEM	Cryo-Scanning electron microscopy
CSP	Cross-species pairs
DIA	Data independent acquisition
eDNA	Extracellular deoxyribose nucleic acid
FITC	Fluorescein isothiocyanate
FRAP	Fluorescence Recovery After Photobleaching
GIT	Gastrointestinal tract
HACM	Human Artificial Colonic Mucus
HEC	Hydroxyethyl cellulose
HNCM	Human Native Colonic Mucus
HPF	High pressure freezing
IBD	Inflammatory Bowel Disease
ITC	Isothermal calorimetry
IVIVC	In vitro-in vivo correlation
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LDA	Linear discriminant analysis
LVR	Linear viscoelastic region
LY	Lucifer yellow
ML	Machine learning
MSD	Mean squared displacement
MST	Microscale thermophoresis
NCM	Native colonic mucus
NET	Neutrophil extracellular traps

PAA	Polyacrylic acid
PACM	Porcine Artificial Colonic Mucus
P_{app}	Apparent permeability
PBPK	Physiologically-based pharmacokinetics
P_{eff}	Effective permeability
PC	Phosphatidylcholine
PNCM	Porcine Native Colonic Mucus
QCM-D	Quartz crystal microbalance with dissipation
QTOF-MS	Quadrupole Time-of-Flight Mass Spectrometry
ROI	Region of interest
SA	Sodium alginate
SH	Sodium hyaluronate
SM	Sphingomyelin
timsTOF-MS	Trapped Ion Mobility Spectrometry with Time-of-Flight Mass Spectrometry
UC	Ulcerative Colitis

Introduction

Colonic diseases existed through history[1–3], with the earliest writings found from Hippocrates' in 400 BC describing chronic diarrhea[4]. Therefore, drugs need to be developed to treat the diseases. However, drug development process is lengthy and involves several stages, starting with drug discovery, followed by drug development, which includes preclinical studies, clinical development, regulatory review, and post-market monitoring. Consequently, drug delivery strategies targeting the colonic region (Fig. 2) faces several challenges (Fig.1). The colon is composed of epithelial cells and goblet cells that form glands secreting mucus, supported by muscle fibers, blood and lymphatic vessels, regulated by both motor and sensory neurons. Colonic mucus consists of a double layer, in contrast to the small intestine, which has only a single layer[5]. The inner mucus layer is typically sterile, with a shedding mechanism that forms the outer layer, which hosts numerous bacteria[6]. Mucus can be classified as a hydrogel and has a high-water content (around 90%). This barrier is highly structured, with its main protein component being mucin, a glycoprotein secreted by epithelial cells for protection. Mucus not only provides physical filtration of biological entities and particles but also interacts with them through complex chemical and molecular interactions[7]. In addition to mucus, tighter junctions in colon compared to small intestine functions as a barrier to protect the body from complex environment and the microbiome[8,9].

This PhD project focuses on experimental study coupled with computational simulation and analysis in preclinical settings to better predict absorption from the colon and potentially replace animal studies. The development and use of biosimilar fluids for drug testing have been studied over the past two decades[10]. Biosimilar fluids provide more in vivo-relevant drug diffusion and dissolution data compared to regular buffers. Biological fluids for studying drug dissolution, diffusion, and complexation can be obtained from cell culture[11], animal or clinical sampling[12], or can be artificially produced[13]. However, using cell culture and animals as sources of biosimilar fluid is limited by fluid quantity and ethical concerns. Sampling procedures, handling, and interspecies and interbatch variability can also affect the samples and the results obtained. To address these issues, artificially produced biofluids, including biological hydrogels such as mucus, offer a promising solution. Artificial mucus is categorized as a biosimilar fluid and hydrogel, with bulk viscosity and composition that mimic native mucus. Since it is artificially produced, standardized protocols can be implemented to minimize variability. Using artificial mucus in studies of drug dissolution, diffusion, and absorption makes screening more efficient.

In the drug delivery process, absorption, distribution, metabolism, and excretion (ADME) of the drug must be considered. This project focuses on absorption from and distribution within the colonic mucus region. For a drug to be absorbed from the colon, the compound must permeate the relatively thick mucus layer. Diffusion and dissolution are complex processes influenced by particle size, drug solubility, matrix viscosity, pH, temperature, and other physicochemical factors. The compound may both dissolve in and diffuse through the mucus. Drug diffusion may be more restricted in this viscous hydrogel compared to water. An often-overlooked role of colonic mucus is its function as a dissolution compartment due to its high-water content. Other components in the mucus may promote solubilization of lipophilic drugs (due to lipids present in the mucus) or limit diffusion through binding (mucin). Therefore, some of these factors are included in the study to produce physiologically relevant mucus.

Another key aspect of in vitro testing is the experimental validation of the models. There is no general consensus on methods for investigating mucus and particle interactions. Therefore, in the first half of the project, robust methods for this process are being developed. Challenges in determining diffusion and dissolution in mucus are mainly due to its inherent viscosity and the complex mixture of components. These properties limit experimental accuracy in conventional pharmaceutical tools such as the Transwell™ setup for permeability and the USP dissolution apparatus, which were designed for larger fluid volumes and lower viscosity. In this project, possible methods for determining complex interactions between mucus and model drug particles were investigated.

Colonic Diseases: The Challenges of Meeting Market Needs

Colonic diseases such as IBD are projected to affect 1% of the Western population by 2030[14], representing a double increase from 0.5% in 2010. Approximately 80% of patients will require hospitalization, with a lifelong, unpredictable relapsing-remitting course[14]. Ulcerative colitis (UC) and Crohn's disease (CD) are the two main types of colonic diseases that fall under the umbrella term inflammatory bowel disease (IBD). Patients' quality of life is affected by chronic inflammation, altered gut transit time, and inadequate public toilet facilities[15]. There is also a higher prevalence of anxiety and depression among patients compared to the healthy population, illustrating the connection within the bio-psycho-social model[15]. Another severe disease with high prevalence arising from IBD is colorectal cancer (CRC). CRC is the third leading cause of cancer death worldwide, with 1.9 million patients diagnosed in 2020[16]. Acute and chronic infections by pathogenic bacteria are also common colonic diseases[17]. Hirschsprung's disease is a neurological disorder of the colon, with a prevalence of 1 in 5,000 live births[18]. Developing drugs to meet these large market needs is important to sustain global public health.

Drug study challenges

In vitro–in vivo correlation (IVIVC) and interspecies variance

The predictability of in vitro testing needs to be enhanced to achieve results similar to the in vivo settings (Fig. 1). Therefore, physicochemical factors which influence drug absorption process from the in vivo setting should be incorporated into in vitro test platforms to improve similarity. To current date, preclinical animal models may help predict drug delivery processes in the human body, but there are limitations to this approach. The genetics of preclinical species differ significantly from humans[19], leading to different physiological structures. In addition, scaling factors based on volume or body mass may miss some mechanical transport aspects[19]. Furthermore, gene regulation and protein expression also differ, resulting in different drug metabolism and transport mechanisms. All these factors would cause study of cross-species pairs (CSP) to be challenging due to complex interaction parameters, which may lead to different conclusions depending on statistical methods and dataset selection[20].

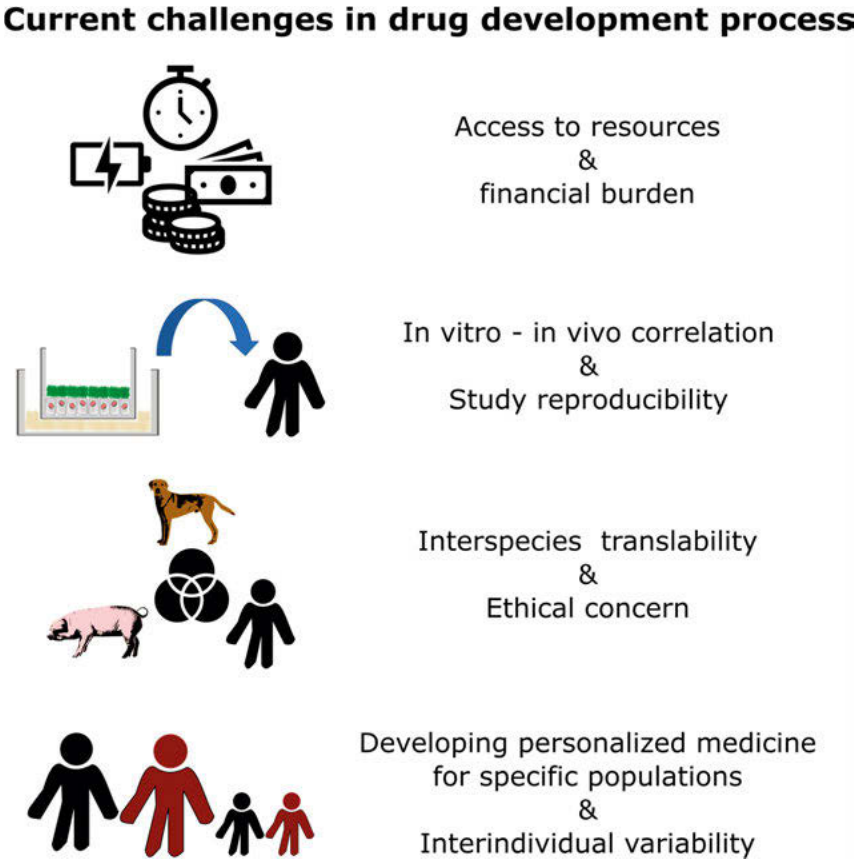

Figure 1. Drug development challenges are multifaceted and complex, ranging from lack of resources to strategies for developing personalized medicine.

Financial burden

Drug discovery and development is a long and arduous process, with an average timespan of 12 years from initial synthesis to regulatory approval[21]. In the drug development pipeline, the largest budget allocation is for the preclinical step[22], from discovery to in vitro testing. The average total cost per drug is estimated to be around 2.5 billion USD[22]. This challenge hinders the process of delivering drugs to patients, and currently there are unmet medical needs for many diseases[23].

Ethical perspectives

Most sampling procedures on animals and humans compromise their activities and quality of life. During colonoscopy, patients must fast and the colon must be cleaned[24]. For animal sampling, gastrointestinal isolation requires euthanized animals[25]. In accordance with the Replace, Reduce, and Refine (3R) principles of animal testing, improved in vitro experiments are important to reduce animal testing. Therefore, inventions that lessen this burden are necessary.

Technical difficulties in sampling

Due to the viscous and sticky nature of mucus, isolation from the colon is challenging. Specialized sampling techniques, such as cytology brushes, are needed to isolate mucus in the colon environment[26]. Sampling from resection materials may not be ideal due to exposure to preoperative antibiotics and operation-associated stress[26], which might influence mucus quality.

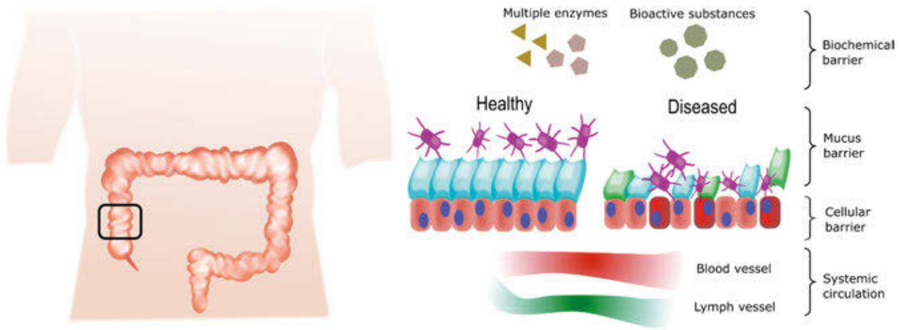

Figure 2. Colon environment with multilayer drug absorption barriers. Depending on healthy or diseased conditions, the environment may change.

Colon environment

The colon is one of the organs that is part of the gastrointestinal (GI) tract in animals (Fig. 2), which located at the terminal end of the GI tract, with the main function of reabsorbing water and forming feces with the help of colon motility and microbes metabolism[27]. Colonic environment is unique due to lower water availability compared to other parts of the GI tract, while it has the thickest mucus layer for protection of the epithelial cell layer and hosts the largest number of microbes in the body. Interaction with these microbes may pose drug delivery challenges but also offers opportunities. For example, Eudragit® is a controlled-release coating that relies on microbes to digest the coating, thus releasing the drug API. A thinner mucus layer that is sterile and free from microbes has also been observed[28]. Colonic fluid is also present in the colon, and the regional fluid volume decreases along the tract. The low amount of fluid present in the colon makes the drug dissolution process challenging. The presence of fluid in the colon (around 20 mL) is about 80% lower than the fluid in the small intestine (around 100 mL)[29]. Colonic mucosal pH has been shown to be around 7.1 to 7.5 in human patients[30,31]. Colon transit time is highly variable depending on the individual[32], making it harder to predict controlled-release strategies.

Mucus as a barrier for drug absorption

Nature has its own way to preserve life. One universal takeaway is that evolutionarily conserved features indicate high importance. Mucus exists in a wide array of organisms, appearing early in Cnidaria, which includes corals[33]. The backbone of mucus may differ depending on the organism[34], but functionally it serves as a barrier for protection against infection[33].

Mucus can be classified as a viscoelastic hydrogel, with mucin as the backbone protein (Fig. 3). The main component of mucus is water, amounting to around 90% of its volume. In addition to mucins and water, there are other components in mucus, such as lipids, proteins, cell debris, and extracellular DNA. These components, other than mucins, play a role in maintaining mucus barrier properties; for example, trefoil factors (TFF) co-regulate mucosal healing with mucin[35]. The mucus serves various functions depending on the tissue location and in colon it also houses microbes.

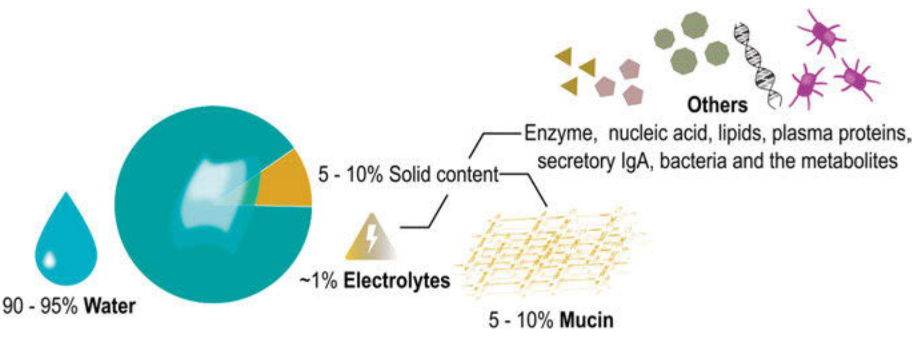

Figure 3. Composition of mucus based on Ichikawa & Ishihara work[36]. Here, the mucus is highlighted as a hydrogel due to water being the main component, with various materials present as solid content.

Mucus acts as a physicochemical barrier due to its high viscosity (Fig. 4). With the network formed by mucin and other biopolymers, it can limit diffusion based on pore sizes. Mucus can also act as an interaction barrier, providing electrostatic and hydrophobic-hydrophilic interactions due to glycosylation around mucin[37]. A matrix environment with multiple components affects the drug dissolution and diffusion process. Lipid and protein content influence the solubility of drugs[38], while viscosity changes diffusivity. Tanaka et al. has elucidated the binding of a small molecule (2,4-diaminopyrimidine) with mucin and mucus-associated biopolymers (DNA and alginate), indicating the influence of hydrophobicity and charge[39]. Physical barrier properties arise from the highly viscous environment of mucus. For example, H^+ ion diffusion across mucus is 4–10 times slower than in water, even though mucus is 85–90% water[40], indicating robust regulation of ion exchange and barrier properties.

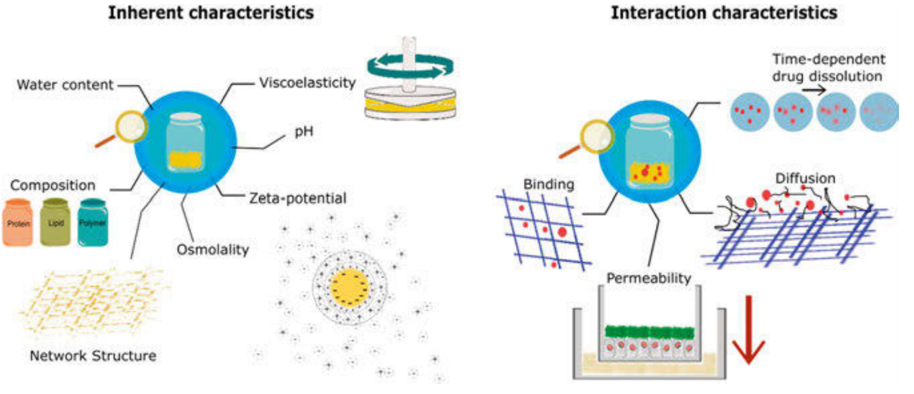

Figure 4. Characteristics of mucus can be defined by multiple parameters. In this study, we classify some physicochemical parameters as inherent characteristics of the mucus itself, and the interaction with compounds as interaction characteristics.

Drugs need to diffuse to reach their target. Diffusion may occur in the mucus environment and is influenced by various factors due to the interaction filtering property. One prominent factor is viscosity, as described by the Stokes-Einstein equation: the more viscous the hydrogel complex, the slower molecules will diffuse[41,42]. A multivariate study suggests that the anomalous exponent taken from curve fitting of the mean-squared displacement is the strongest predictor of effective diffusion[43]. Effective diffusion spans seven orders of magnitude, from 10^{-5} to 10^{2} $\mu m^2/s$, indicating a wide range of diffusivity depending on the particle–mucus interaction[43].

Mucus properties in healthy conditions vs. disease progression

In the respiratory tract, the color of mucus or phlegm can indicate health status[44]. In the gastrointestinal (GI) tract, due to its concealed location, this is more difficult to assess. Endoscopy or colonoscopy studies have reported differences in the mucus layer between healthy and inflamed conditions[45]. During inflammation, secretion of neutrophil extracellular traps (NETs), which are composed of extracellular DNA and histones, changes mucus composition and physicochemical properties[46]. In healthy conditions, mucus and microbes coregulate each other. This interaction is demonstrated by a thinner mucus layer and lower Muc2 gene expression in germ-free mice[47]. Furthermore, coregulation between mucus and microbes has been well studied, with bacterial secretion of short-chain fatty acids (SCFAs) elevating mucin type-2 (MUC2) protein production, which is dependent on fiber intake and mucin glycans that serve as nutrient sources for bacteria[47,48]. In pathological conditions, such as inflammatory bowel disease (IBD), infection occur more frequently[49].

In colorectal cancer (CRC), the mucus layer is thinner, with increased sialylation and decreased sulphation[48]. Significant differences were found between normal mucosa and cancerous tissue, with median thicknesses of 100.7 μm and 48.9 μm, respectively[50]. Mucus thickness in Crohn's disease (CD) patients is higher compared to healthy controls, while in ulcerative colitis (UC) it is relatively lower in the ascending, descending, and rectal regions[51]. Altered glycosylation of MUC2 in colitis may explain this phenomenon, resulting in faster degradation of glycans and mucin due to bacterial digestion[52]. Another study of rectal mucus reported a different trend, with CD patients having a thinner mucus layer (15.4 μm) compared to normal (31.5 μm). The severity of UC also affects mucus thickness, ranging from 27.6 μm in quiescent UC to 12.0 μm in severe UC[53]. In terms of viscoelastic properties, CD patients' ileal mucus has significantly higher viscoelastic moduli. Cryo-SEM also showed a tighter network in CD patients' ileal mucus[54]. Mucus barriers are also altered in Hirschsprung's disease (aganglionosis), highlighting abnormalities in the enteric nervous system[18,55]. All these changes in pathological conditions must be considered when designing mucus mimics as in vitro drug study platforms.

Interspecies mucus variability – challenges in translation

Each species has its own phenotypic and genotypic characteristics. These traits are closely linked to lifestyle, survival strategies, and ecological roles[56]. Such complex traits pose challenges for translating research findings from animals to human patients[19]. Gastrointestinal physiological features differ between species, even among commonly used preclinical animal models (murine, porcine, canine). Varum et al. study highlight interspecies variability in mucus thickness, showing differences among leporine, porcine, and murine models[57]. Mucus thickness also differs between mice and humans, which is intuitive given body size differences[58]. Although some pathways are associated with increased or decreased mucus production, it remains unknown whether these signaling pathways are species-specific[58]. However, there is no clear comparison of colonic mucus properties among canine, porcine, and human models.

Artificial mucus – a biosimilar medium mimicking native mucus

The concept of a biosimilar medium (Fig. 5) to predict in vivo dissolution has been explored for some time[10]. Simulated gastric, intestinal, and colonic fluids are available on the market, with compositions based on physiological characteristics[59]. Improved in vitro–in vivo correlation is the main goal of using these media. Then again, GI tract fluids have been widely studied[24,59,60]. Based on content characterization, artificial fluids for fasted and fed conditions have been developed. Due to controlled manufacturing, these assays are reproducible even in cross-laboratory settings for drug dissolution studies[61].

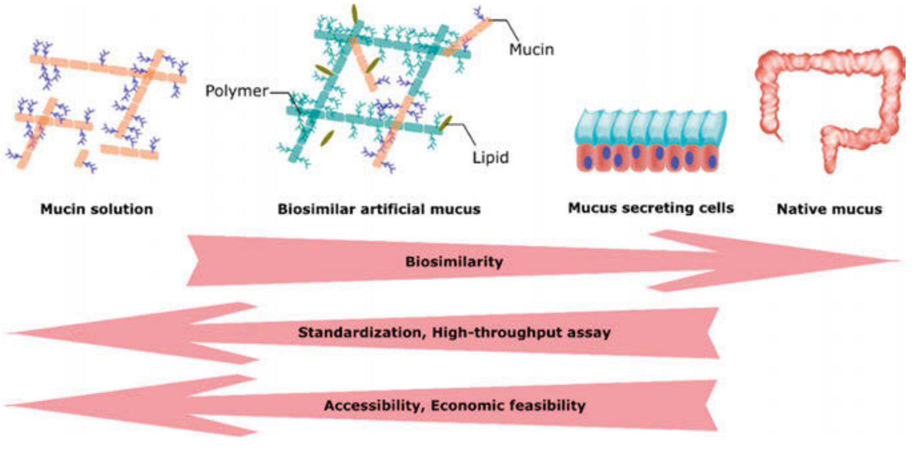

Figure 5. Mucus model for studying drug interaction. Different categories were sorted based on the level of biosimilarity to in vivo conditions, ability for standardization and high-throughput assays, and accessibility or economic feasibility.

The development of biosimilar mucus to mimic small intestinal mucus has also been studied[62–66]. Research by Boegh et al. reported cytotoxicity of native porcine intestinal mucus toward Caco-2 cells[62]. Therefore, designing biocompatible artificial mucus is necessary to ensure an appropriate permeability profile across the mucosal layer. With the FDA Modernization Act 2.0, alternatives to animal testing that provide predictive capability are continuously endorsed[67]. Thus, the development of artificial colonic mucus is a promising alternative that enables the study of drug absorption while mitigating challenges posed by usage of native colonic mucus.

Absorption process in mucus as a hydrogel

The drug absorption process is a crucial step in delivering drugs to their target. According to the current Biopharmaceutics Classification System (BCS), drugs are classified based on their solubility and permeability[68]. The Developability Classification System (DCS) further classifies BCS class II drugs by distinguishing between dissolution rate-limited drugs (class IIa) and solubility-limited drugs (class IIb). In both classification systems, most current studies focus on class II (low solubility and high permeability) and class IV (low solubility and low permeability) due to the challenges and opportunities to improve absorption through formulation. Current drug formulations use controlled release (CR), modified release (MR), and extended release (ER) strategies to target the colon[69]. These approaches allow for supersaturated drug dosage forms to improve drug dissolution and absorption.

Molecules in solution are transferred from one place to another depending on various factors. This process is called diffusion, which depends on several factors as described by the Stokes-Einstein equation (Eq. 1). Passive diffusion occurs as a mass transfer phenomenon from higher concentration to lower concentration.

$$D = \frac{kBT}{6\pi\eta r} \qquad \textbf{(Eq.1)}$$

Where D is the theoretical mass diffusion coefficient ($\mu m^2/s$), kB is the Boltzmann constant, T is the temperature (^oK), η is the fluid viscosity (Pa·s), and r is the hydrodynamic radius of the particles (nm).

Particle movement in a medium can be random or influenced by other factors. These movements are referred to as Brownian motion and non-Brownian motion[70]. In this thesis project, the colloidal medium is native colonic mucus or biosimilar mucus. As described in previous studies[71,72], mucus is a complex structure with pores that hold water pockets. Depending on their proximity to the mucus network, particles may interact with the mucus, thus influencing diffusion.

Diffusing particles moving in mucus yield 3D trajectories. These trajectories can be characterized by mean squared displacement (MSD)[43], which quantifies particle movement over a defined time period. From displacement over time, the dominant mode of particle movement can be classified. Common practice is to classify movement based on the slope of curve fitting (α) or the anomalous diffusion coefficient. Based on this analysis, particles can be classified as immobile, subdiffusive, diffusive, or superdiffusive (active transport).

Mucus, as a complex network environment, has its own physicochemical properties. Depending on the charge of the mucus and particles, transient or prolonged binding may occur as a result of electrostatic interactions. This binding phenomenon can be utilized as a drug delivery strategy, specifically in the design of mucoadhesive drug carriers. Entrapment of particles may also occur due to size filtering[73]. Due to diffusion and binding interactions in the mucus environment, particle permeability through the mucus is affected.

Connecting in vitro experiments and computational analysis

The study of drug administration, distribution, metabolism, and excretion (ADME) in the human body often faces challenges such as patient variability and the need for non-invasive sampling. Therefore, the development of a "digital twin" system containing physiological information could help predict drug ADME (Fig. 6). Moreover, the screening of drug kinetics also aids in designing better drug delivery strategies[74].

To fully utilize data from in vitro experiments, computational models can simulate in vivo data. Physiologically based pharmacokinetics (PBPK) is one approach that enables incorporation of complex parameters, ranging from compound formulation and gut physiology to pharmacokinetic parameters. Among various PBPK models, there are compartmental and non-compartmental models. These models differ in parameter complexity, and both can be used to validate each other[75].

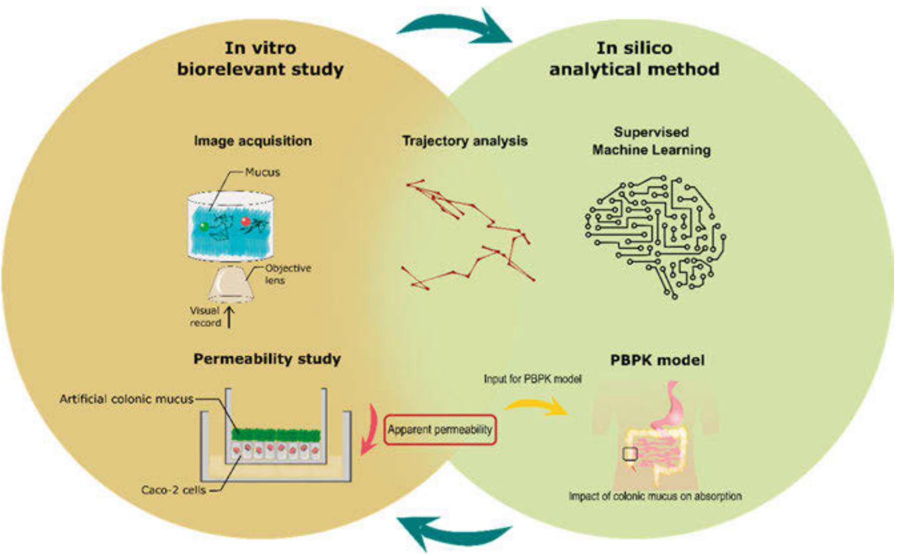

Figure 6. In vitro–in silico interdependency provides input for one another. Example of application in the projects is illustrated.

There is no specific PBPK software endorsed by the FDA; however, applicants must report details of the software version, model structure, and parameter value sources according to the Physiologically Based Pharmacokinetic

Analyses - Guidance for Industry by the FDA (August 2018)[76]. In the context of GI tract modeling and colon drug delivery, the current model with GastroPlus™ Advanced Compartmental and Transit (ACAT) enables modeling of drug delivery formulations. Recent studies have developed models with parameters that enable simulation mimicking clinical pharmacokinetic studies[77,78]. Therefore, input from improved permeability experiments can be used to simulate dosing in pharmacokinetic models.

Importance of connecting in vitro data with in silico analysis is also demonstrated by observation of particle diffusion in mucus yields complex data with multiple parameters. Regression-based analysis of curve fitting may introduce error due to selection of the timeframe[79]. Therefore, the application of machine learning algorithms to classify multiple particle tracking data based on feature analysis is a promising approach[80].

Aims of the thesis

The overall aim of this thesis was to develop an in vitro platform that enables investigations of the role of colonic mucus in drug absorption, with a focus on drug diffusion, binding, and permeability (Fig. 7).

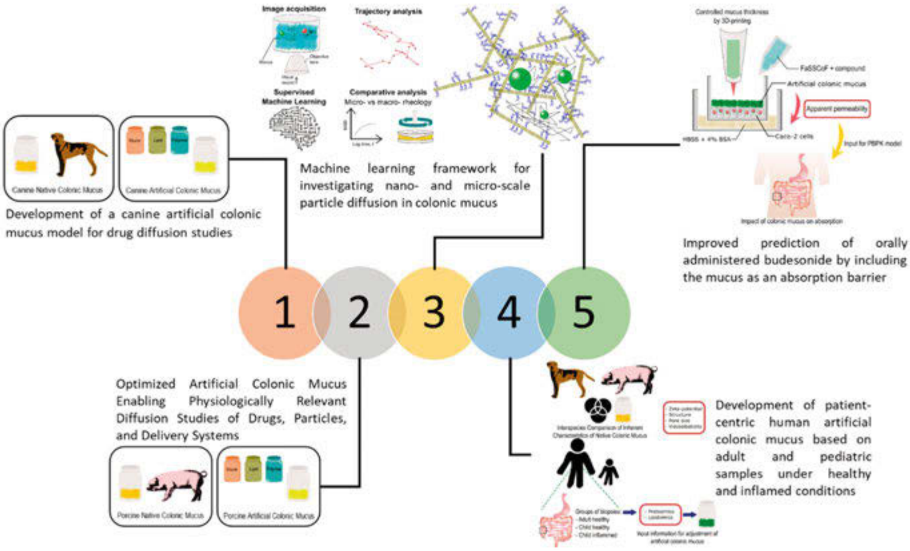

Figure 7. Project summaries with illustrations of graphical abstracts.

The specific aims of the thesis based on the projects were:

- To develop canine artificial colonic mucus (CACM) that captures the properties of native canine colonic mucus as a barrier to drug diffusion (**Paper I**).
- To improve the similarity of a previously developed porcine artificial colonic mucus (PACM) to native porcine colonic mucus for drug diffusion studies by polymer substitution (**Paper II**).
- To identify the artificial mucus model that best replicates the key diffusion behaviors observed in the native system, providing insights into the development of improved mucus-mimicking materials for drug delivery applications (**Paper III**).
- To study human colonic mucus properties and make interspecies comparisons with animal models (dog and pig for translational purposes (**Paper IV**).
- To design an improved PBPK model of colon absorption, taking drug diffusion and dissolution in colonic mucus into consideration (**Paper V**).

Methods

Isolation of mucus & Ethical statement

Depending on the sample type, the mucus collection strategy also differs. Tissue samples allow for direct scooping of mucus with a spatula, as described in **Papers I & II** for canine and porcine colon tissue. However, for patient biopsies (**Paper IV**), the tissue size is too small to isolate mucus with a spatula. Therefore, resuspension of the mucus with buffer is necessary for isolation. Research investigations by Dubbelboer et al. (2022) and Barmpatsalou et al. (2022) have detailed the process of mucus collection[12,25] for canine native colonic mucus (CNCM) and porcine native colonic mucus (PNCM) respectively.

For **human native colonic mucus (HNCM)** sampling, samples from the colon of adults under 40 years and children under 18 years were taken from patients undergoing investigation for suspected IBD at participating units. The patient group represents a large proportion of newly diagnosed patients who have not yet received treatment and usually do not have other underlying diseases. Ethics approval for this study was granted by the Swedish Ethical Review Authority (codes 2022-01570-01 and 2025-03918-02) under the project title "Studier av inflammatoriska mag-tarmsjukdomar hos barn, ungdomar och unga vuxna; Kartläggning av mekanismer kring uppkomst, diagnostik, sjukdomsförlopp och effekt av behandling, för vidareutveckling av metoder för diagnostik, uppföljning och behandling," or "Studies of inflammatory gastrointestinal diseases in children, adolescents and young adults; Mapping of mechanisms surrounding the onset, diagnosis, disease course and effect of treatment, for further development of methods for diagnosis, follow-up and treatment." Examinations are routinely performed under general anesthesia in children and adolescents. The physician examines the gastrointestinal mucosa and routinely takes tissue biopsies from 16–18 sites for clinical microscopic assessment by a pathologist. For patients who agreed to participate, 2–4 additional tissue samples from the intestinal mucosa were taken for the study during this routine examination. Each biopsy is approximately 1 mm^3 in size, compared to tissue from porcine (4 – 6 m)[81] and canine (around 0.6 m)[82]. Sampling extended the examination and possible anesthesia by approximately 10 minutes. Samples were coded and stored in biobank freezers (−80°C).

Formulation of artificial colonic mucus

Formulations of the PACMs (**Paper II**) were adapted from published work[12], with modifications to the polymer type and concentration. Polyacrylic acid (PAA), the polymer used in the previously established PACM, was used for comparison.

Polymer candidates were selected through a literature review, considering their proposed use in artificial mucus models and their likelihood of providing (i) a gel-like structure, (ii) a net negative charge at colonic pH (pH 7.3–7.5[12]), and (iii) a mesh microstructure (porous due to crosslinks of polymer). Polymers selected in this study were chosen to enable production of artificial mucus in a standard laboratory, hence, commercially available polymers were focused on. Based on these criteria, five polymers were selected (**Paper II**). PAA and hydroxyethyl cellulose (HEC) have been proposed as suitable polymers for artificial mucus, to mimic the mucus barrier of the porcine intestine[13] and horse respiratory tract[64], respectively. Sodium hyaluronate (SH) and a porcine stomach mucin type III mixture have also been proposed, where the high molecular weight SH modulates mucin nanostructure and mesh size[83]. Sodium alginate (SA) has been used to hinder diffusion in small intestinal mucus by providing barrier properties to mimic the mucus[84]. Pectin mixed with mucin has been shown to enhance the mucin network through electrostatic interactions, resulting in increased viscosity[37].

An initial screening of the required polymer amount was performed through visual inspection, by serially adding the polymer (1% to 5% w/v) until a gel-like structure similar to the PACM PAA model was obtained. The appropriate amount was confirmed by viscosity sweep and storage modulus measurement, with targets to mimic PNCM values (**Paper II**). In a second step, the polymer (HEC) that produced a zeta potential (for measurement settings, see below) similar to PNCM was selected and further optimized. At this stage, the buffer was changed to achieve a PACM pH similar to that of the PNCM. MES in the mucus buffer was replaced with N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), and the HEC zeta potential was compared to the control (PACM PAA in BES). In a third step, the concentration of HEC was optimized to produce interactions between the drug molecules and the mucus mesh similar to those observed in PNCM.

Mucus physicochemical inherent characterization

Bulk rheology measurement

An ARES-G2 strain-controlled rheometer (TA Instruments, Delaware, USA) with Advanced Peltier System (APS) accessory was used for rheology measurements. The equipment had a A 25-mm diameter stainless steel upper geometry (0.0174 rad cone angle and 0.021 truncation gap) and a 60-mm diameter lower geometry with hardened chromium surface and quick-change system. Visual observation after each measurement confirmed samples were properly loaded in between geometries. The cone and plate geometry were selected to provide more grip on the sample and reduce the sample size needed. Mucus sample rheology was profiled at 37°C in a solvent trap to prevent evaporation. The apparent viscosity (η) of mucus samples was continuously measured by ramping the shear rate from 0.1 to 500 s^{-1}. The linear viscoelastic region (LVR) was identified in an amplitude sweep from oscillation strain 0.01 to 100.0%, at 1 Hz frequency. Finally, the storage (G') and loss modulus (G'') were measured in a frequency sweep from angular frequency 0.63 to 60 rad/s at 0.5% oscillation strain. The settings were within the LVR to prevent structural damage, and 0.5% was chosen to accommodate for both native and artificial mucus without getting to the lower limit of detection. The rheological profiles of the artificial mucus samples were compared to the native ones established by Barmpatsalou et al.[12].

Structural analysis with Cryo-SEM

Native mucus samples were kept on ice without freezing (to prevent ice crystal formation) during transport to the microscopy facility, then submerged in liquid nitrogen (-200ºC) with a Leica EM ICE high pressure freezer (Leica, Wetzlar, Germany) at 325 milliseconds and 2200 bar. Samples were placed in 200-micron deep gold-plated planchets with flat planchet tops. Prior to imaging, the flat top planchet was removed. PACM samples were prepared one day before high-pressure freezing and handled in the same manner as above.

Samples with planchets were stored in liquid nitrogen until imaged. At the beginning of the imaging session, samples were sublimated inside the Aquilos 2 cryo-FIB-SEM for 17 minutes at -110 ºC and 5 x 10^{-7} mbar. Following sublimation, the Aquilos 2 was cooled to -190 ºC and the samples were sputter-coated with platinum to prevent charging. Scanning electron microscopy images were obtained using an Aquilos 2 cryo-FIB-SEM (Thermo Fisher Scientific). Images were collected using an Everhart-Thornley secondary electron detector with 3 kV, 13 pA, and 1 μs dwell time. These settings were chosen to reduce sample damage.

Images were analyzed with FIJI software using the image binarization process. This process involves pixel thresholding (0 to 75 fixed values, the same for all images) to distinguish pores and mesh. Scale of measurements were set based on a micrograph scalebar. The "analyze particles option" was chosen, and size range was set at minimum 0.1nm (to eliminate noise) to infinity. Default circularity was chosen (between 0-1). Feret maximum and minimum diameters were obtained for comparison with the theoretical mesh size obtained from the storage modulus. The Feret measurement was also performed manually, to compare with the binarization.

Surface charge

One of the factors influencing diffusion and binding interactions is surface charge. To determine the surface charge of a porous material (Fig. 8), the current standard is electrokinetic measurement by streaming potential current[85]. This method has been used in various porous films. Mucus is a hydrogel with pores; therefore, this technique was studied by using the SurPASS electrokinetic instrument (Anton Paar GmbH). Unfortunately, the measurement was unsuccessful because the mucus between the clamps was carried away by the streaming electrolytes. Another idea, using atomic force microscopy (AFM), was also explored[86]; however, the probe explored was not compatible with studying mucus topology due to binding. Considering that mucus is a complex hydrogel with both hydrophobic and hydrophilic components, this issue requires further investigation. Since other approaches are not feasible for characterizing mucus surface charge, DLS was used as described in **Papers I–IV**.

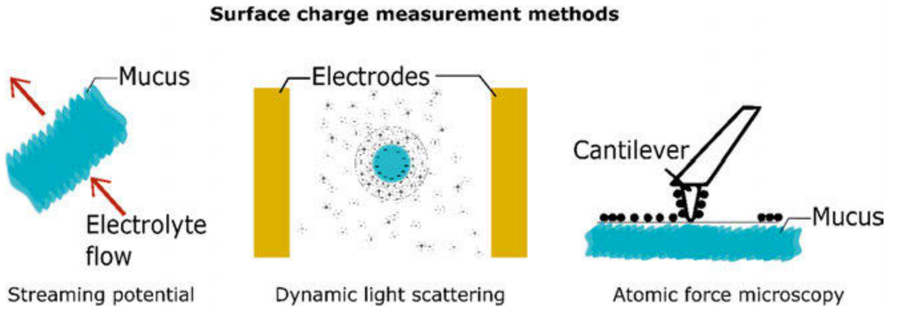

Figure 8. Various surface charge measurement methods explored in this project.

The dynamic light scattering (DLS) technique for measuring hydrogel size has been reported by Wang & Sande[87], with additional studies investigating the measurement of hydrogel zeta potential in buffer suspension[88]. Zeta potential was measured at 37°C with Litesizer 500 (Anton Paar) using an Omega cuvette with Smoluchowski approximation. Before each of the measurements, the Omega cuvette was tested with the Applied Microspheres (Anton Paar) as a control. Samples were prepared as a dispersion of mucus 0.05%-0.07% (w/v) in MilliQ-water. Each sample was measured five times following the assay guidelines developed in collaboration with Anton Paar, and three replicates were performed.

pH, osmolality, density, and water content

The pH, osmolality, and water content of the material may influence hydrogel structure. pH measurements were performed within 1 h after sample collection, using a micro-electrode (Orion Sure-Flow, Thermo Fisher Scientific).

Osmolality of the artificial colonic mucus was measured using the Fiske 210 Micro-Osmometer (Advanced Instruments, Norwood, MA, USA). This single-sample, freezing-point depression micro-osmometer uses a sample size of approximately 100 µg. Experiments were performed in triplicates. To study density of mucus, fluid density of mucus was measured with a pycnometer. For the pycnometer (BRAND™ BLAUBRAND®) measurement, the container and sample mass were weighed on a Sartorius ENTRIS 224i-1s electronic micro-balance. The density was calculated based on volume of the provided calibrated vessel (5.316 cm^3) at 20 ºC. For water content analysis, approximately 200 mg of porcine native and artificial colonic mucus (PNCM and PACM) each were weighted on tared vials, sealed with parafilm perforated with a few holes, and frozen at -20°C overnight. Freeze drying was carried out for 48h using a Flexi-Dry MP, FTS systems from CiAB, Sweden at -80°C. Water content was calculated from the weight difference.

Proteomics

Mucus suspension were generated by vortex mixing and resuspending of colonic biopsies with 300µL of KREBS buffer (without glucose). RIPA buffer were used to digest the mucus suspension together with vertical tip sonication for one minute. Then filter aided sample preparation (FASP) was implemented. In brief, ultrafiltration membrane was used for retaining proteome content, while smaller contaminant molecules and interfering buffers were removed. By utilizing the membrane as solid support, further steps of reduction, alkylation, and digestion of proteins were performed with ultrasound energy to speed up the process [89]. Followed this step, 200ng of peptides were utilized in 45 minutes run of LC–MS/MS Analysis.

LC-MS/MS analysis are performed using a High Resolution Trapped Ion Mobility timsTOF HT mass spectrometer coupled to a nanoElute 2 LC system via a CaptiveSpray ionization source (Bruker Daltonics). 200 ng of peptide mixture sample reconstituted in 0.1 % FA will be loaded of reverse-phase C18 HPLC column (PepSep XTREME with dimensions of 25 cm x 150 µm x 1.5 µm, Bruker Daltonics) and peptides will be separated in 45 min with a gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as follows: from 2% B to 17% B over 25 minutes; from 25 % B to 37% B over 35minutes; from 37 % B to 95% B over 45 minutes a flow rate of 400 nl/min and a column temperature of 50°C.

All data were acquired under the dia-PASEF mode with a MS1 scan range of 100-1700 m/z, and the collision energy was linearly interpolated between 1/K0 values, from 20 eV at 0.6 Vs/cm2 to 59 eV at 1.6 Vs/cm2, keeping constant above or below.

Peptide and protein identification were performed using DIA-NN version 2.0.2. for academic use[90]. Default setting were applied with exception of MS accuracies set to 15. Protein group summary which generated at 0.01q values was used for further data analysis. Data overview was performed using Amica software[91], whereas Venn diagram tool and Panther GO database [92] were applied for functional annotation of the dataset. Limma differential analysis package were used to analyse the data of gene expression [93].

Lipidomics

Lipid profiling of the mucus suspension were performed with UHPLC/QTOF-MS. Standard procedure of chloroform:methanol (2:1) extraction were performed[94], followed by UHPLC separation of lipids based on polarity / fatty acids lengths using reverse phase column (C18). Internal standards were added to all samples before extraction process and were included for normalization and evaluation purposes. Chromatography separation protocol[95] was followed, with gradient elution optimized for plasma lipid extracts start (53:13:34) (Acetonitrile:isopropanol:water) and final (11:89) (Acetonitrile:isopropanol). All mobile phases included 10mM NH4COOH & 0.1% HCOOH. An Acquity UPLC CSH 2.1x50 1.7um C18 column from Waters and an instrument QTOF 6546 instrument ("ODIN") from Agilent were used. Electrospray ionization (ESI) MS scan 70-1700 m/z were used, collecting 4 scan/s in positive mode. Data processing was done by quality assurance of the analysis (internal standards) and targeted data analysis using in-house databases of Swedish Metabolomics Centre, Umeå.

Mucus physicochemical interaction characterization

Diffusion of particles in mucus

To investigate the diffusion coefficient of particles in mucus, several methods can be used. In **Papers I–II**, the fluorescence recovery after photobleaching (FRAP) technique was used to determine diffusivity. Also time-gated Raman spectroscopy[96] to fingerprint the diffusion coefficient was explored, with further optimization currently taking place to tune the laser pulse to reduce background signal.

Fluorescence Recovery After Photobleaching (FRAP)

Fluorescently labelled model drugs were used to study diffusion in mucus. The fluorescent intensity of a hydrogel system depends on the fluorophore attached to the drug model. Depending on the excitation and emission of the fluorophore, along with the extinction coefficient, recovery after intentional bleaching using a confocal microscope's laser can be measured. A control region should be selected to monitor nonspecific or off-target bleaching. Following bleaching after laser exposure, diffusion occurs during the recovery process. By correlating fluorescence intensity, the diffusion rate can be calculated.

Fluorescence recovery after photobleaching (FRAP) experiment was carried out as described in **Paper I & II**. Mucus samples (300 µg) were prepared with 3.75µL of 10mg/mL FITC-dextran stocks to achieve a final concentration of 0.125mg/mL in the mucus. The FITC-dextran loaded-mucus samples were put onto a glass slide with stacked cover slips set up to ensure sample thickness of 100 µm. Experiments were conducted in a 37°C temperature chamber. Images were recorded with a confocal microscope Zeiss CLSM 780 NLO and data collected using ZEN Black software (Carl Zeiss GmbH, Jena, Germany). At the beginning of experiments, samples were photobleached with maximum laser power for 3 seconds. Bleaching areas and control areas with circular shapes (20 µm diameter) were made inside the rectangle-shaped frame to increase the speed of data acquisition.

Diffusivity values were calculated with FRAPanalyzer[97] using double normalization settings:

$$I_{norm}(t) = \frac{I_{ref\_pre}}{I_{ref}(t) - I_{back}(t)} . \frac{I_{frap}(t) - I_{back}(t)}{I_{frap\_pre}} \qquad \textbf{(Eq.2)}$$

where,

I (*t*) *norm* - normalized intensity;

I (*t*) *frap* - measured average intensity inside the bleached spot;

I (*t*) *ref* - measured average reference or whole studied compartment (cell, nucleus, etc.) intensity;

I (*t*) *back* - measured average background intensity outside the cell;

Subscript *pre* means the averaging of intensity in the corresponding region of interest (ROI) before bleach moment after subtraction of background intensity;

and a diffusion model for the circular spot, based on modified Bessel functions:

$$FRAP(t) = a_0 + a_1 . e^{-\frac{\tau}{2(t - t\ bleach)}} . \left(I_0 \left(\frac{\tau}{2(t - t\ bleach)} \right) + I_1 \left(\frac{\tau}{2(t - t\ bleach)} \right) \right) \qquad \textbf{(Eq.3)}$$

$$\tau = \frac{w^2}{D} \qquad \textbf{(Eq.4)}$$

where τ is the diffusion time (s), *w* is the radius of bleach spot (µm) and *D* is the diffusion coefficient (µm²/s). Two normalizing coefficients (a_0, a_1) are introduced to account for the non-zero intensity at bleach moment and incomplete recovery.

Particle tracking

In recent years, nanoparticles have emerged as a promising drug delivery strategy. To evaluate nanoparticle diffusion in mucus, particle tracking methods have been developed[98,99]. Diffusion of fluorescently labeled polystyrene nanoparticles[100], as a water-insoluble compound, provides predictive information about penetrability[101].

During the particle diffusion process, several types of particle movement can occur. Although there are multiple opinions regarding classification, movement is generally categorized as dominantly immobile, subdiffusive, diffusive, or superdiffusive (active transport)[102,103]. The mean square displacement (MSD) of the trajectories is analyzed, and the diffusion coefficient can be inferred from the MSD. Diffusion of particles in soft matter, such as mucus, is highly dependent on the mucus environment[104]. Therefore, particle tracking experiments can also be used to probe matrix rheology, which, depending on the probe's surface area, can be classified as nano- or microrheology[104,105].

Nanoparticles movement were investigated using a custom-built spinning disk confocal microscope based on an Eclipse Ti2 body (Nikon) using a 100×/1.42 NA Plan Apo Lambda objective (Nikon). Latex beads were studied in triplicates for each setting: polystyrene particles with sizes of 0.1, 0.2, 0.5, and 1 μm (carboxylate modified and hence, negatively charged) and 0.1, 0.2, 1 μm (amine modified and hence, positively charged) were studied in PACM PAA, PACM HEC, and PNCM. The suspended latex beads were sonicated following the manufacturer's instructions and thereafter 0.3 μL of the particle suspension was mixed with 40 mg of mucus. Dilution volume was minimal (less than 1% of the mucus) to avoid influencing mucus viscosity. Each time lapse video would be at least 100s in length with images acquired at 1 Hz in triplicate. Samples were incubated in a humidified chamber at 37ºC. To avoid thermal drift samples in 18 well μ-Slides (Ibidi, Gräfelfing, Germany), samples were equilibrated for approximately 45 minutes before recordings were taken[106].

Time lapse movies were analysed with the FIJI[107] plugin MosaicSuite[108] with particle tracker[108]. For each type of particles, radius settings (in pixel units) were optimized to ensure particle detection. The cut-off and percentile were also set for determining stringency and signal intensity detection, respectively. Following the particle tracking process, images were obtained and trajectory files saved, which were then analysed with MPTHub[102]. Trajectories of at least 100 particles were analysed for each group of experiments. Following the image acquisition, following input were used: lag time (Δt) 1000 ms, width of the full frame 182.83 μm, and a pixel size of 0.114 μm.

Mean squared displacement (MSD) values $\langle \Delta r^2(\tau) \rangle$ were determined by

$$\langle \Delta r^2(\tau) \rangle = [x(t+\tau) - x(t)]^2 + [y(t+\tau) - y(t)]^2 \qquad \textbf{(Eq.5)}$$

where x and y denote coordinates between consecutive τ intervals[102].

Derivation of particle diffusivity from the calculated MSD value was obtained through

$$D_{eff} = \frac{\langle \Delta r^2(\tau) \rangle}{2n\tau} \qquad \textbf{(Eq.6)}$$

$$D_w = \frac{kBT}{6\pi\eta r} \qquad \textbf{(Eq.7)}$$

$$\langle \Delta r^2(\tau) \rangle = 4D_0\tau^{\alpha} \qquad \textbf{(Eq.8)}$$

where n refers to dimensionality; D_w is the theoretical diffusion coefficient of spherical particles in water (µm^2/s), kB is the Boltzmann's constant (1.38×10^{-23} m^2 kg s^{-2} K^{-1}), T the temperature (°K), η the fluid viscosity (Pa.s), and r the hydrodynamic radius of the particles (nm). D_0 is the time-independent diffusion coefficient[102].

Analysis times (AT) were determined to be 100s and 10s (10% of the whole video, as suggested by Plaza-Oliver et al. to reduce error[109]). Frame filters (FF) were used to determine particle availability in a given number of frames to qualify for analysis. Here 1FF, 10FF, 50FF, and 100FF were selected meaning only particles that were detected in 1, 10, 50, and 100 frames, respectively, were included in analysis.

There is no general consensus regarding data analysis and filtering parameters to obtain a good fit of anomalous coefficient from an mean squared displacement (MSD) log slope[102,109]. Some studies report that increasing the time interval increases the error rate[79]. For determining the anomalous coefficient (α), 10% of the total timeframe were chosen, corresponding to the MSD log slope (see Fig. S1, **Paper II**). To classify α into ratio of transport mode, suggestion by previous study[102] by Gabriel et al. were referred:immobile ($0.0 < \alpha < 0.199$), subdiffusive ($0.2 < \alpha < 0.899$), diffusive ($0.9 < \alpha < 1.199$), and active transport ($1.2 < \alpha < \infty$). To reconfirm the trajectories analysis, machine-learning based classifiers were applied (**Paper III**, or see section 'Computational method & Data analysis').

Mucus binding assay

During diffusion, there is a possibility of interaction between drug particles and mucus. Considering the properties of mucus, which is negatively charged, binding between cationic particles and anionic mucus can be observed. Several assays have potential to measure this phenomenon (Fig. 9).

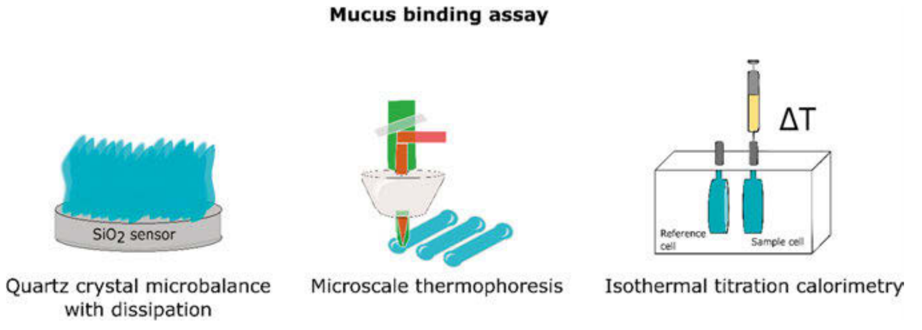

Figure 9. Various mucus binding assays explored in this project.

To study drug binding, isothermal titration calorimetry (ITC, Malvern Panalytical®) was used, which is a titration-based method with a thermal sensor. Depending on the caloric change, one can determine whether there is an exothermic or endothermic reaction between two compounds[110]. An endothermic reaction would suggest a binding interaction. Preliminary studies indicated that some interaction was detected. However, the system also produced a high background, reducing the signal-to-noise ratio, and if this method is to be used, significant method development will be required to increase reliability.

Another approach explored was the quartz crystal microbalance with dissipation (QCM-D, Biolin Scientific®), a nanogravimetric mass balance system. By pumping mucus suspension onto a silica-based disc, binding interactions can be detected based on frequency shifts. This frequency shift can be extrapolated to a mass balance, providing the amount of mass either being deposited to or being transported away from the system. Unfortunately, preliminary experiments showed that extensive optimization would also be necessary here to achieve stable binding of mucus as the baseline (Fig. 10).

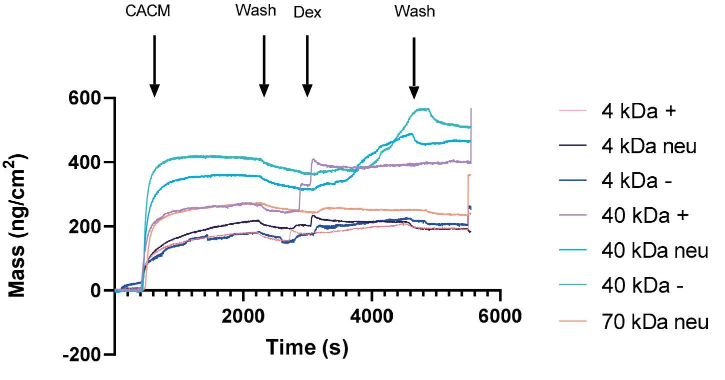

Figure 10. Mass of mucus (Canine artificial colonic mucus; CACM) suspension attached to a silica-based disc coupled with various sizes (4, 40, and 70 kDa) and charges (cationic (+), neutral (neu), and anionic (-)) of dextran (dex), measured using the nano-gravimetry technique by quartz crystal microbalance with dissipation.

Microscale thermophoresis (MST)

The interaction between FITC-dextrans and three mucus samples (PACM PAA, PACM HEC, and PNCM) was studied using a microscale thermophoresis (MST) fluorescent detector (Monolith NT.Automated) with capillary chips from NanoTemper Technologies GmbH (Germany). A stabile temperature of 25 °C was maintained during recording of the fluorescence levels of FITC-dextran and mucus samples at designated capillary positions. FITC-DEAE-dextran were used as ligand, after being dissolved in 10 mM MES buffer pH 6.5 at concentration of 2 μM based on a pretest. A pretest was performed to determine the detectable concentrations of FITC-dextran, using a Nano-Blue detector with λ_{ex} 495nm and λ_{em} 519nm (the recommended settings for FITC). Maximum concentration for each sample was determined through preliminary testing. Mucus samples were dispersed in 10 mM MES buffer as stock solutions (4; 25; and 25 mg/ml, respectively). The targets were prepared by serial dilutions of the mucus samples stock solutions with 10 mM MES buffer containing 0.3% Tween-20 (resulting final concentrations ranging from 0.0024 to 5 mg/ml) to prevent adsorption of the samples to the capillary chips. Mixtures of ligands and targets were inserted into the chips, followed by readings with 10% excitation power and low MST power for 3s pre-MST, 10s during MST, and 1s post-MST. All buffers and samples were degassed by sonication beforehand to avoid air bubble formation and experiments performed in triplicate.

The normalized fluorescence was plotted as a function of concentration of the target to determine the dissociation constant (K_d). The K_d indicates the equilibrium of the ligand-target complex and describes the concentration-dependent binding interaction. Reports and analysis of K_d-fit were generated using MO.Affinity Analysis (NanoTemper)) software.

Mucus permeability assay

To reach the systemic circulation, drugs must permeate the mucosal layer. One component of the mucosal layer is mucus, which acts as a barrier. In pharmacokinetics, in vitro permeability is defined as apparent permeability (P_{app}), which translates to effective permeability (P_{eff}) in vivo[68].

The current approach for in vitro permeability studies uses cell monolayers such as Caco-2 cells. However, as demonstrated in **Paper V** and a study by Butnarasu et al.[111], the lack of mucus may lead to underestimation or overestimation of compound permeability. In **Paper I**, demonstration of how the mucus components would influence permeability were investigated. The permeability studies were carried out in 6-well Costar® Snapwell™ plates. The Costar® inserts, containing 12-mm diameter and 0.4-μm pores polycarbonate membrane with a support ring, were selected.

Prior to applying the mucus layer, the apical side of the Snapwell™-cultured cells was gently washed with pre-warmed (37°C) Hank's balanced salt solution (HBSS; pH 7.4) and allowed to equilibrate for 15 minutes. Following this, the transepithelial electrical resistance (TEER) was measured using the Millicell® ERS-2 system (Merck, Sweden) to assess monolayer integrity before mucus application. The plates were then transferred to 3D-print the mucus (see section 2.3). The 2-ml receiver solution, HBSS with 4% BSA, was added to the lower compartment after the printing process. The plates were equilibrated at 300 rpm and 37°C for additional 10 minutes. An 11-mm diameter nylon filter (11µm mesh size, Millipore®) was gently placed on top of the mucus layer to keep the layer remain intact during the entire permeability experiment. The 300-µl donor solution, which contained budesonide, enalaprilat, and lucifer yellow in fasted state simulated colonic fluid (FaSSCoF, pH 7.8) at 116.5µM, 5µM, and 50µM was slowly added over the nylon filter and mucus layer. The plates were transferred to a plate shaker and shaken at 500 rpm for 3 hours in an incubator kept at 37°C. Samples of 400 µl receiver solution were collected every 30 minutes, and the same amount of preheated (37°C) receiver solution was immediately added into the lower compartment to maintain the volume. The donor solution and the applied mucus were collected at the last time point.

Apparent permeability was calculated by using the Equation 9):

$$Papp = \frac{dQ}{dt} \times \frac{1}{A \times C_0} \qquad \textbf{(Eq.9)}$$

Where dQ / dt is the transport rate across the membrane (µg/s), A is the surface area (113.1 mm^2), and C_0 (µM) is the initial concentration of compound in the donor solution.

3D printing

To deposit mucus, a viscous material, a technique that allows precise control is needed. There are several types and variations of 3D printing, and extrusion-based 3D printing is often chosen for bioprinting because it is suitable for hydrogels[112]. A study by Li et al. shown that hydroxyethyl cellulose, the polymer used in the ACM formulation, has been shown to be beneficial as a formulation additive, enabling tuning of extrusion printability and scaffold properties by altering viscoelastic properties[113]. Mucus thickness is a key factor influencing permeability. The current experimental approach using Transwell/Snapwell™ studies poses challenges in maintaining consistent mucus thickness. Therefore, in **Paper V**, method using 3D printing (BIO X, Cellink®) was assessed to better control mucus thickness, which in turn affects permeability.

To control the thickness of mucus on Snapwell™ setup, a gel extrusion-based printer method was used. The artificial mucus was printed on top of the inserts by using BIO X 3D printer, (CELLLINK, Sweden). The 3D-printer template was designed by using TinkerCad web-based software (Autodesk, USA) and two cylindrical shapes of 11.5-mm diameter (corresponding to the diameter of cell culture inserts) with a height of 0.2 or 0.8-mm height were created. The printer used a pneumatic print head with rectilinear infill pattern. The print head was manually calibrated. The 3D-printing parameters—print speed, pressure, nozzle type and infill density— were fine-tuned for each thickness (Table 1). Percentage of infill density would correspond to the material deposition and time needed for printing [114], therefore in this study, optimization of it was performed to observe mucus mass and thickness on Snapwell™. Following the printing process, weight of the added mucus layer was recorded. The mass was used together with the measured density (Table S2 in **Paper V**) and cylinder geometry (Eq. 10 and Eq. 11), to calculate the volume and thereby the thickness of the printed mucus layer. The calculated thickness was confirmed with a digital microscope to measure the thickness. In **Paper I**, mucus was applied on Snapwell™ by direct addition. Comparison with previous setup of 200 mg direct addition of mucus was done[115] to check for thickness and evaluate the importance of 3D-printing step.

$$\rho = m/V \qquad \textbf{(Eq.10)}$$

with volume of cylinder is:

$$V = \pi r^2 h \qquad \textbf{(Eq.11)}$$

Where ρ is density (g/cm^3), m is mass (g), V is volume (cm^3), *r* is radius of cylinder structure (6 mm), *h* is the height (mm) or in this case the mucus thickness.

Table 1. Parameters of mucus 3D-printing using BIO X.

	Parameters			
Target thickness (mm)	Tips (gauge/mm)	Pressure (kPa)	Speed (mm/s)	Infill (%)
0.2	27/0.20	55	9	40
0.8	22/0.41	40	9	42

Compound detection and quantification

Depending on the model compound used to test interaction characteristics with mucus, different quantification methods can be employed. For the robust barrier marker lucifer yellow (LY), permeability is widely measured spectrophotometrically using plate readers. For FITC-dextrans (large macromolecules), fluorescent plate readers can be used. For budesonide, sensitive methods based on chromatography and mass spectrometry (Waters® UPLC-

MS/MS) are important to ensure accurate quantification. The LC-MS/MS system used herein was a Waters Xevo TQ MS electrospray ionization coupled to an Acquity UPLC system (Waters, Milford, MA) and a Waters BEH C18 column, 2.1 × 50 mm (1.7µm). The column was kept at 60°C, with a 2 min gradient and a flow rate of 0.5 mL/min. Solvent A consisted of 5% acetonitrile and 0.1% formic acid in water, and solvent B consisted of 0.1% formic acid in acetonitrile. The LC run used a linear gradient from 0.5 to 1.2 min starting at 5% of solvent B and ending at 90%, followed by a hold from 1.2 to 1.6 min and a linear gradient to return to the initial conditions at 1.7 min. Samples were kept at 10°C until injection. For normalization, 10 nM warfarin was used as internal standard.

Computational methods & Data analysis

Image analysis

Microscopy visual data must be processed to be quantified. Using FIJI® software, images can be processed to reduce background noise, as observed in the particle tracking experiments (**Papers II–III**). There are multiple approaches and algorithms to detect particles for tracking their trajectories. In principle, images need to be restored and enhanced, then particle locations must be detected. Next, refinement of these locations ensures correct labeling of the particles, and noise signals must be distinguished from actual particles. Finally, locations are linked into trajectories, and error estimation is performed to gauge the brightness-weighted centroid estimation[116].

Image analysis also enables structural analysis from electron micrographs, as described in **Papers I & IV**. In principle, pore sizes were detected and quantified based on contrast thresholding. After detecting the pores, Feret's diameter was calculated to estimate the effective mesh size. Using these estimates and Eq. 12, the storage modulus of the mucus can be determined.

$$\xi = \left(\frac{k_B \times T}{G'}\right)^{\frac{1}{3}} \quad \textbf{(Eq.12)}$$

Where ξ is the effective mesh size (nm), kB is the Boltzmann constant (1.38×10^{-23} m^2 kg s^{-2} K^{-1}), T is the absolute temperature (ºK), and G' is the storage modulus (Pa). This equation estimates the mesh size assuming a cubic lattice[117,118].

Machine-learning-based algorithms

Nanoparticles in the mucus system, which is a polymer network with complex compositions, experience various interactions. Thus, the movement of the particles cannot be characterized by simple Brownian motion. Anomalous diffusion refers to any type of diffusion that deviates from classical Brownian motion[119]. Complex trajectory analysis with MSD-based fitting may introduce error depending on the selected timeframe[79,99]. Therefore, advanced trajectory analysis using machine learning classifiers based on features of diffusion characteristics has been developed. Application of machine learning algorithms to classify multiple particle tracking data has been used in recent years[80]. Machine learning algorithms can be classified based on complexity, starting from linear discriminant analysis (LDA) (logistic regression and Fisher's LDA), classification-based methods (SVM and kNN), and ensemble methods that combine both classification-based and regression-based approaches (decision tree, random forest, GradBoost)[120,121]. Pinholt et al. highlight the top two performing algorithms in classifying nano- and micro-particle diffusion trajectories as random forest and gradient boost systems[121].

Random forest

Random forest methods construct several decision trees from the same training dataset. Predictions are aggregated from these trees and involve a bagging process. The bagging process repeatedly selects random samples with replacement from the training dataset and fits trees to the selected samples. To retain independence and avoid correlations between the trees, for each tree, a random subset of features is selected[99].

Gradient boost system

For gradient boosting, the trees are built sequentially by learning from previous mistakes. Its main difference compared to the random forest method is the interdependence of trees[99]. Furthermore, the gradient boosting method was highlighted for its classification accuracy of particle track data and its ability to interpret which statistical features contribute most to the predictions[80].

Physiologically-based pharmacokinetic (PBPK) model

In Gastroplus®, the implemented model is the Advanced Compartmental Absorption and Transit (ACAT) model. The ACAT model uses compartments to describe drug states: unreleased (solid), undissolved (particles), and dissolved. Only when the drug exists in dissolved form can it be absorbed and interact with enzymes and transporters. This model allows exploration of dissolution-rate-limited absorption and the effect of formulation release rates on pharmacokinetics[122]. The ACAT model calculates absorption based on fluid volume and local drug concentration (lumen and enterocytes). A limitation of the ACAT model is that the volume used is static, meaning it does not account for changes in the dynamic GI tract environment, and the driving gradient for absorption depends more on the amount of drug available. Another point mentioned by Yu et al. concerns fluid volume, which is unclear how it is determined in the ACAT model and is confounded by the absorption scale factor

(ASF)[123]. ASF is a coefficient multiplier used for scaling absorption rate-determining parameters such as pH and surface-to-volume ratios with the effective surface area[124]. Model of drug dosing and compartment were made based on literature data. Clinical pharmacology and biopharmaceutics review document from FDA Center for Drug Evaluation and Research were studied and graph's data were digitized with PlotDigitizer™ (PORBITAL).

Gastroplus™ 9.8.3 with gastrointestinal absorption (ACAT™) were used for modeling. First, new drug database (.mdb) were created. Then molecular structure of budesonide (.mol) was uploaded, with predicted physicochemical features calculated by the software Gastroplus™ 9.8.3. Controlled release data in vitro were taken from a study by Borra et al [125] and created as input file (.dsd). Input data were gathered from literature study (Table 3; **Paper V**), with the exception of apparent permeability that were obtained from the experiments performed herein (Fig. 30). Clearance values were predicted from IV data (**Paper V**), and simulation were run for 48h corresponding to experimental data for budesonide UCERIS® [126,127].

Statistical analysis

GraphPad Prism 9.0 (GraphPad Software, CA, USA) was used for data visualization and statistical analyses. An unpaired t-test was used to evaluate differences between two groups, whereas a one-way ANOVA followed by Holm-Sidak's multiple comparisons test was used to evaluate differences between more than two groups. P values less than 0.05 were considered statistically significant. The ANOVA nonparametric test (Kruskal–Wallis test) was used for data assumed to have a non-normal distribution (pore size data). Wilcoxon paired t-tests were used for comparison of Feret diameters. Data cleaning (compiling values and converting units) was performed in Microsoft Excel. For the MST results, analysis and graph plotting were done with MO.Affinity Analysis (NanoTemper, Germany) software

Results and Discussion

The different studies performed in this thesis, with focus on inherent characterization and formulation of mucus, interspecies differences in colonic mucus and the role of mucus in drug interactions and absorption are summarized in Fig. 11.

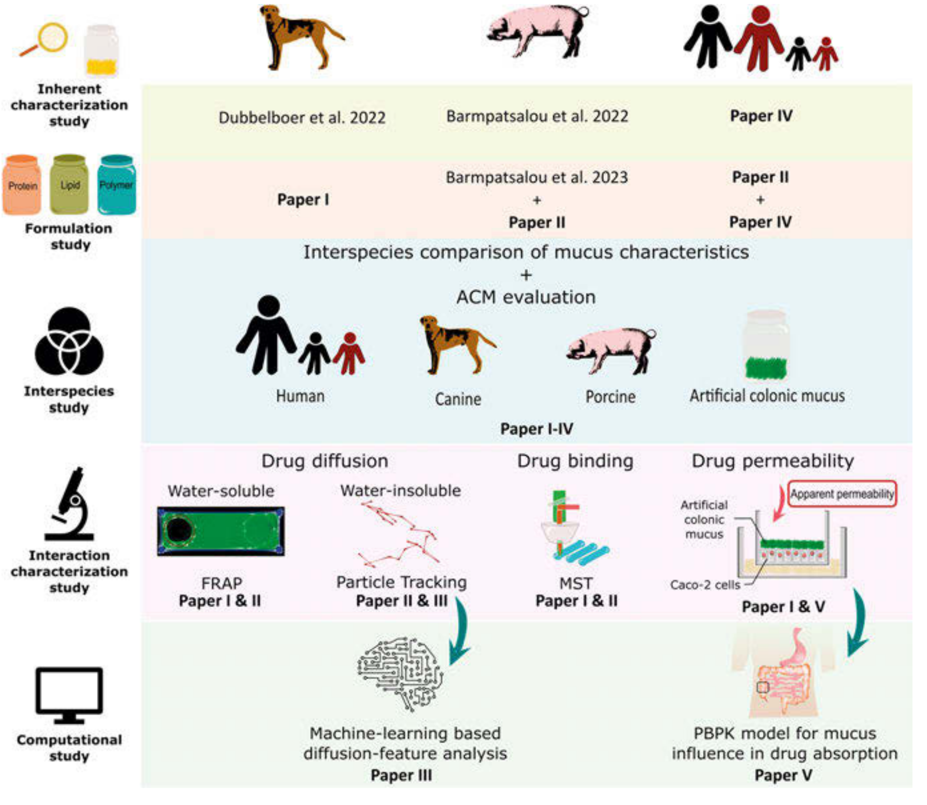

Figure 11. Overview of research workflow based on the five subprojects of the thesis. Initially, characterization of the native colonic mucus was performed to formulate artificial colonic mucus (ACM). This is followed by comparisons of interspecies differences and differences in ACM models. From a drug delivery perspective, drug interaction studies with mucus were performed and the obtained results was used in computational modelling and simulation.

Human native colonic mucus show slight differences in structural and physicochemical properties, as well as protein composition, between groups.

Human native colonic mucus has a porous structure similar to that of canine[25,115] and porcine[12,63] mucus, forming a heterogeneous, tortuous medium with non-circular pores (Fig. 12). This porous structure exhibits a range of Feret's minimum diameters, from 6 to 520 nm, across the studied groups (children inflamed, children healthy, and adult healthy individuals; **Paper IV**).

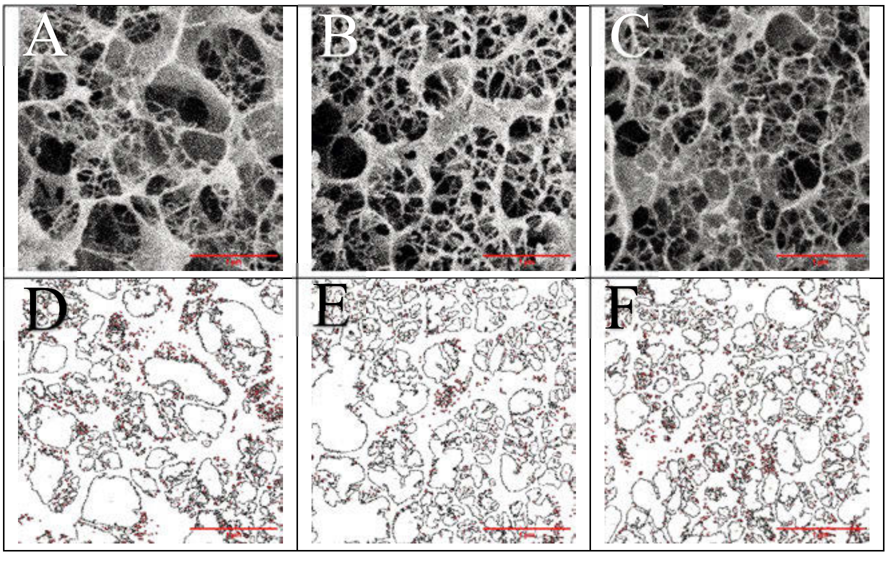

Figure 12. Representative Cryo-SEM micrographs of native human colonic mucus: inflamed child (A), healthy child (B), and healthy adult (C). Images resulting from the binarization process to allow detection of the pores are shown in figures D-F.

After acquiring the mucus images, calculation of the storage modulus from extrapolation of pore size can be performed by using Eq. 12 (**Paper II**). A slightly higher median pore size was observed in the inflamed children group (25.41 nm) compared to the healthy children (20.72 nm) and healthy adults (20.89 nm) groups (Fig. 13). This corresponds to a lower storage modulus in the inflamed children group (0.0002544 MPa) compared to the healthy children (0.0003079 MPa) and healthy adults (0.0003058 MPa) groups. This finding aligns with the reported structural weakening of colonic mucus in the early stages of ulcerative colitis[128]. Zeta-potential values (Fig. 14) showed that the adults healthy group has a lower value of -24.55 ± 3.654 compared to children inflamed (-19.19 ± 6.520) and children healthy (-18.52 ± 8.039).

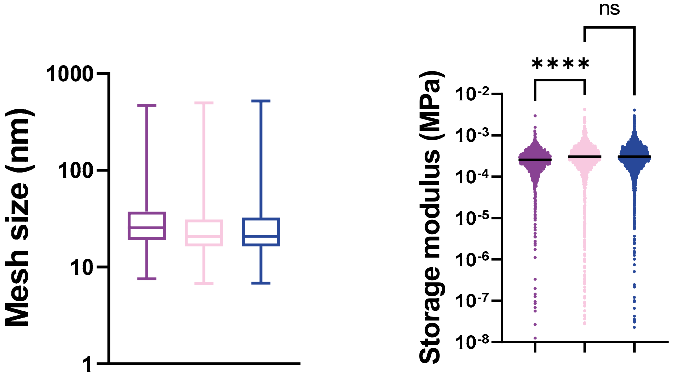

Figure 13. Mesh size and storage modulus values of colonic mucus: inflamed child (purple), healthy child (light pink), and healthy adults (blue) (n=3).

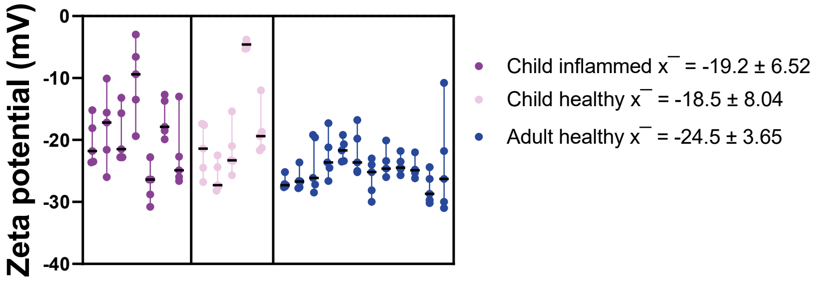

Figure 14. Zeta potential of human native colonic mucus (NCM) samples dispersed in Milli-Q water. Note the interindividual variability in most samples. Child inflamed NCM in purple (n=7), child healthy NCM in light pink (n=5), and adults healthy NCM in blue (n=12). Each sample was measured with five technical replicates, mean values are provided with standard deviation.

Global data-independent acquisition (DIA) proteomics analysis revealed similarity in the majority of protein composition, with 7,379 types of same proteins present in each group. Whereas other study manages to detect only around 900 proteins[128]. Visual observation by the clinician was performed during biopsy sampling to determine the healthy and inflamed region of the colon. In healthy adults, membrane-bound proteins, CYP (cytochrome P450 enzymes), and SLC (solute carrier transporters) were detected, indicating maturation of the mucosal region and healthy xenobiotic detoxification function[129]. In the healthy child group, unique types of mucins (MUC6, MUC3A, and MUC17) were detected, indicating developmental progression of mucin expression between children and adults. Furthermore, the results of proteomics further verified inflammation status, as pro-inflammatory cytokines (TGF-β, IL-37, and ANGPTL2) were detected only in the inflamed child group (Fig. 15).

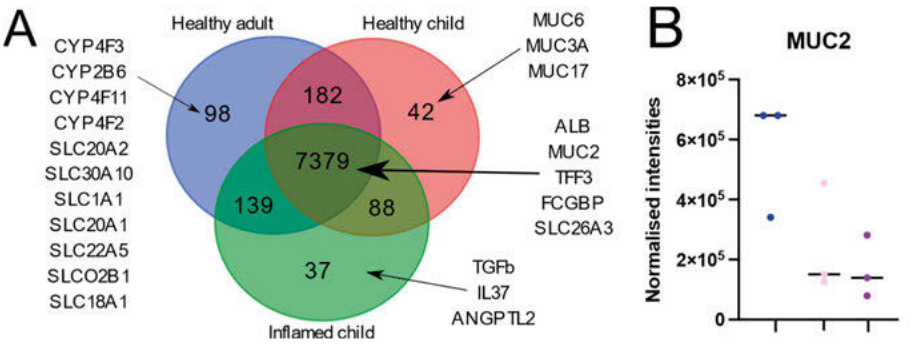

Figure 15. Proteomic data obtained from mucus samples. (A) Venn diagram of the identified proteins among healthy adult, healthy child, and IBD-diagnosed pediatric groups. Data from three or four different donors were used per condition, and a protein was considered identified if detected in at least one sample per group. Biologically meaningful findings are highlighted for each group. (B) Normalized expression values for mucin proteins detected in the dataset: inflamed child (purple), healthy child (light pink), and healthy adult (blue) (n=3). Data are presented as mean values ± SD. Based on limma differential expression analysis, no statistically significant differences were detected.

The unique proteins identified in inflamed children are TGF-β, IL-37, and ANGPTL2. These proteins are associated with IBD progression[130–132]. Other proteins that differed between the inflamed and healthy children group were MUC6, MUC3A and MUC17 that only were present in the healthy children group. Earlier study have investigated the importance of MUC17 in protection against Crohn's disease[133]. Expression of MUC6 and MUC5AC may play a role in epithelial wound healing or have protective effects on the mucosal layer[134]. MUC2 expression in the colon is dominant under normal conditions[135,136], and this finding is consistent with its higher abundance in healthy adults compared to children (Fig. 15). Macierzanka et al. have also describes a more robust mucus barrier in adult pigs compared to piglets[103].

One interesting highlight is the high albumin content discovered in all three groups of colonic mucus. This finding seems to be in agreement with the findings in canine[25] and porcine[12] model, along with findings by Van der Post et al. from human colonic mucus taken from biopsies[137]. The findings provide the basis for the formulation of artificial colonic mucus (**Paper I-II**), while albumin influence on mucus barrier property was demonstrated (Fig. 25).

Types of lipids present in the mucus are important to identify, since the lipid present will influence the mucus properties, including the zeta potential of the hydrogel formed[138]. Based on the analysed samples, 10 groups of lipid classes were identified from healthy adult and healthy children (Table 2). Three larger groups of lipids were present across the samples (Fig. 16); phospholipids (PL), sphingomyelins (SM), and triacylglycerides (TG).

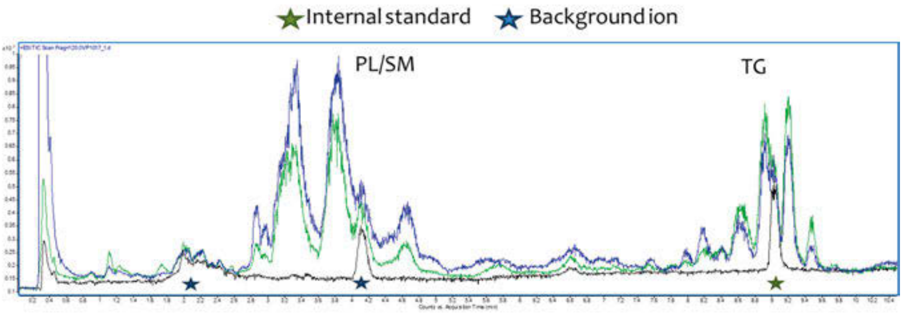

Figure 16. Total ion chromatogram showing detected peaks from colonic mucus suspension samples of adult (blue) and child (green), both in the healthy condition.

Based on the structural analysis, physicochemical properties (storage modulus and zeta potential), proteomics-lipidomics study, it can be concluded that human native colonic mucus shows similarity between healthy adults and healthy children, as well as between healthy and inflamed children.

Interspecies differences in the physicochemical properties of porcine, canine, and human colonic mucus

As described in the introduction, interspecies differences account for challenges when translating in vitro and in vivo drug absorption results obtained under different conditions. Therefore, it is important to understand the similarities and differences in the characteristics of canine, porcine, and human native colonic mucus (NCM). Visual observation of the Cryo-SEM images confirms the heterogeneous porous network of colonic mucus in canine (**Paper I**), porcine (**Paper II**), and human (**Paper IV**). Results are compiled in Fig. 17. Based on image analysis, the storage modulus of mucus was calculated (Fig. 18; **Paper II**). This analysis was performed due to challenges and limitations in sample volume from human biopsies, and alternative methods for rheological assessment were developed based on image analysis. Comparing the median storage modulus between porcine NCM obtained through rheology (9.384×10^{-3} MPa) and porcine NCM obtained through Cryo-SEM image analysis (1.029×10^{-2} MPa) validated this technique. The storage modulus of human colonic mucus across groups (2.544×10^{-4} to 3.079×10^{-4} MPa) is in the lower range of porcine NCM (4.5×10^{-5} to 2.6×10^{-2} MPa), while canine NCM storage modulus is lower than both human and porcine (4.299×10^{-5} MPa; from rheometer).

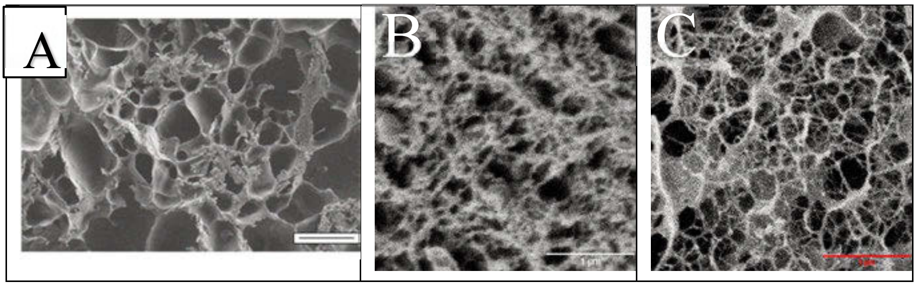

Figure 17. Representative Cryo-SEM images of colonic mucus porous structure. (A) Canine native colonic mucus, (B) porcine native colonic mucus, and (C) human native colonic mucus. Note that due to differences in sample preparation steps for the canine samples (direct liquid nitrogen vitrification) versus porcine and human samples (high-pressure freezing), quantitative comparisons would be inaccurate. Different magnifications apply; scale bar for canine: 10 µm; scale bars for porcine and human: 1 µm.

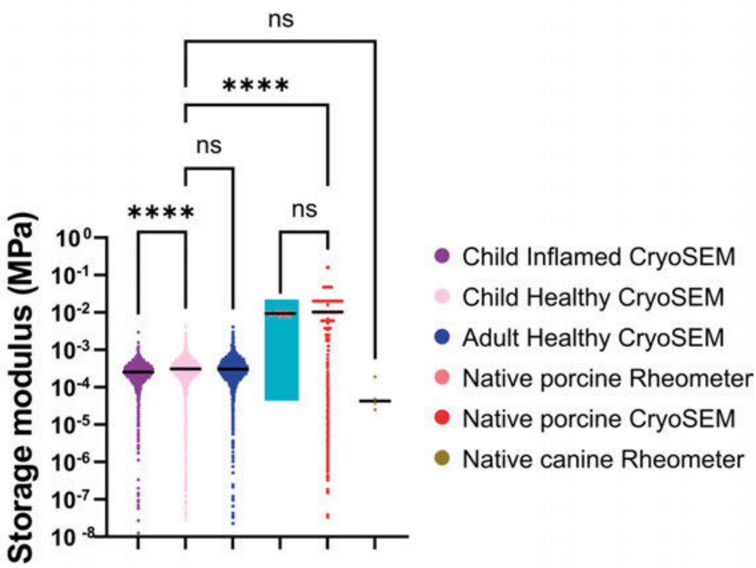

Figure 18. Storage modulus values of colonic mucus. (A) Comparison between pig native colonic mucus (PNCM) (n=3) with two methods (rheometer (light orange) and Cryo-SEM (red)) from published study[12,139], along with canine native colonic mucus (CNCM) (n=4) with rheometer (light brown) from published study[25], inflamed children (purple), healthy children (light pink), and healthy adults (blue). (B) The storage modulus of formulated artificial colonic mucus (orange), which is similar to human native colonic mucus (n=3).

From the calculation of storage modulus (Fig. 18), human NCM overlapped with the lower range of porcine mucus, whereas canine mucus had an even lower storage modulus range. This may be because pigs consume larger amounts of food compared to humans and may therefore need a thicker barrier to protect the colon. In addition, the porcine colon hosts a 10- to 100-fold higher density of microbes compared to the human colon (porcine 10^{10}–10^{11} per gram[140]; healthy human average 10^9 per mL[45]). When comparing zeta potential of the mucus obtained from different species, similarities were found between pig NCM and all human samples (Fig. 19).

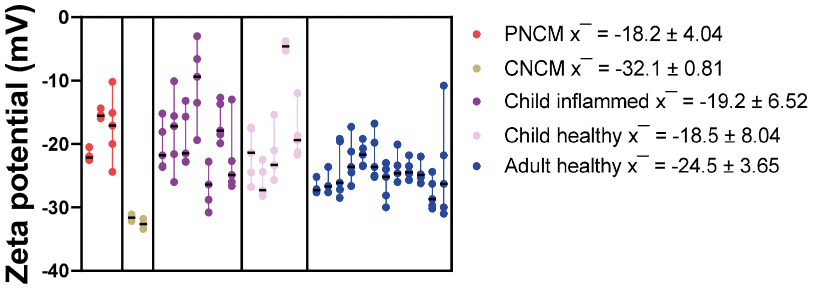

Figure 19. Zeta potential of native colonic mucus (NCM) samples dispersed in Milli-Q water. Porcine NCM (red)[139], Canine NCM (light brown)[25], adults healthy NCM (blue), children healthy NCM (light pink), and children inflamed NCM (purple). Each sample was measured with five technical replicates, mean values are provided with standard deviation.

Table 2. Lipids classes detected on the samples

Abbreviation	**Lipid class**	**Fatty acids (FA)**	**Polarity**
PL	phospholipid	2*ester	
LysoPL	Lyso means 1*FA and free alcohol	1*ester	
TG	triacylglyceride	3*ester	Pos
DG	diacylglyceride	2*ester	Pos
PC	Phosphatidylcholine / lecithin	1-2*ester	Pos
	PC(O-X:n)	1-2*eter	Pos
SM	sphingomyelin	alcohol+amide	Pos
Cer	ceramide	alcohol+amide	Pos
PE	phosphatidylethanolamine	1-2*ester	Pos
PS	phosphatidylserine	1-2*ester	Pos
PI	phosphatidylinositol	1-2*ester	Neg

The group of lipids detected in human colonic mucus samples are also found in porcine and canine colonic mucus. Glycerophospholipids are the main group detected in both canine[25] and porcine[12], while in human phospholipids were one of the groups detected (Table 2). Sphingomyelin group is also detected in PNCM[12]. This highlights similarity between interspecies colonic mucus. Lipids composition would influence diffusion of particles[141,142], and heterogeneous nanostructures corresponds to lipids may cause anomalous diffusion[143]. Taken together, mucus from canine model is the least similar compared to porcine and human. Whereas human colonic mucus storage modulus is at the lower range of porcine colonic mucus.

Artificial colonic mucus can capture the inherent characteristics of native colonic mucus.

Physicochemical properties influence the diffusion process. To mimic native mucus barrier properties, characterization of both native and artificial mucus is crucial. After characterization and optimization, the developed porcine artificial colonic mucus (ACM) closely resembles human NCM. The porous structure of mucus can be observed in native colonic mucus (**Paper I, II, IV**). The structure of native colonic mucus can be replicated by artificial colonic mucus mimics.

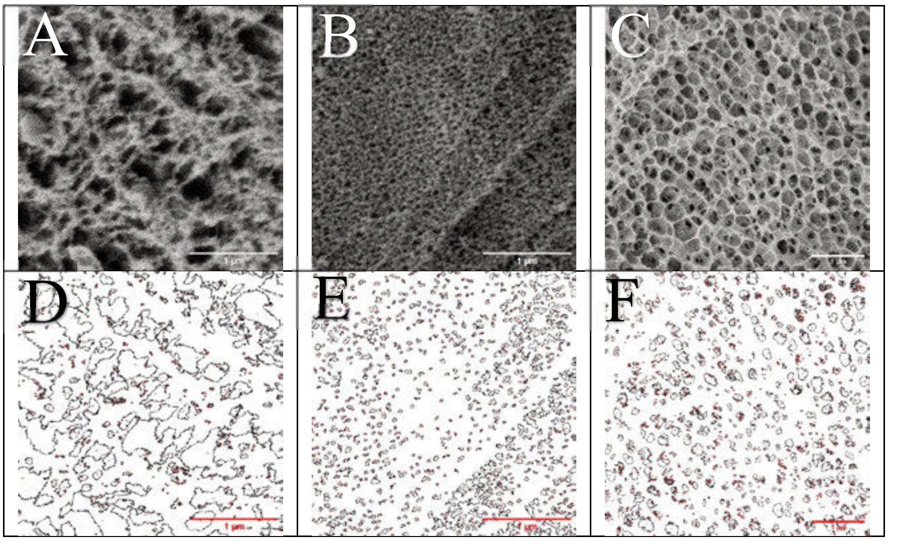

Figure 20. Representative Cryo-SEM micrographs of native mucus (A, D), PACM-PAA (B, E), and PACM-HEC (C, F). Resulting images from a binarization process allow detection of the pores (D, E, F). Scale bars: 1 μm.

Consistently, the pore size and storage modulus of ACM can mimic human NCM values (Fig. 20 & 22). The viscoelastic properties are also replicated, showing higher storage modulus than loss modulus, confirming the gel characteristics in both native and artificial colonic mucus (Fig. 21). This finding is in alignment with current literature[46,62,144,145], which confirms that mucus is a viscoelastic hydrogel.

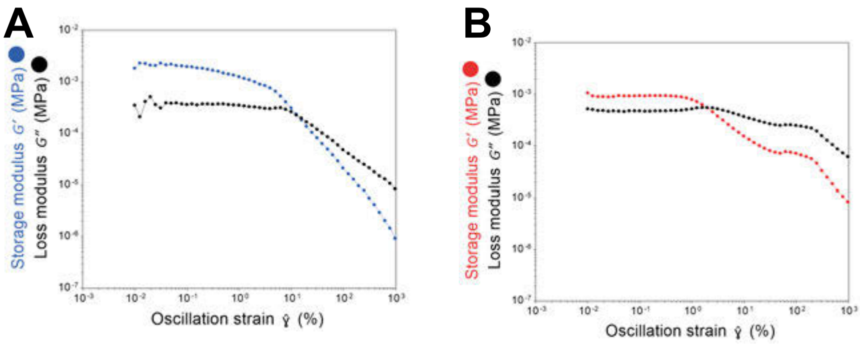

Figure 21. Amplitude sweep measurements showing viscoelastic behavior and the linear viscoelastic region. Black circles indicate the loss modulus; colored circles indicate the storage modulus. (A) Porcine native colonic mucus, blue; (B) PACM HEC, red.

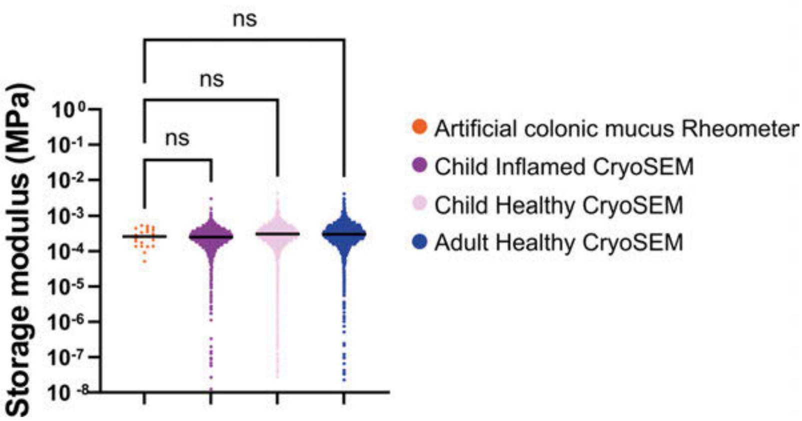

Figure 22. Storage modulus values of colonic mucus. Comparison between inflamed children (purple), healthy children (light pink), and healthy adults (blue). The storage modulus of formulated artificial colonic mucus (orange) is similar to human native colonic mucus (n=3).

Following structural characterization by Cryo-SEM imaging and storage modulus measurement, the zeta potential of the mucus (Fig. 23) was determined. Zeta potential influences interaction characteristics as described (**Paper II**). The formulated ACM-HEC captured the zeta potential of human NCM. It closely matched both children groups and was in the range for the healthy adults, although the latter group generally had a slightly lower zeta potential value.

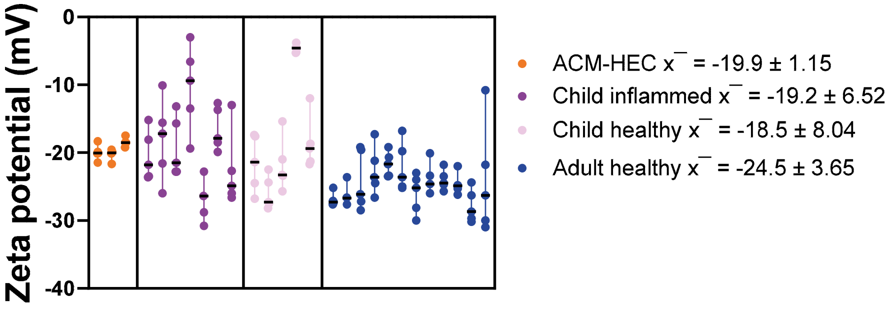

Figure 23. Zeta potential of artificial colonic mucus (ACM; orange) samples dispersed in Milli-Q water contrasted with human groups. Note the interindividual variability in most samples, adults healthy NCM (blue), children healthy NCM (light pink), and children inflamed NCM (purple). Each sample was measured with five technical replicates.

Microrheological analysis based on particle tracking, performed in **Paper III**, reveals that the formulated ACM can also capture the microrheological properties of NCM (Fig. 24). This was demonstrated by the similar range of elastic-to-viscous modulus ratios (Fig. 24 (d), (h), (l), and (p)) across different sizes of nano- and microparticles. Cosine similarity analysis (**Paper III**) showed that PACM-HEC and PACM-PAA are more similar to each other than to PNCM, which is logical given the nature of artificially formulated mucus. However, PACM-HEC is more similar to PNCM compared to PACM-PAA, possibly due to charge properties (Fig. 26). It has been shown that similarity in macrorheology between mucus mimics may not translate to similarity in microrheology[71]. Microrheology values provide insight into the diffusion barrier experienced by nano- and micro-sized particles[146], which is important in the context of drug delivery. It has been shown that mucus microstructure plays a role in determining microrheology values[64] due to porous structure, which observed in Fig. 16 and Fig. 19. Lower viscosity values (elastic-to-viscous ratio) are expected in smaller particle sizes group[64] since smaller particle size would have less contact with the network structure.

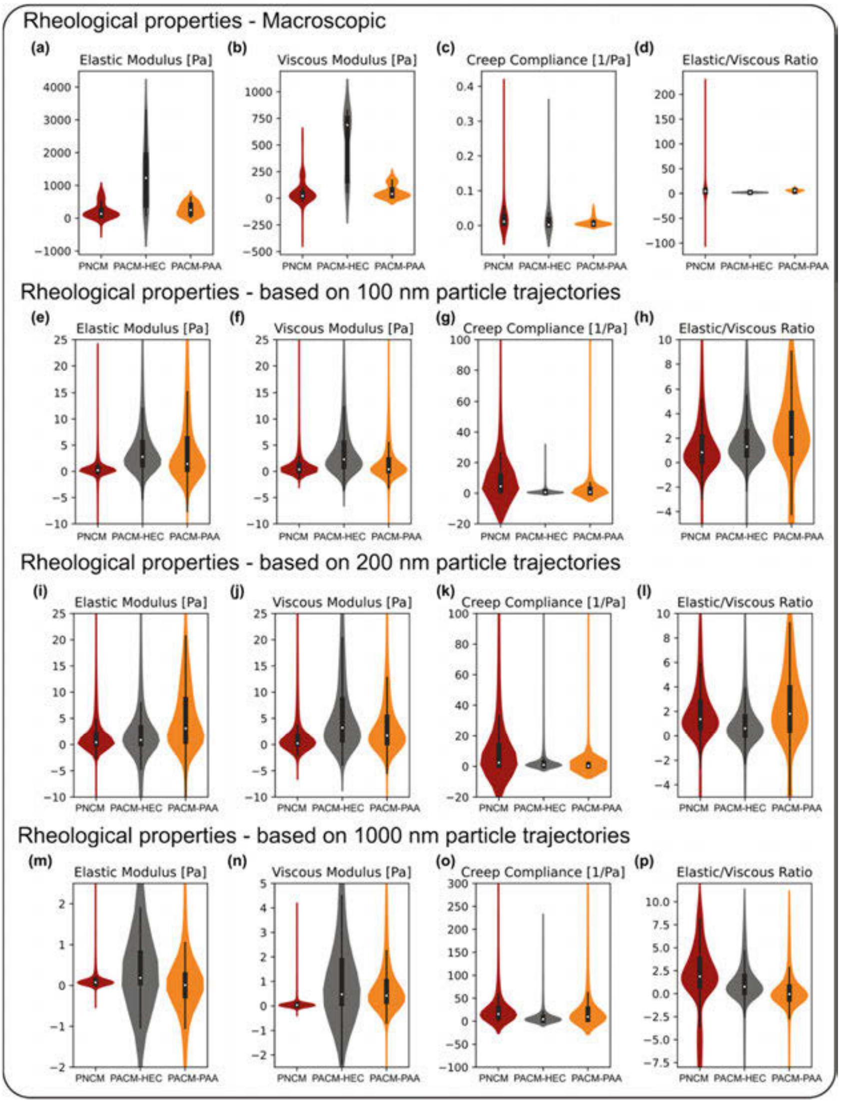

Figure 24. Comparative analysis of rheological properties—including elastic modulus, viscous modulus, viscosity, and creep compliance—across different mucus types: PNCM, PACM-HEC, and PACM-PAA. Violin plots combined with box plots are used to visualize the data. The first row (a–d) shows experimental bulk measurements, while the second (e–h), third (i–l), and fourth rows (m–p) present values calculated from particle trajectories of 100, 200, and 1000 nm particles, respectively. Each plot combines features of a box plot and a kernel density plot. The white dot at the center of each violin plot represents the median value. The box surrounding the dot indicates the interquartile range, covering the middle 50% of the data. The lines extending from the box represent the data range, excluding outliers, and help visualize overall data spread and variability. The outer violin shape illustrates the full distribution density, showing where data points are more concentrated or spread out.

The inherent physicochemical properties of mucus determine its interaction characteristics.

In the previous investigation by Barmpatsalou et al., artificial colonic mucus mimicking porcine native colonic mucus was formulated with optimized protein and lipid contents[13,63]. Integration of the main mucus components (mucin, BSA, and lipids) (Fig. 25) is necessary for robust barrier properties (**Paper I**). Exclusion of mucin, bovine serum albumin (BSA), or lipids in the study resulted in increased permeability (Fig. 25). As shown in the figure, absence of mucin in the ACM led to a 3-fold increase in the permeated amount for 4K and an 8-fold increase for 40K and 70K neutral dextrans. Exclusion of BSA also increased dextran permeability. Lipid composition was less important in for the compounds explored (hydrophilic dextrans), but may significantly interact with lipophilic compounds[147]. It may also influence other properties, such as altering zeta potential[148].

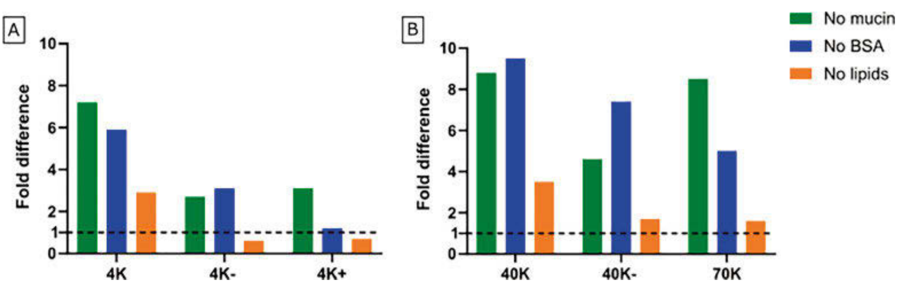

Figure 25. Impact of components in artificial mucus on diffusivity. The difference in mean permeated amount of FITC-dextrans in CACM is shown: without mucin (green), without BSA (blue), or without lipids (orange) for 4K FITC-dextrans (A) and 40K and 70K (B). n = 3–6. The dotted line represents the CACM baseline containing all three components (mucin, BSA, lipids).

In **Paper II**, it was demonstrated that the zeta-potential properties of mucus differ depending on the choice of polymer backbone (Fig. 26A), which is of importance for different interaction patterns with cationic compounds (Fig. 26B). This finding provides insight into the crucial factors that make up the mucus barrier, showing it depends on multiple components and not only on mucin. Binding experiments with cationic 4K FITC-dextran showed that by altering the zeta-potential of the ACM, binding kinetics more similar to PNCM were achieved (Fig. 26B).

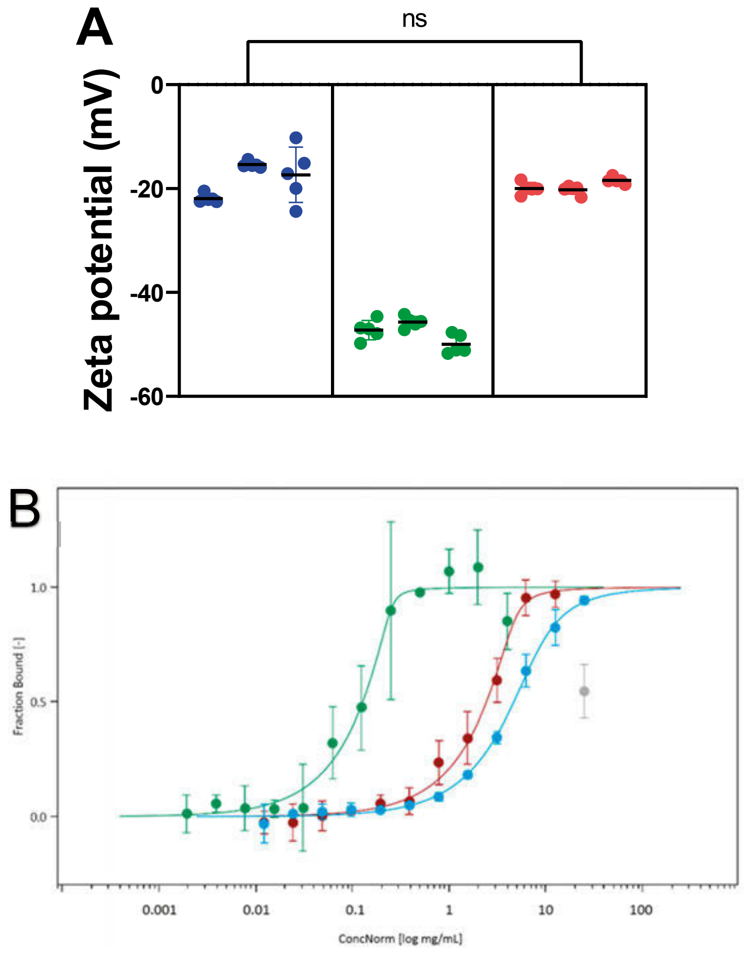

Figure 26. Mucus polymer selection influences zeta-potential and binding interactions. Circles represent data points. (A) Zeta-potential of mucus samples dispersed in MilliQ water. Note the interindividual variability in PNCM (blue). Green: polyacrylic acid (PAA); Red: hydroxyethyl cellulose (HEC). Each sample was measured from three batches with five technical replicates. (B) Binding profile between cationic FITC-dextran 4K and PACM PAA (green), PACM HEC (red), and PNCM (blue). Mucus samples were diluted to create a series of gradient concentrations suspended in MES buffer.

Size and charge of the particles influence diffusion, binding, and permeability in colonic mucus

Through **Papers I–III**, the inherent properties of mucus, along with interaction properties, were investigated. In **Paper I**, it was demonstrated that only cationic dextrans have binding interactions with the mucus (Fig. 27), which further was observed in nano- and microparticle studies in **Papers II & III**. Cationic particles bound to the mucus, resulting in more immobile and sub-diffusive particles in the mucus compared to anionic particles (Fig. 28).

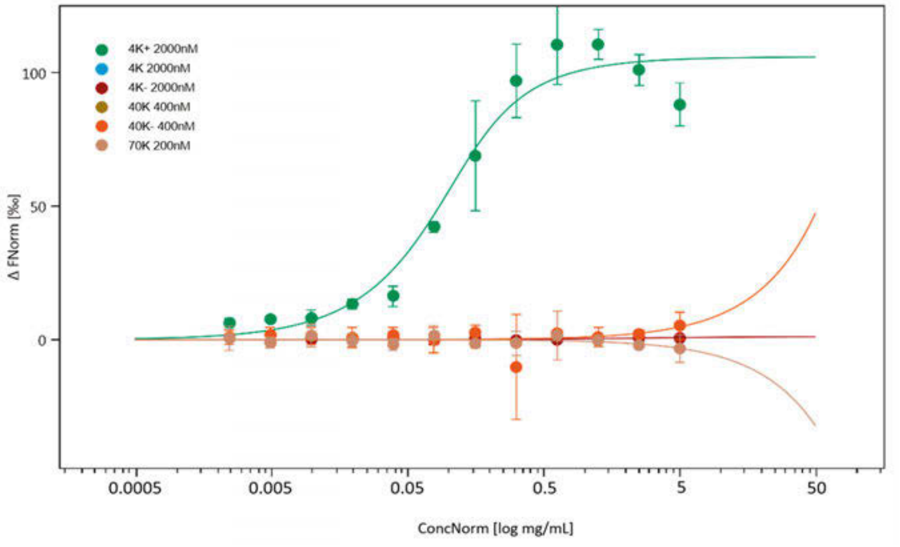

Figure 27. Binding profile between FITC-dextran with different sizes (4K, 40K, and 70K) and charges (cationic, neutral, and anionic) with CACM. Mucus samples were diluted to create a series of gradient concentrations suspended in MES buffer.

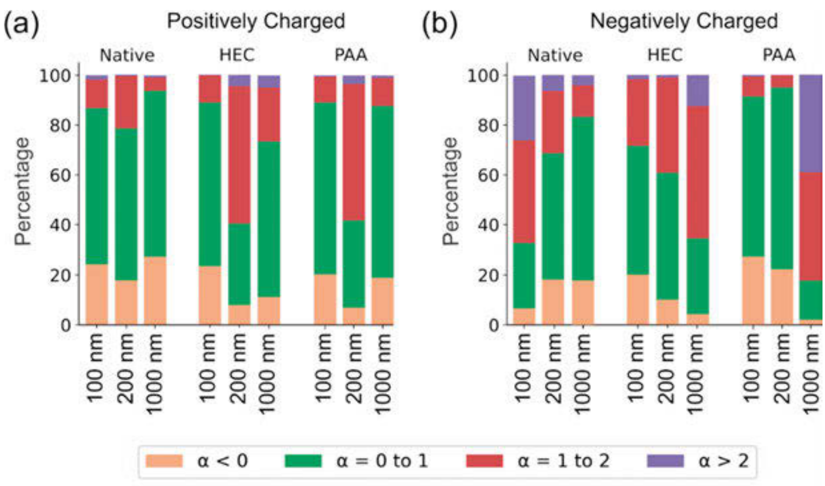

Figure 28. Diffusion modes classified based on α values from MSD power-law fitting of particle trajectories. Panels (a) and (b) show results for positively and negatively charged particles, respectively, across three mucus hydrogels and three particle sizes. This figure presents stacked bar plots illustrating the percentage of diffusion modes classified by exponent α in the MSD power-law fitting equation. The classification includes immobile ($\alpha < 0$, orange), subdiffusive ($0 \leq \alpha < 1$, green), diffusive ($1 \leq \alpha \leq 2$, red), and active ($\alpha > 2$, purple).

Using FRAP (**Paper I – II**) and particle tracking (**Paper II – III**), particle diffusivity in mucus can be measured, demonstrating that artificial colonic mucus replicates the diffusion barrier properties of the native colonic mucus (Fig. 29). The formulated ACM was found to capture the diffusivity in native mucus of both water-soluble and water-insoluble particles; however, solubility-dependent particle diffusion remains to be investigated[149]. The observed differences in diffusivity in colonic mucus highlight the influence of particle size and charge, consistent with Kirch et al. and Hansing et al. findings[150,151]. This also relates to the mucus structure described in Figs. 12, 16, and 19. Smaller particles experience less viscosity[64], and therefore diffuse faster (Eq. 1).

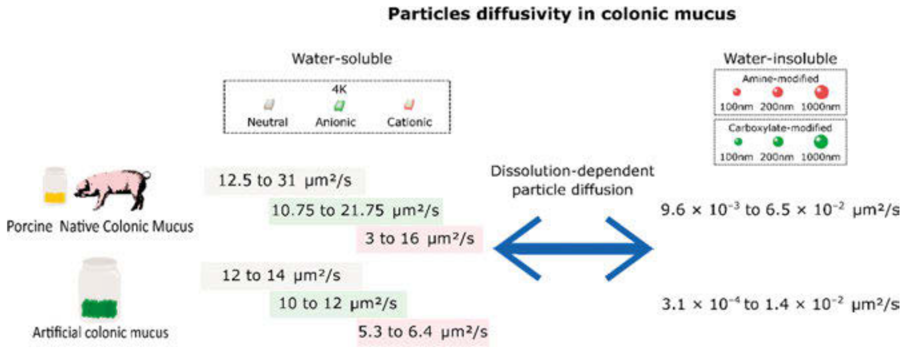

Figure 29. Diffusivity of water-insoluble and water-soluble particles in colonic mucus. Data are compiled from previous study[63,139].

In vitro experimental setup using 3D-printed mucus provide more accurate input values for PBPK modelling

Based on a study of intestinal motility by Naranjani et al. (2025), a mechanistic model was generated, and SHAP feature analysis was performed to identify prominent factors influencing absorption. The most prominent factor identified was mucus thickness[152]. Mucus permeability experiments were conducted in **Paper I** to determine the influence of mucus composition. In **Paper V**, we examine the integration of the mucus model (Fig. 30) with the Caco-2 permeability model, which is the current gold standard for permeability studies.

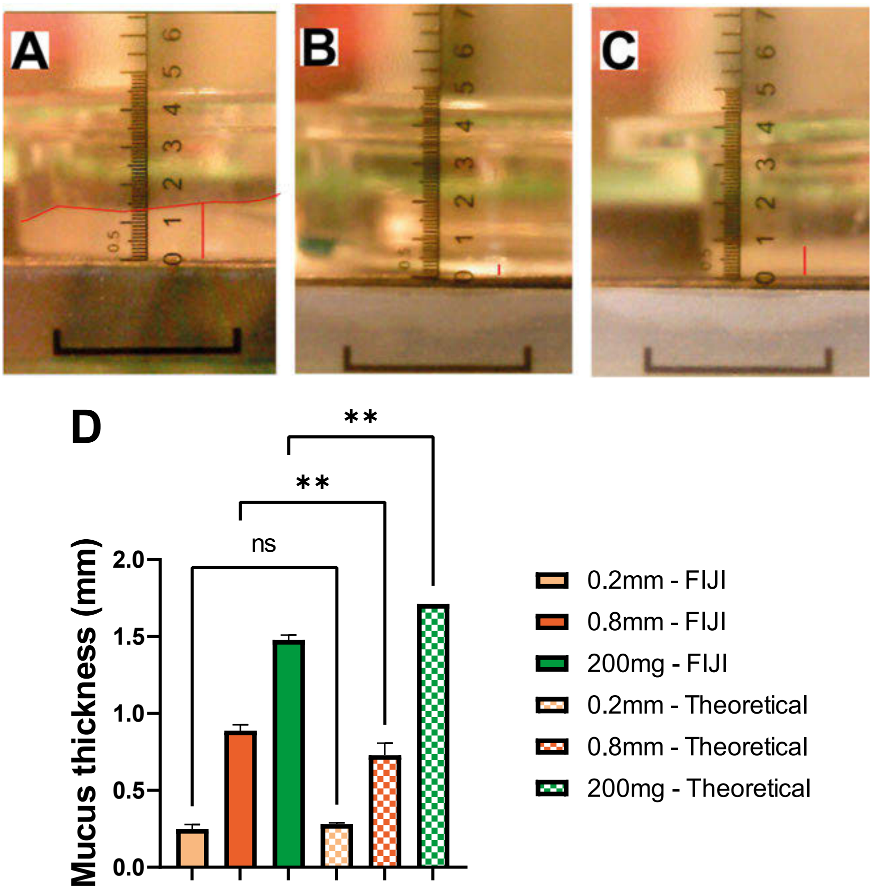

Figure 30. Mucus applied on top of a Snapwell™ filter. (A) Direct manual addition of 200 mg of artificial colonic mucus using a spatula; the red horizontal line highlights the uneven thickness. (B) 3D printing targeting a 0.2 mm thickness. (C) 3D printing targeting a 0.8 mm thickness. (D) Comparison of mucus thickness in Snapwell™ by measurement using light microscopy – FIJI used for image processing (solid color) and theoretical calculation from mass and density (checkered color). Different methods of addition yield different thicknesses: 3D-printed 0.2 mm thick mucus (light yellow), 3D-printed 0.8 mm thick mucus (orange), and direct addition of 200 mg mucus (green).

Apparent permeability values from the experiments (Fig. 31) showed that mucus thickness influences budesonide permeability. Reports have describes that mucus acts as a barrier to drug permeability[73,111]; however, to the best of our knowledge, this is the first study to highlight the influence of the thickness parameter on permeability. This finding is further supported by the PBPK model, where the absorption profile of budesonide (Fig. 32; Table 3) was shown to depend on the incorporation of P_{app} – P_{eff} values based on mucus thickness. Previous study highlights the challenge of translating P_{app} to P_{eff}

values[153], and to date there is no established correlation between Caco-2 + mucus P_{app} values and in vivo P_{eff} values.

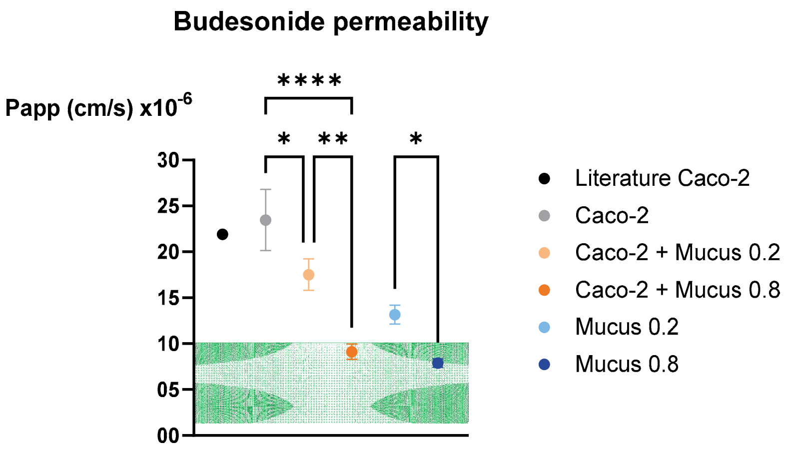

Figure 31. Apparent permeability of budesonide dispersed in fasted state simulated colonic fluid (FaSSCoF) buffer. The value from the literature (black) was used for comparison[154]. Different barrier complexities influence budesonide permeability: Caco-2 only (gray), Caco-2 with 0.2 mm thick mucus (light yellow), Caco-2 with 0.8 mm thick mucus (orange), only 0.2 mm thick mucus (light blue), and only 0.8 mm thick mucus (blue). The green area corresponds to the intermediate permeability range, which is between 1–10 × 10^{-6} cm/s[155,156]. Each group was measured in triplicate, and data are presented as mean ± standard deviation.

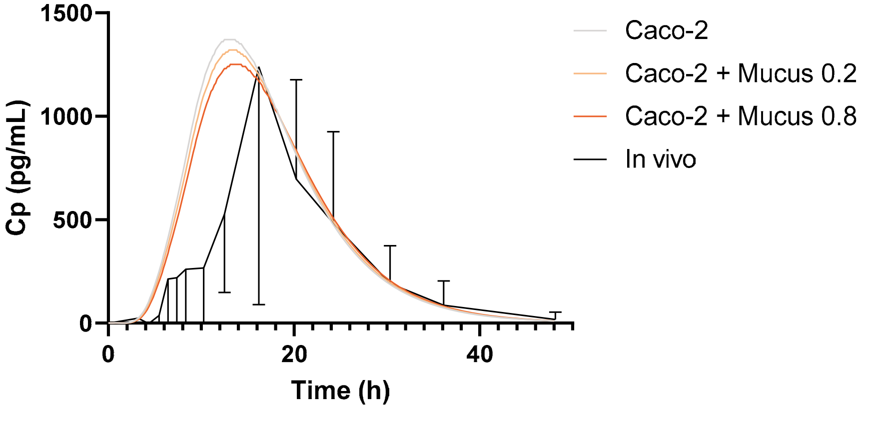

Figure 32. Simulated pharmacokinetics of controlled-release budesonide with apparent permeability values under the following setups: Caco-2 with 0.8 mm thick mucus (orange), Caco-2 with 0.2 mm thick mucus (light orange), Caco-2 only (gray), and experimental in vivo data (black).

Table 3. Summary of simulated values vs experimental data of controlled release budesonide.

	Caco-2	Caco-2 + 0.2	Caco-2 + 0.8	Observed	Reference
Fa%	77.55	75.64	73.73	69	[157]
FDp%	27.143	26.47	25.8	28 (rat)	[158]
F%	18.9	18.53	18.06	10 – 15; 9 – 21	[126], [159]
Cmax (pg/mL)	1388.1	1320	1250	1237.7 ± 1013.5	[127]
Tmax (h)	13.1	13.43	13.75	13.3 ± 5.9	[127]
$AUC_{0\text{-}inf}$ (pg.h/mL)	21270	20740	20220	16431.2 ± 10519.8	[127]
$AUC_{0\text{-}t}$ (pg.h/mL)	21210	20680	20150	13555.9 ± 7816.9	[127]

Conclusions

This thesis investigated the development of artificial colonic mucus and conducted an interspecies comparison of colonic mucus. Methods for studying drug absorption in colonic mucus were explored to support the improvement of in vitro preclinical studies, making them more predictive of clinical outcomes.

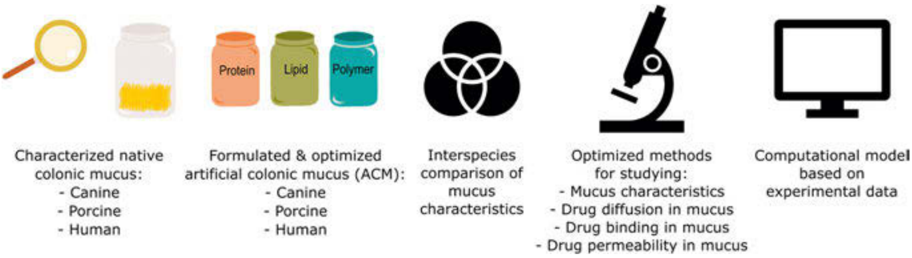

Figure 32. Visual summaries of the conclusions in this thesis, covering characterizations, formulations, comparative analysis of mucus characteristics, and model generation.

The major findings of this thesis can be summarized as follows:

- Artificial colonic mucus formulations that capture the biophysical characteristics of native colonic mucus from large preclinical animal models (**Papers I–II**) and human patients (**Paper IV**) were developed.
- The inclusion of mucins, lipids, and albumins in artificial colonic mucus was found important if the barrier properties of native mucus are to be mimicked (**Paper I**).
- Artificial colonic mucus can replicate the micro- and macro-rheological profile of native colonic mucus, corroborated by deep analysis using an ML-classifier of particle tracking trajectories (**Papers I-III**).
- Artificial colonic mucus formulated based on native colonic mucus properties is useful for drug binding studies (**Papers I–II**), drug particle diffusion studies (**Papers I–III**), and drug permeability studies (**Paper V**).
- Compounds and particles tested for interaction characteristics in artificial mucus included water-soluble FITC-dextrans (4K, 40K, and

70K with cationic, neutral, and anionic charges) (**Papers I–II**), water-insoluble polystyrene beads (100, 200, and 1000 nm with cationic and anionic charges) (**Papers II–III**), insulin and permeation enhancers (Naranjani et al. 2025[152]), and budesonide (**Paper V**), demonstrating the capacity of artificial mucus to capture native mucus barrier properties.

- For low volume samples volume, exemplified with the human colonic mucus, the relationship between the effective mesh size of the mucus porous structure and viscoelastic properties from bulk rheology (**Paper II**) was proven useful for predicting the storage modulus (**Paper IV**).
- Microrheology analysis highlights differences in the storage modulus of mucus compared to macrorheology, showing dependence on the size and charge of the probe particles. Artificial colonic mucus can capture the microrheological characteristics of porcine native colonic mucus (**Paper III**).
- The revealed interindividual similarities and differences in pig colonic mucus properties (**Paper II**) and human colonic mucus (**Paper IV**), along with interspecies differences in zeta potential and storage modulus (**Papers I–II, IV**), provide a framework for how to use artificial colonic mucus for more standardized drug absorption studies.
- Pediatric colonic mucus under inflamed conditions shows a lower storage modulus than the healthy group, while the zeta potential remains similar. Inflammatory cytokine markers were detected in the inflamed colonic mucus group. Different protein expression patterns were also found between the healthy pediatric and adult groups (**Paper IV**).
- In vitro cell-based permeability assays based in Caco-2 were compatible with artificial colonic mucus (**Paper I**) and the mucosal lining mimicking the colon was further optimized in vitro by controlling mucus thickness using 3D printing (**Paper V**) resulting in improved PK prediction with PBPK model.

Future Perspectives

Biomimicry studies based on native colonic mucus provide data that enable the development and optimization of species-specific artificial colonic mucus for studying drug absorption. For the canine ACM in **Paper I**, further optimization could be performed to better mimic the zeta potential of canine NCM. Although the importance of mucus components (lipids, BSA, mucins) in permeability across the mucus was investigated, it would be interesting to examine how mucus composition influences particle diffusion in the mucus, as explored in **Papers II & III**. Investigating the correlation between diffusion in mucus (seconds to minutes timeframe), permeability (hours timeframe), and colon transit time (hours timeframe) would also be valuable.

In this study, numerous methods for characterizing mucus properties have been applied. The limitations of analytical methods should be considered, such as the inability of MST readings to handle high-viscosity samples. Therefore, applying different methods to cross-check characteristics can help validate results, as demonstrated by comparing pore sizes and storage modulus from Cryo-SEM images with rheometer data in **Paper II**, and mucus thickness from image analysis with theoretical mass-density-volume calculations in **Paper V**. Comparing the same parameter values derived from different methods is important for validation, which could be applied, for example, to diffusion coefficients by FRAP versus time-gated Raman spectroscopy and binding coefficients by MST versus SPR/QCM-D.

Mucus structure reveals heterogeneity within the network. In our study, water pockets were demonstrated by both imaging and microrheology. However, as shown in **Papers II & III**, there are different filament thicknesses composing the mucus structure. These findings indicate different levels of size and interaction filtering properties of the mucus, which would be interesting to investigate further. As shown in our study, mucus pore size ranges from the nanometer to micrometer scale. Therefore, a study of nano-rheology would be beneficial to elucidate the nano-viscosity of water pocket fluid in the mucus.

In **Paper IV**, colonic mucus properties from human patients are contrasted with porcine and canine data. These interspecies comparisons indicate that porcine colonic mucus characteristics are closer to human than canine. Intraspecies comparisons between age groups (pediatric vs. adult) and health status (healthy vs. inflamed) reveal different storage modulus values and biomarkers present in colonic mucus. Including more patient and population samples could reveal additional patterns and protein markers, which could be beneficial for designing population-based or disease-based artificial colonic mucus.

Permeability studies are useful for understanding compound permeation rates. Even though apparent permeability (P_{app}) is useful for studying permeation in vitro (**Papers I & V**), these values need to be translated to effective permeability (P_{eff}) in vivo for proper scaling. Future studies should investigate the correlation and scaling factors between P_{app} through mucus and translation to P_{eff} to further improve the PBPK model.

Popular scientific summary

There are diseases in the human body, which is why we develop medicines. Most people prefer to take their drugs by mouth, a method known as “oral administration.” In the digestive tract, the large intestine (colon) is located at the terminal end. The colon contains the largest amount of the human microbiome and has a lower water content compared to the small intestine. The colon is shaped like a tube, with gels that protect the lining from infection and help form feces. Due to its location and specific conditions, drug delivery and absorption in the colon are challenging. One important factor is the colonic gels (mucus layer), which are thicker than in other parts of the body and may hinder drug absorption.

In this thesis, the characteristics of mucus and how it may influence drug absorption was studied. To treat colonic diseases, drugs must reach the affected area. For example, when a drug is in tablet form, it must dissolve before the body can absorb it. Therefore, understanding the properties of mucus helps us understand how it interacts with drugs.

Before drugs are tested in humans, animal testing is often conducted. But is it really necessary? Are the organs or tissues of animals the same as those of humans? If not, can the information gained from animal testing be applied to humans? This is why species comparison studies are important for understanding the similarities and differences in colonic mucus properties. In this thesis, it was found that pig mucus is more similar to human mucus than dog mucus.

Even after we better understand colonic mucus properties, challenges remain. Each mucus collection yields only a limited amount. The collection process may require sacrificing animals, and human patients may experience discomfort. To address this, formulation technology allows us to create artificial colonic mucus. This artificial mucus is a biosimilar gel with properties similar to native colonic mucus, reducing the need for animal studies. We have demonstrated that formulated artificial colonic mucus enables the study of drug-mucus interactions in a scalable, economically feasible way, producing robust results similar to those with native mucus. This technology allows us to study particle movement in mucus, using machine learning to analyze movement and identify which particles move fastest, depending on their size and surface charge. Another application is studying how mucus thickness affects drug absorption. By using 3D printing, the mucus thickness can be controlled, enabling reproducible results. The drug absorption studies that are performed on the lab bench can then feed data into computational simulations based on human data, with the aim to predict drug concentrations in different body parts. This approach is called physiologically based pharmacokinetic (PBPK) simulation. In the thesis it was demonstrated that mucus influences the absorption profile and contributes to changes in drug concentration in the blood.

Populärvetenskaplig sammanfattning

Det finns sjukdomar i människokroppen och det är därför vi utvecklar läkemedel. De flesta föredrar att dricka eller svälja sina läkemedel vilket kallas "oral administrering". Tjocktarmen (kolon) utgör den sista delen av matsmältningskanalen. Kolon innehåller den största delen av människans mikrobiom och har en lägre vattenhalt än tunntarmen. Kolon är formad som ett rör med ett slem på rörets inre yta som skyddar från infektioner och hjälper till att bilda avföring. På grund av tarmens fysiologiska karaktär är läkemedelsleverans och absorption i tjocktarmen utmanande. En av de viktiga orsakerna är att slemskiktet är tjockare jämfört med andra delar av kroppen och kan hindra läkemedelsabsorptionen.

I denna avhandling karaktäriserades slemegenskaperna och hur slemmet kan påverka processen för läkemedelsabsorption studerades. För att kunna behandla sjukdomar i kolon måste läkemedlet nå det drabbade området. Till exempel måste ett läkemedel i tablettform först lösas upp för att kunna absorberas av kroppen. Därför skulle förståelse av slemmets egenskaper göra det möjligt för oss att förstå slemmets interaktion med läkemedlet.

Innan läkemedel testas på människor utförs ofta djurförsök. Men är det verkligen nödvändigt? Är djurens organ och vävnader desamma som människors? Om inte, är informationen som fås från djurförsök tillämpningsbar på människor? Det är därför viktigt att jämföra likheter och skillnader i egenskaperna hos kolonslem mellan olika arter. Resultaten från avhandlingsarbetet visar att grisslem är mer likt människans slem än hundens.

Även om vi förstår kolonslemmets egenskaper bättre återstår fortfarande utmaningar. Vid varje insamling av slem finns det bara en begränsad mängd tillgänglig. Under insamlingsprocessen kan djur behöva avlivas och patienter kan uppleva obehag. För att hantera utmaningarna kan formuleringsteknik användas för att skapa artificiellt kolonslem. Det artificiella kolonslemmet är en biosimilar gel med egenskaper som liknar naturligt kolonslem vilket minskar behovet av djurstudier. Det formulerade artificiella kolonslemmet som utvecklats under avhandlingsarbetet möjliggör studier av läkemedelsinteraktioner med slem på ett skalbart och ekonomiskt genomförbart sätt, vilket ger robusta resultat som liknar de för naturligt kolonslem. Denna teknik möjliggör studier av partikelrörelser i slem och genom att använda maskininlärning kan rörelsemönster analyseras och informera om vilka partiklar som rör sig snabbast beroende på deras storlek och ytladdning. En annan tillämpning av artificiellt kolonslem är att studera slemtjocklekens inverkan på läkemedelsabsorption. Med hjälp av 3D-skrivning kan kontrollerad tjocklek uppnås vilket möjliggör reproducerbara resultat. De erhållna absorptionsdata kan användas i datorsimuleringar baserade på mänskliga data för att förutsäga läkemedelskoncentrationer i olika kroppsdelar. Denna metod kallas fysiologiskt baserad farmakokinetik. Avhandlingsarbetet har visat att slem påverkar absorptionsprofilen och faktiskt bidrar till förändringar i läkemedelskoncentrationen i blodet.

Ringkasan ilmiah populer

Terdapat penyakit dalam tubuh manusia, dan itulah sebabnya kita mengembangkan obat-obatan. Kebanyakan orang lebih suka minum atau mengonsumsi obat mereka, yang kita sebut sebagai "administrasi oral". Dalam konteks saluran pencernaan, usus besar (kolon) terletak di ujung terminal. Lingkungan kolon juga menampung mikrobioma manusia dalam jumlah terbesar dan memiliki kadar air yang lebih rendah dibandingkan dengan usus halus. Kolon berbentuk seperti tabung, dengan lapisan lendir yang melindungi tabung dari infeksi sekaligus membentuk feses. Karena faktor lokasi dan kondisi kolon, penghantaran dan penyerapan obat ke kolon menjadi sulit. Salah satu faktor yang menarik untuk diteliti adalah lapisan lendir (lapisan mukus) kolon, yang lebih tebal dibandingkan bagian tubuh lainnya dan dapat menghambat proses penyerapan obat.

Dalam tesis ini, penelitian berfokus tentang karakteristik mukus, dan bagaimana hal itu dapat memengaruhi proses penyerapan obat. Ketika kita ingin mengobati penyakit kolon, obat harus mencapai area tersebut. Bayangkan, ketika obat dalam bentuk tablet kering, obat tersebut perlu dilarutkan agar dapat diserap oleh tubuh. Oleh karena itu, pemahaman tentang sifat-sifat mukus akan memungkinkan kita memahami interaksi mukus dengan obat.

Sebelum obat diuji pada manusia, pengujian pada hewan sering dilakukan. Namun, apakah hal ini benar-benar diperlukan? Apakah kondisi organ atau jaringan hewan sama dengan manusia? Jika tidak, apakah informasi dan pengetahuan yang kita peroleh dari pengujian pada hewan dapat diterapkan pada manusia? Inilah mengapa studi perbandingan spesies penting untuk mengetahui lebih lanjut tentang persamaan dan perbedaan sifat-sifat mukus kolon. Dalam studi kami, kami menemukan bahwa mukus kolon babi lebih mirip dengan manusia daripada anjing.

Setelah kami memahami lebih lanjut tentang sifat-sifat mukus kolon, masih terdapat beberapa tantangan. Untuk setiap pengumpulan mukus, jumlah yang tersedia terbatas. Selama proses pengumpulan sampel, hewan mungkin perlu dikorbankan dan pasien manusia akan merasa tidak nyaman. Untuk mengatasi masalah ini, dengan teknologi formulasi, kami dapat membuat mukus kolon buatan. Mukus kolon buatan secara konseptual merupakan gel yang serupa mukus asli, yang memiliki sifat-sifat mukus kolon asli. Oleh karena itu, studi pada hewan akan lebih sedikit diperlukan. Dalam penelitian ini, telah ditunjukkan bahwa formulasi mukus kolon buatan memungkinkan orang untuk mempelajari interaksi obat dengan mukus, dalam hasil yang terukur, terjangkau secara ekonomi, dan struktur yang mirip dengan mukus kolon asli. Teknologi ini memungkinkan kami untuk mempelajari pergerakan partikel dalam mukus, yang digabungkan dengan pembelajaran mesin (machine le-arning) untuk menganalisis pergerakan partikel dan menemukan tren partikel mana yang akan bergerak paling cepat, tergantung pada ukuran dan muatan permukaan partikel. Aplikasi lain dari mukus kolon buatan adalah untuk mempelajari pengaruh ketebalan mukus terhadap penyerapan obat. Dengan menerapkan teknologi pencetakan 3D (3D-printing), ketebalan yang terkontrol dapat dicapai, memungkinkan hasil penelitian yang dapat direproduksi. Dalam mempelajari penyerapan obat, kita dapat menggunakan simulasi komputasi berdasarkan informasi manusia untuk membantu memprediksi konsentrasi obat di sekitar bagian tubuh kita. Pendekatan ini disebut simulasi farmakokinetik berbasis fisiologis (Physiologically based pharmacokinetic -PBPK). Di tesis ini, penelitian telah menunjukkan bahwa keberadaan mukus di kolon akan memengaruhi profil penyerapan, dan memang berkontribusi pada perubahan konsentrasi obat dalam darah.

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For being another home in Uppsala (or anywhere in the world!), organizing fun events, and saving me from constant instant noodles. **Bu Juju** & **Stefan** Ekberg, for always hosting the Indonesian community and keep the fraternity spirit alive. **Jerry**, the sultan which brings people together, grateful for your support to promote closeness for all of the Indonesian and the constant julidness. **Amel**, for all the cheer and cooking session tryouts, may you find the happiness you are seeking for. **Angel**, as the translator of Gen Z and keeping me up with the pop culture shenanigans. **Farah**, as the bestie, Gen A-Z great advisor, supplier of konditori stuff, and the moral compass for all people (me especially). **George**, for occasional lunch & fika, fellow masseuse specialist, hope we can open it as the side business. **Isak**, for fun times with the doggos and karaoke session. **Odi**, for all of the laughs. Master **Biyanto**, for all of the wise thoughts. **Rifa** & **Aini**, for your support and being understanding besties. **Rosi**, your small suitcase is extremely useful for travelling and sample handling 😊 **Devi A.**, for taking care of my pants’ & jackets’ hole. **Olin**, for all fun afterwork session. Habib **Suraj**, one of the trustworthy Habib with plenty of wisdom. **N. Alvin**, the spirit of East Java and all of the swear words. **Gaby** (cwk), for all of the supports and deep talks, and **Gaby** (cwk), for all of the cooking session. **H. Diva**, hope the ghost in your apartment is now tamed, for always providing a meeting place. **Diaz** & **Daniel**, another fellow PhD student. **Bella** Salma Salsabila, may your next concert in Svalbard be successful as always. **Yuda & Fathia**, for being the best snack supplier & successor to Hasanah Tjahaja Konditori AB. **Mbak Dr. (cand.) Hj. (cand.) Nden** & **Dr. H. (cand.) Reza Fachrizal**, for being a role model of a balance between many different aspects of life, and always being kind towards all people. **Mbak Octa**, leader of Indonesian in BMC, **Mas Manu**, for all of the amazing songs playlist. **Mas Mumtaz** & **Mami Faikha**, for hosting us so many times. **Obie**, for sharing the same interest around "cultured taste" of things. **Aldrian**, for the tempeh supply and haircut training session. Angelina (**Aling)**, for a random hiking session and a guided tour, deep philosophical talks, looking forward for more 👣. Lord **Alfa**, Gus **Luthfi**, **Alvin W**iwiet(berg), **Om Wandi** & fam, **Nath**aniel **Pono**, **Averina**, **Siska**, **Oline**, **Fahri** & **Resti**, **Hikma**, **Reza** potatis, **Galih**, **Vera**, Hj. **Anil**, the legendary **Mas Abdul** & fam, **Raufzha** & **Dina**, **Farid**, **Ayu**, **Yussy**, **Nisrina**, **Mila**, **Teh Ira** & fam, **Vigha**, **Devi N.**, **Celine** & **Randy**, **Raras**, **Said**, **Ko Daniel** & **Ci Desis**, Mas **Dwiyan** – 4D family, Mas **Moko** & fam, **Thomas**, **Dantie**, **Erina**, **Lukas**, **Hikma**, **Mbak Tri**, **Stephen**, **Mas Damar**, **Bang Doly, Kak Jenny & Kak Lanny, Bli Komang, Rheza, Edward T.** and many more people which interacted with me, such a surprise eh Indonesians are everywhere 😎

For high school nakama, never-ending school moment with you guys, **Fani**, **Pipin**, **Ipon**, **Andre**, **Marcel**, **Lina**, **Yesti**, **Chrysta**, **Clifford**, **Ricky**, **Mele**, for all of the reassurance, cheering, and moral support. **Nicho Benito**, we are pursuing our goals together, one as an artist, and the other as a scientist. As this is your stage name, let it be more famous 😊 **Glenis**, **Wira**, **Om Vava**, **Livy,** for all memes and IG reel-a-tionships. Grateful to everyone for staying with me and grow together into each of our own direction.

For **Biotech Atma Jaya Univ. / Biomedical Engineering Chongqing Univ. / COVID Lab UnTar** friends, which stays connected (partially / fully) with me through this learning journey. Especially *for those struggling in the further postgrad - PhD studies* too 😂 **Vicky Vania**, for being one of the initial motivations when I started this journey. May you get the happiness you are wishing for. **Febrilina**, for wisdom and in-depth conversation as always. **Lisda**, for sharing the struggles as PhD student, best of luck and you are doing great! **Isna**, for welcoming me in Leuven, best of luck! **Natassa**, for showing me around Leiden - Netherlands, talk about life, sharing the struggles in EU. **Lena**, for all late-night talks and life questions. **Steffanus**, for sharing stories and research journey. **Bos Yuu**, for showing me around Philly, catching up with life, and many more brainstorming of great questions! **Alex & Ian**, for all shares of PhD struggles and geopolitical situation updates. **Rika**, **Andy**, **Muflih**, **Visakha**, Habib **Mayer**, **Brian, Edwin, Vincent A.** for life updates and encouragement every now and then.

In this random mixed up life journey, I get to meet and click with different people. **Oza**, a friend since grade school & shared occasional talks. **Chyntia**, sharing same dreams of working abroad. **Wendy**, RPG discussion friends and emotional support. **Parya**, for the fika time and talks. **Yaren**, **Kaelan,** and **Yifan** for being a nice corridor friend, reminder for a good food every now and then. **Daniel A**. for being a professional innebandy to the extend you can help grab my key!

For my family, **Papi** which drills and trains into me the business & marketing principles, which surprisingly can be applied universally even in life science research. For giving freedom of choosing life paths, and keep on believing that your child is indeed doing something useful, though it is a bit weird and sort of unknown for "regular people". **Mami** for taking care of plenty of things in the house and keep on praying for us. I still remember how you are trying your best to teach me about the science of solar & lunar eclipse which is a core memory for me. **Marcia**, for being one of the motivations for me. Life will always find the way; may your smile keeps blooming every day as always. **Marcella**, which takes over plenty of duties around the house, and still manages to splendidly develop yourself. Hope you keep on pursuing your dream to study abroad and travel the world. To **Cimmy** and **Cookie**, for always cheering me up, loving me unconditionally, teaching me how to enjoy life and make the best out of every situation. And of course, for all of the prayers, supports, constant check-in, story sharing, giving a role model of "adulting" and encouragement from **Kongkong** & **Ama**, **Iie Silvie**, **Om Thomas**, **Noah**, **Kuku** & **Tante Arum**. **Iie Nagari**, which always been around and cheering us. The huge **谢 family**, including **Cek Lok**, the uncle which I just knew exist in NJ, USA, which welcome me dearly. Recently passed away **Be Cek Kong**, which has an inspiring career of eastern – western medication, opening up my view of the world though our interaction is relatively short.

For anyone struggling with low self-esteem, PTSD, depressive episodes, and apathy towards life: There is always hope for better days. I see your pain, and trust me that you are not alone. There are people celebrating your existence and your struggles would never be in vain.

You might reach the end now, and not finding your name… doesn't mean you are forgotten, simply because I might unintentionally find it challenging to fit you here (It's not you, it's me! See how I would qualify as a politician? 🤣). I swear I want to write more about you guys, but… this kappa has reached more than 100 pages, I might need a Thor's hammer and a Longinus' spear to nail it (literally – wait for the video). So, you can fit yourself here: ….

And I would like to say thank you for your contribution and reminding me that I am indeed not perfect, and need to learn continuously! Deeply grateful for our friendship. Terima kasih! I have received your love / affection indeed.

To myself, which continuously improving and learning, though it is indeed quite hard. Bra jobbat! Jättebra! Writing this thesis makes me realize plenty of things that I have undergone through in these 4.5 years-ish + 7 years of education in bio- field, seeing how much I progress in terms of knowledge, way of thinking, together with handling perilous situations and emotional challenges. Crossing across "different research fields" is indeed challenging, or maybe there is no such label or concept as "a field" right? It is just one big biology – the science of life!

"…Then I may learn to love myself
A little more than before…" – "Towards a dream" by Jasmine Ikari Webb

"…私は 私を好きになれるよ…" – "願いへ" by Daisuke Kaneda
From Persona 5 Strikers (one of the best games I've ever played!)

Thanks for reading! Hope it will be useful somehow, happy learning 😎

Uppsala, Summer – Autumn 2025 (Extreme write-a-thon tiki-taka!)
Sincerely (as brave as always, as honest as I strive to be),
Leonardo Paolo Marco Tjakra 谢俊吉

つづく

おまけ

Omake Project

- The side stories -

Literally it means bonus/extra, commonly known as “bloopers” in the film industry.

May this unusual addition which is part of my acknowledgement makes you feel joyful, surprised, and inspired, just like scientific discovery!

“***The whole world is a product of cognition****... ...not just the Metaverse. It can be* ***freely re-made****... The same goes for you, and everyone else. Soon a new world will come. One where mankind isn't held captive. The world will shine brightly as long as you hold hope in your hearts. Remember... There's no such thing as the "real" world. What each person sees and feels— Those are what shape reality. This is what gives the world infinite potential. Even if you feel that only darkness lies ahead... As long as you hold hands together... See it through as one... ...the world will never end! The world exists within all of you...!” – Morgana, Persona 5 –*

Figure O1. Life is a school and so is the people that you meet! Anything and anyone can be a chance to learn! 📖

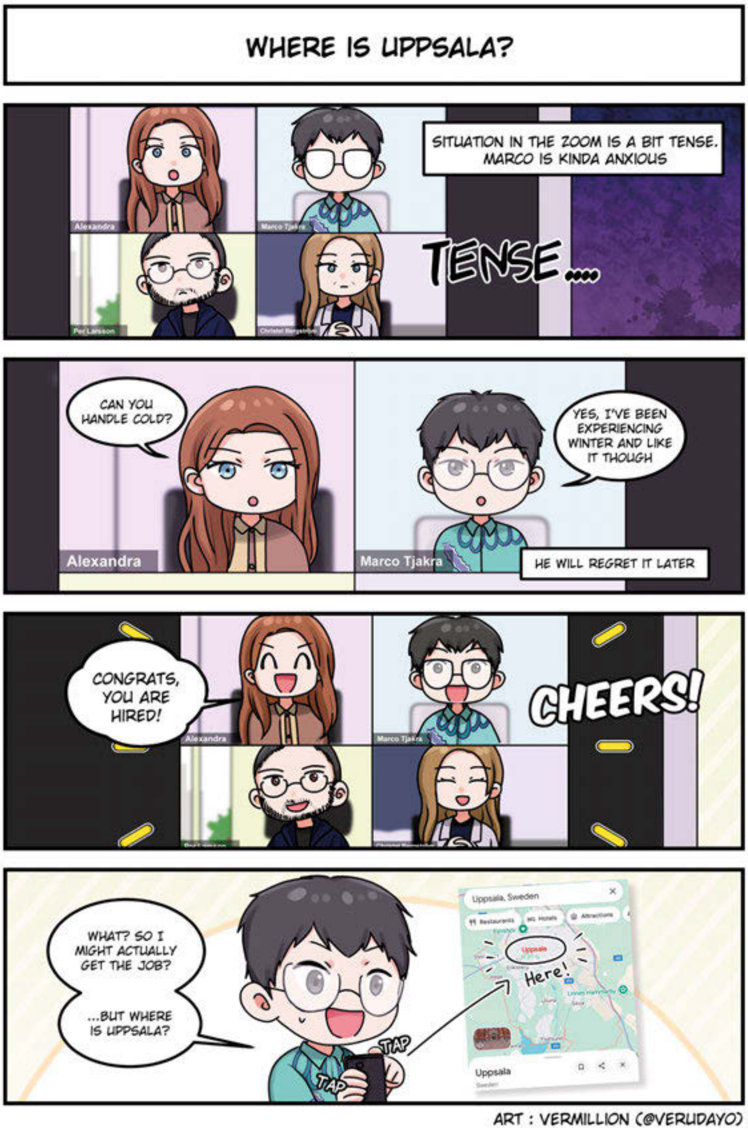

Figure O2. Check the job description thoroughly before you apply! 🤣 Memorable 3rd interview of the recruitment process with Kicki, Alexandra, and Per L.

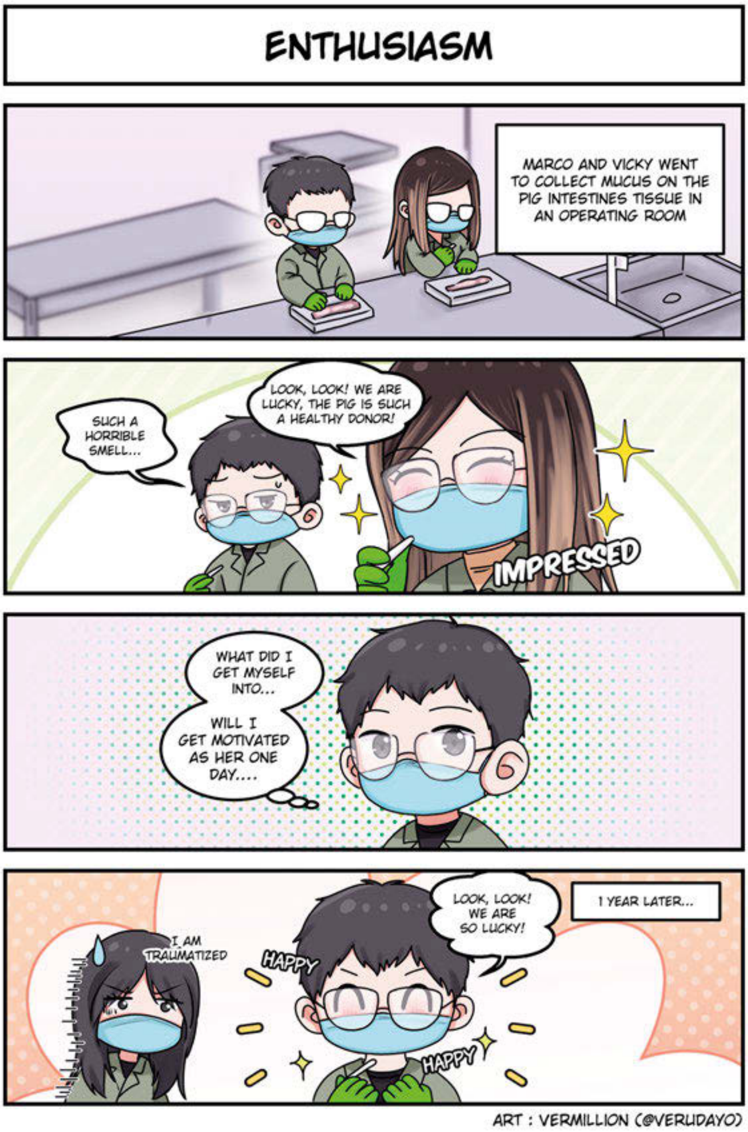

Figure O3. Give things some time, oddly enough you will get enthusiastic and find joy in it! Hope you will have more mucus collection session Mingjun 🤪

Figure O4. Take some break every now and then, give your body chance to rest and recover 😊 but I agree with you Nopdanai, somehow (purified) mucin smells tasty 🤣

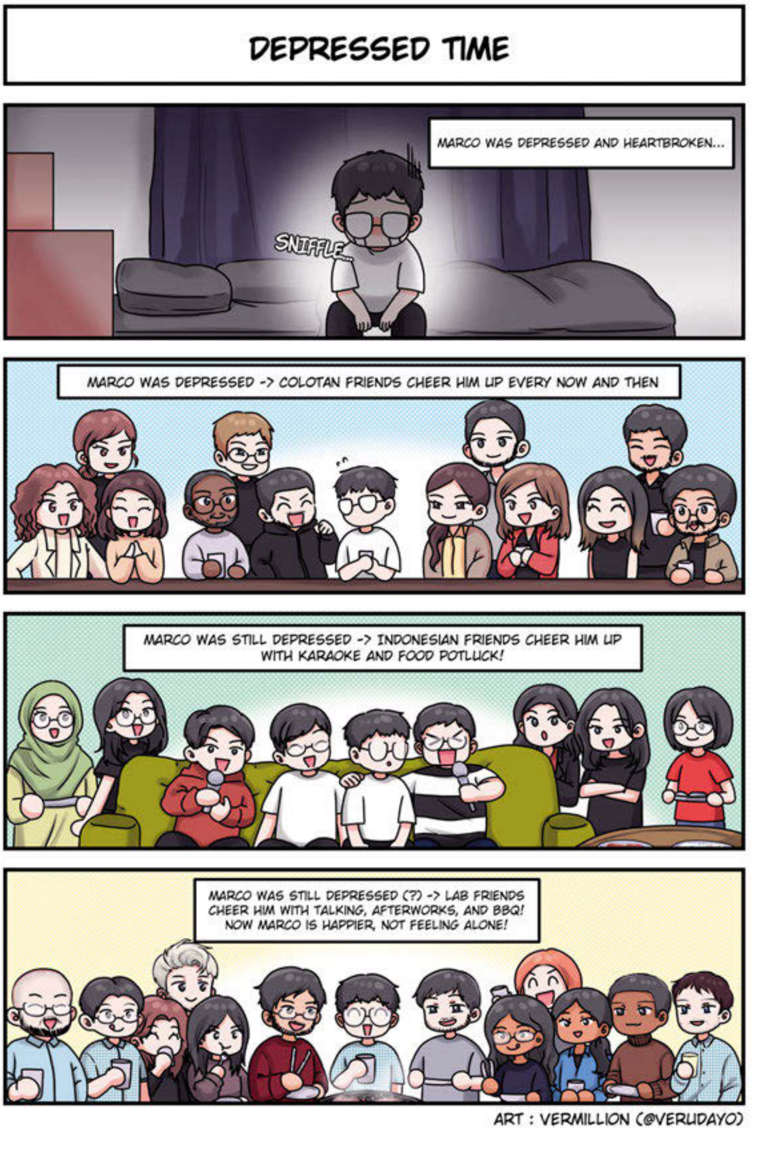

Figure O5. Sometimes life doesn't go as planned – and that is okay! That is why it is so important to surround yourself with supportive people and community, build a *social scaffold* for yourself 🌏🌏🌏 Be brave to acknowledge vulnerabilities and things might be improving! Honestly, I am a bit afraid to share this part of my journey, but I sincerely hope this will help others with a similar problem around depression, that they are not alone.

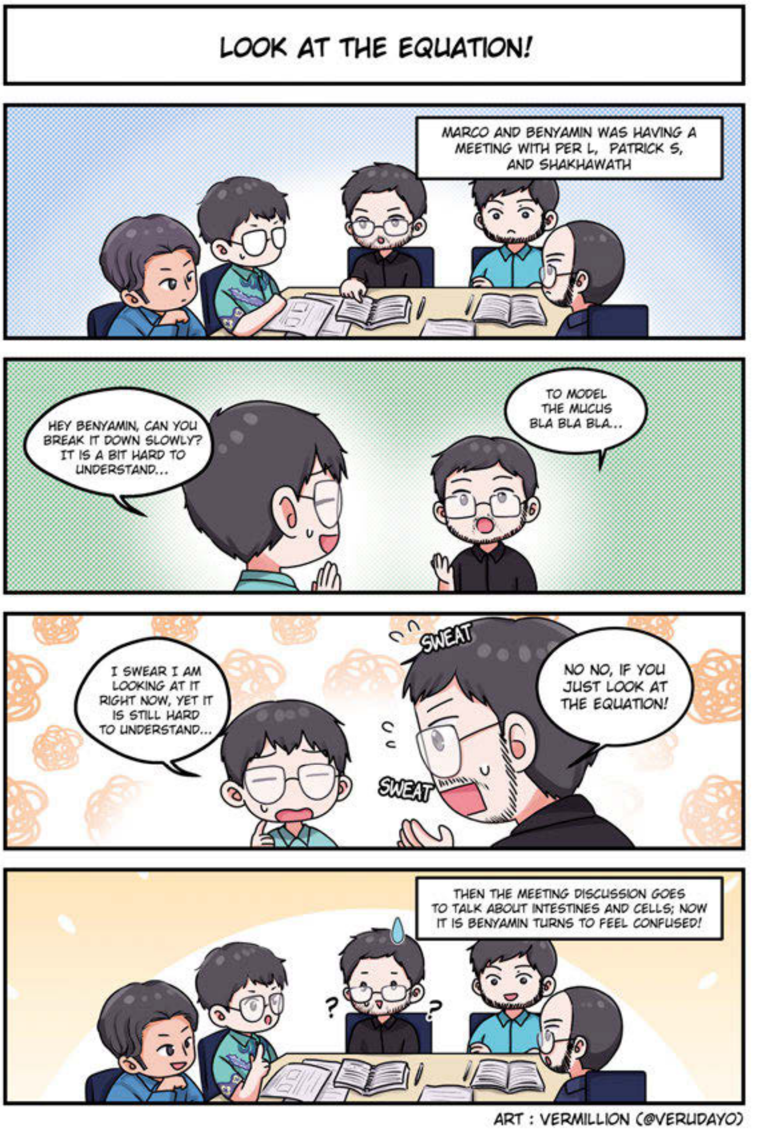

Figure O6. Interdisciplinary research is challenging, mostly because we use different terminology to talk about things. I think the key is communication and patience, the result would be rewarding! 👨‍🔬👩‍🔬💊⚙️🛠️

Figure O7. Enjoy the process and sometimes it is good to do stuff spontaneously! 🍾 Never lose the sense of joy! Any guess how do Benji get the champagne?

Figure O8. Conference time is a fun time to explore the world and connect with more people! If it feels scary, you can chill anyway ⛷️🌭 It is so cool to see the “mucus team” expanding with different directions and focuses!

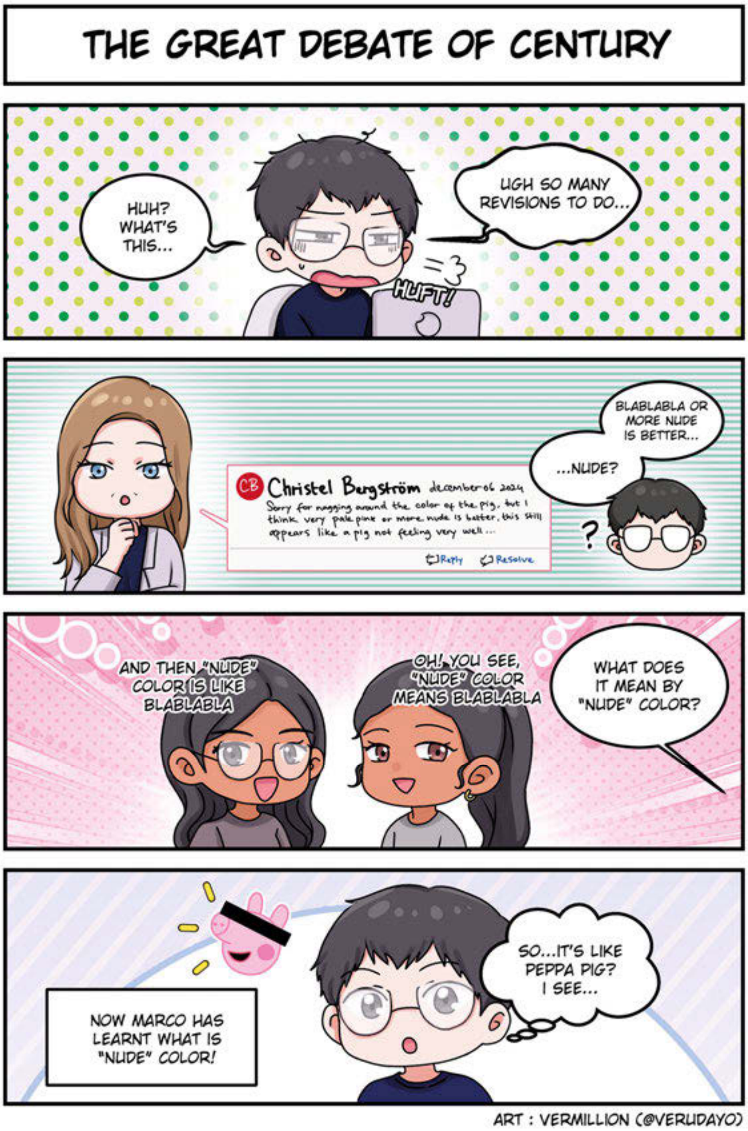

Figure O9. Color is such a relative label, but it is fun to learn more about its' term 🌈

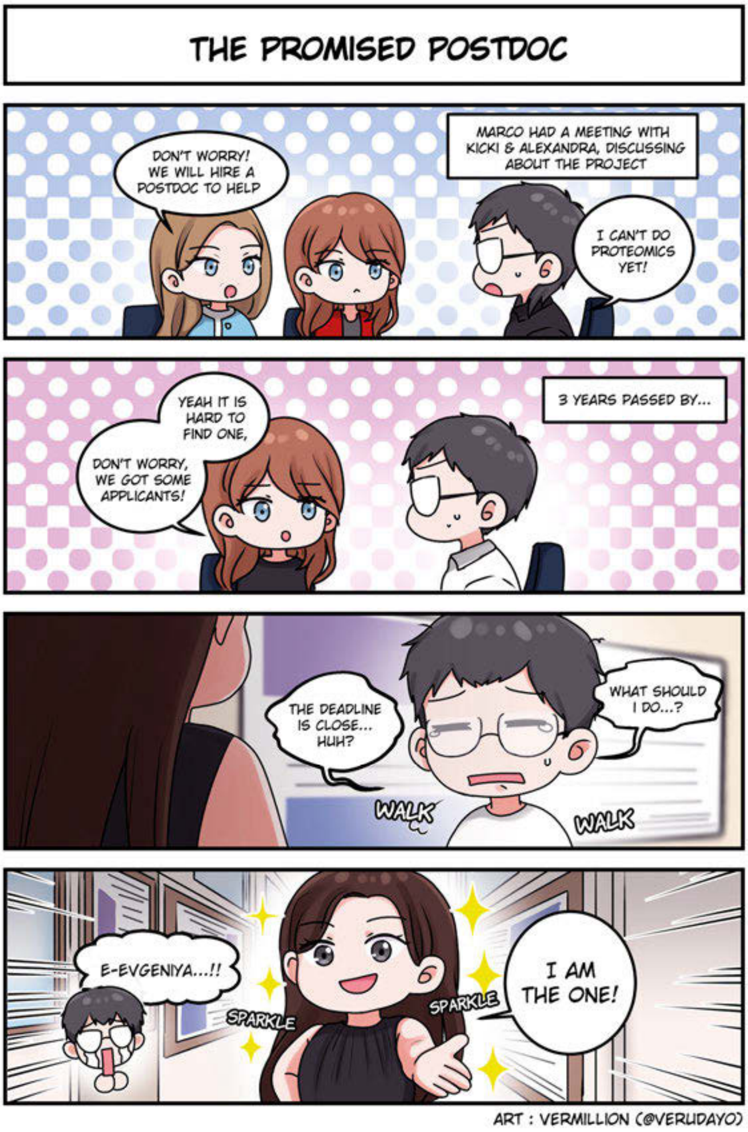

Figure O10. Never ever give up, help surely will come! One way or another, life will find a way! 😝 So is this omake project, under such a tight deadline, we (notably @Verudayo) manage to push through!

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