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Native Gastrointestinal Mucus: Models and Techniques for Studying Interactions with Drugs, Drug Carriers, and Bacteria

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Abstract

Gastrointestinal mucus plays essential roles in modulating interactions between intestinal lumen contents, including orally delivered drug carriers and the gut microbiome, and underlying epithelial and immune tissues and cells. This review is focused on the properties of and methods for studying native gastrointestinal mucus and its interactions with intestinal lumen contents, including drug delivery systems, drugs, and bacteria. The properties of gastrointestinal mucus important to consider in its analysis are first presented, followed by a discussion of different experimental setups used to study gastrointestinal mucus. Applications of native intestinal mucus are then described, including experimental methods used to study mucus as a barrier to drug delivery and interactions with intestinal lumen contents that impact barrier properties. Given the significance of the microbiota in health and disease, its impact on drug delivery and drug metabolism, and the use of probiotics and microbe-based delivery systems, analysis of interactions of bacteria with native intestinal mucus is then reviewed. Specifically, bacteria adhesion to, motility within, and degradation of mucus is discussed. Literature noted is focused largely on applications of native intestinal mucus models as opposed to isolated mucins or reconstituted mucin gels.

Graphical Abstract:

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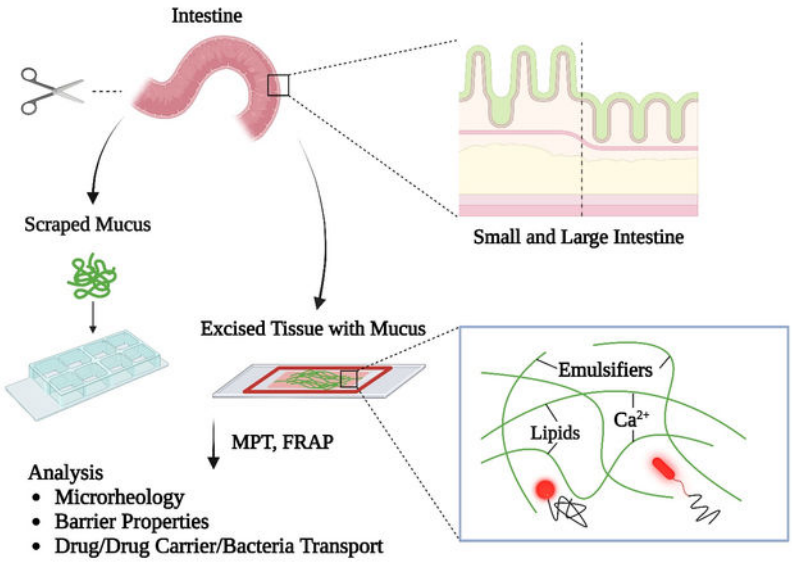

Keywords

Native intestinal mucus; Mucus structure; Mucus composition; Mucus barrier properties; Multiple particle tracking; Fluorescence recovery after photobleaching; Mucosal drug delivery; Bacteria-mucin interactions

1. Introduction

Mucus lining the gastrointestinal tract provides a barrier protecting the underlying tissue against bacterial invasion and controlling particulate and nutrient diffusion to intestinal epithelial cells [1–4]. The gut microbiota has been increasingly recognized to be tremendously significant to human health through multiple functions including secretion of metabolites affecting the host, modulation of immune function [5], and modulation of pharmaceutical compound efficacy both directly through drug metabolism and indirectly through changes in host function (e.g., in immunotherapy) [6–8]. The role of mucus-bacteria interactions in the impact of the microbiota is increasingly appreciated [9]. Further, as oral drug delivery remains the preferred route of administration for most drugs, and all drugs and drug carriers must pass through the intestinal mucus before reaching the site of absorption, intestinal mucus is highly significant in consideration of design of both traditional and novel drug delivery systems, including bacteria which can serve as probiotic supplements and have themselves been explored as novel carrier systems [10, 11]. Due to the inherent challenge of analyzing the intestinal mucosal interface in situ, it is important to consider experimental systems and analytical methods that can be applied to study intestinal mucus, including its rheological and associated barrier properties, and specific interactions with drugs, drug carriers, and microbes. There have been multiple excellent reviews relevant to mucus and mucus models [4, 12–17]. In this review, we focus specifically on experimental systems and analytical methods useful for analysis of gastrointestinal mucus as relevant to drug delivery and interactions with bacteria. First, the composition and structure of gastrointestinal mucus, as is relevant to its barrier properties and interactions with drugs, drug carriers, and bacteria,

as well as the suitability of different mucus models in capturing relevant aspects of mucus *in vivo*, are reviewed. This information is provided in part to assist the reader in evaluating the suitability of different experimental systems for capturing relevant features of the native mucosal barrier as important in their particular scientific inquiry. Next, different experimental methods for studying native intestinal mucus and specifically its role in drug delivery are presented, again with the practical goal of assisting the reader in assessing tradeoffs between different experimental techniques which may be useful for answering specific questions relevant to drug delivery. Given the emergence of bacteria as potential therapeutics and the role of the gut microbes in impacting drug delivery, particular attention is afforded to bacteria-mucus interactions. This review focuses on information relevant to models and techniques used to study the intact intestinal mucus barrier, and conversely does not focus on methods or properties of purified mucin gels, which have recently been reviewed [18].

2. Features to Capture in Mucus Models: The Composition and Properties of Mucus Along the Gastrointestinal Tract

2.1 Components of Gastrointestinal Mucus

Gastrointestinal mucus barrier properties are largely determined by composition. Gastrointestinal mucus is comprised of water (over 95%), heavily *O*-glycosylated mucins, lipids, salts, antimicrobial peptides (e.g., defensins), other proteins secreted by the intestinal epithelial cells (e.g., IgG Fc-binding protein (FCGBP), Trefoil factor 3 (TFF3), Resistin-like molecule (RELM) β)) and cellular debris [19–21]. A comprehensive proteomic, lipidomic, and metabolomic analysis of composition canine mucus along the GI tract revealed significant differences in composition along the GI tract [22]. The principal structural component of mucus is mucin, a highly *O*-glycosylated protein. MUC2 mucin is the main gel-forming mucin in the intestine, while gastric mucus is mainly composed of MUC5AC mucin [23]. In addition to secreted, gel-forming mucins, transmembrane mucins can also be found at the intestinal epithelial apical surface [24–26]. This review mainly focuses, however, on models and studies of gel-forming mucins, and predominantly MUC2 mucin, as it comprises the bulk of the barrier experienced by drugs, drug carriers, and bacteria within the intestinal lumen. Mucins interact with each other covalently (e.g., via disulfide bonds) and non-covalently (e.g., via hydrophobic, hydrogen bond, and ionic interactions). Interactions between mucin molecules, as well as between mucins and other mucus components, regulate the viscoelastic properties of intestinal mucus and control drug/particulate diffusion and microbe penetration through the mucus layer [27, 28].

Purified mucins and gels formed via interactions of purified mucins have proven to be highly useful tools to study the biology of mucus and its role as a barrier to drug delivery [18, 29–32], but one major consideration in their use is to what degree interactions between purified mucins recapitulate those in native mucus. It has been noted that the purification process can result in degradation of the mucins, as reflected in differences in rheological properties of native mucus gels relative to reconstituted gels formed from purified mucins [2, 33]. Even the mechanical forces exerted while collecting native mucus from tissue can alter its structure and associated barrier properties [17]. One advantage to using native mucus models

is thus that molecular interactions are inherently more intact, and non-mucin components that may be important in network formation are present. Non-mucin components that impact intermolecular interactions within mucus include proteins, lipids, and salts. FCGBP has been proposed to covalently interact with mucins to create a mesh-like structure [34, 35], although these interactions have been debated [36]. Lipids constitute up to 2% of mucus mass and impact mucus viscoelastic properties through covalent and hydrophobic interactions with mucin molecules [17]. Murty et al. have demonstrated that removal of lipids associated with glycoproteins from canine gastric mucus resulted in 80–85% decrease in viscosity [37]. Mineral salts account for up to 1% of mucus mass and can directly change mucus barrier properties by modulating the electrostatic interactions within the mucus network [17].

One advantage to using reconstituted mucin gels is the ability to directly investigate the impact of mucus components like salts on mucin interactions and gel properties via their inclusion in rehydration medium. Lieleg et al. demonstrated that a high concentration of sodium chloride significantly increased amine-functionalized particle motility through a reconstituted 1% mucin gel at pH 3, perhaps due to a shielding effect weakening the electrostatic interactions between positively charged amine groups and negatively charged mucins [31]. In a recent study by Wagner et al., reconstituted MUC5AC gels were found to have increased viscoelastic moduli in the presence of a high concentration of sodium chloride at neutral pH [38]. The enhanced ionic strength appears to weaken the electrostatic repulsion between mucins, which permits stronger interactions between hydrophobic entities of mucins, thus altering the viscoelastic moduli of the mucin gel. These findings support the concept that changes in interactions between mucus components can modulate mucus barrier properties as relevant to drug delivery.

The intermolecular interactions and high water content within gastrointestinal mucus render it a viscoelastic hydrogel with dynamic and heterogeneous rheology reflective of variations in composition (mucins, mucin glycosylation, non-mucin components, etc.) with anatomical position (e.g., stomach vs. colon) as well as age, diet, and the presence of specific commensal and pathogenic microbes [17]. In studying the intestinal mucus barrier to drug delivery as relevant to specific disease states, it is important to consider how changes in mucus composition and mucus rheology associated with certain physiological states impact drug and drug carrier diffusion. For example, patients with Hirschsprung’s disease (HD) were found to have reduced MUC2 mucin production [39] and altered mucin glycosylation, with an increased ratio of neutral mucins to acidic sialomucins in their intestinal mucus [40]. These changes might contribute to altered intestinal mucus barrier properties measured in a mouse model of HD, as reflected in changes in nanoparticle and bacteria transport rates through intestinal mucus on harvested tissue [41]. Exposure to food-associated stimuli can alter mucus viscoelasticity, modifying the mucus barrier to particulate species and bacteria [42, 43]. Yildiz et al. demonstrated that rodent ingestion of soybean oil reduced mucosal transport of nanoparticles across intestinal mucus, perhaps due to hydrophobic interactions between mucins and lipids and increased cross-linking within the mucus network [42]. Consideration of fat content in the intestinal lumen associated with the fed state, as well as lipids in drug delivery systems, may thus be important in analysis of drug carrier transport through intestinal mucus. These examples highlight the importance of considering how relevant states of disease and/or health may impact the mucus barrier itself.

Glycosylation of mucins is highly significant in interactions of drugs, drug carriers, and bacteria with mucins, as mucins are 85% carbohydrate by weight. While there are *N*-glycosylation sites on MUC2 mucin, *O*-glycosylation accounts for the bulk of the carbohydrate side chains that emerge from the mucin protein backbone and impart an elongated, bottlebrush-like structure to portions of the molecule. All mucins contain at least one domain abundant in proline, serine, and threonine amino acids (PTS domain), providing *O*-glycosylation *O*-linkage sites for N-acetylgalactosamine (GalNAc) [1]. Varying numbers of glycan residues, including galactose (Gal), N-acetylglucosamine (GlcNAc), and GalNAc, extend from the GalNAc to construct oligosaccharide side chains typically ranging from 2–12 sugar residues, and these chains may include and often terminate with fucose, sialic acid, or sulfated sugar (Fig. 1) [44]. Sialic acid and sulfate residues impart a net-negative surface charge which is significant in interactions with drugs and drug delivery systems. Terminal ends of the glycan chains can form significant antigenic structures including the ABO blood group determinants and Lewis antigens [45]. Sugar residues on mucins are crucial for regulating bacteria-host interactions by providing attachment sites and nutrient sources [45, 46]. As mucin carbohydrates present binding sites similar to those on epithelium, pathogen binding with mucins can prevent contact with epithelial cells [47].

2.2 Intestinal Mucin Domains

Human MUC2 mucin has around 5200 amino acids that constitute multiple distinct domains. These domains appear in the following order from the N-terminus to the C-terminus: von Willebrand D1 (VWD1), VWD2, VWD´ (an incomplete Von Willebrand D domain), VWD3, first Cysteine-rich domain (CysD), short PTS domain, second CysD, long PTS domain, VWD4, VWB, VWC, and Cystine knot (CK) [48] (Fig. 2). MUC2 mucins are dimerized after translation in the endoplasmic reticulum through formation of disulfide bonds covalently linking CK domains at C-terminal tails [49, 50]. Subsequently, MUC2 mucin dimers are delivered into the Golgi apparatus where *O*-glycosylation of serines and threonines in the PTS domains occurs. The mesh-like structure of mucus is then rendered by disulfide bonds covalently linking VWD3 domains at N-termini to form trimeric structures [51]. After polymerization, MUC2 mucins are compactly stored in the regulated secretory granules of goblet cells. The packing and release of MUC2 mucins is a sophisticated process controlled by pH and calcium ion concentration. Folded MUC2 is densely packed in the secretory granules where the pH is around 5.2 and calcium ion concentration is high. After secretion into the neutral pH, lower Ca^{2+} concentration environment of the intestinal lumen, MUC2 expands and forms a stratified mucus layer covering the intestinal epithelium [52].

2.3 Macroscopic and Microscopic Structure: Mucus Organization Along the Gastrointestinal Tract

The structure of mucus varies along the gastrointestinal tract (Fig. 3). The stomach has two layers of mucus consisting mainly of gel-forming MUC5AC mucin [23] and serving as diffusion barriers to protect the underlying epithelium against the acidic lumen environment [54]. The inner layer of gastric mucus is firmly adhered to the epithelium, while the outer layer is unattached and can be aspirated [55]. Unlike the stomach and colon, the small intestine contains a single layer of mucus comprised of MUC2 mucin. Jejunum mucus was demonstrated to exhibit stratified MUC2 staining, especially close to the epithelial surface

[56]. Small intestinal mucus is not fixed to the epithelium, and can be easily removed [55]. The mucus layer in the jejunum has been reported to be the thinnest along the gastrointestinal tract (~123 μm in rats) [57]. Mucus in the colon, like that in the stomach, consists of two layers: an outer loose mucus layer that can be easily removed and an inner dense mucus layer that is firmly attached to the epithelial surface [53, 58]. The approximate thicknesses of the colonic inner and outer mucus layers in a rat are 116 and 830 μm, respectively [57].

Small intestinal mucus aids in protecting underlying epithelial cells from bacterial invasion, although the mucus layer itself is penetrable by bacteria [21, 55]. The intermolecular interactions within mucus render a porous hydrogel network. The pore size of gastrointestinal mucus has been reported to range up to approximately 100–200 nm, with some pores larger and some smaller in size [59, 60]. Electron microscopy of canine intestinal mucus along the GI tract indicated the pore size of mucus is smaller in the small intestine than in the colon [22]. Since the small intestinal mucus is not adherent to the epithelial surface, it moves with the peristaltic waves into the large intestine. Goblet cells, especially those residing in the crypts, secrete MUC2 mucins to replenish the mucus layer. Paneth cells and enterocytes secrete antibacterial peptides and proteins such as RegIIIγ into the mucus to create a gradient of factors controlling bacterial invasion from the crypts to the villi, helping to confine bacteria at villus tips [61, 62]. The barriers provided by mucus, antibacterial peptides, and other proteins work collectively to hinder bacterial contact with small intestinal epithelial cells.

In contrast to the relatively low levels of bacteria in the small intestine, the human colon harbors ~10^{14} commensal bacteria [63]. The two distinctive mucus layers that exist in the colon help maintain the balance between this large number of bacteria and host cells [58]. The inner dense mucus layer is firmly attached to the epithelium and impenetrable to bacteria [58]. Together with antibacterial peptides and proteins, this physical barrier protects the epithelial cells from bacterial contact. In contrast, the outer loose mucus layer is permeable to bacteria and serves as the habitat for commensal microbiota [53]. Both mucus layers in the colon are mainly comprised of highly *O*-glycosylated MUC2 mucins. Since the two mucus layers in the colon share the same protein components, it is hypothesized that the outer mucus layer results from transformation of the inner one. Although the mechanism of the transition from the inner mucus layer to the outer layer has not been entirely elucidated, Johansson et al. demonstrated that proteolytic cleavages of MUC2 mucins are necessary for the formation of the loose mucus layer [58]. Proteolytic cleavage within the cysteine-rich domains of MUC2 allows the colonic outer mucus layer to expand its volume by four times compared to the inner mucus layer. It is assumed that endogenous proteolytic enzymes are largely responsible for this process, as germ-free mice still possess a loose mucus layer [58]. Overall, the variability in mucus structure along the GI tract and the non-isotropic nature of mucus structure motivate analysis of intact mucus on tissue or cell culture models.

3. Experimental Systems for Studying Gastrointestinal Mucus

3.1 Advantages and Disadvantages of Different Experimental Systems

Various mucus models [12], including collected native mucus [2, 3, 42, 56, 64–71], excised tissue and animal models [41, 64, 72], purified mucin hydrogels [2, 18, 29–32], conventional *in vitro* cell culture models [73, 74], and organ-on-a-chip systems [75], have been used to study gastrointestinal mucus properties. These different systems offer distinct advantages for conducting unique analyses. Collected native mucus, excised tissue, and animal models, as well as some *in vitro* cell culture models offer the advantage of maintaining the complex composition that includes non-mucin components also significant in mucus properties, as described above. However, mucus in these systems is inherently complex and undefined in composition, creating challenges in controlling mucus properties between experiments and understanding molecular phenomena contributing to observed properties. In contrast, purified mucin gels are inherently defined and controlled, but do not reflect the *in vivo* mucus composition. Collected native mucus as well as excised tissue and animal models offer the ability to collect mucus from different areas of intestine or from disease models to reflect inherent anatomical and disease-related changes in mucus, as noted above. Excised tissue and animal models as well as *in vitro* mucus-producing cell culture models maintain the spatial variation in mucus properties that exists from the intestinal epithelial apical surface to the lumen, as described above, whereas collected native mucus and purified mucin gels do not.

Analyses of viscoelastic and barrier properties of mucus and the diffusion of drug carriers are some of the principal experiments carried out in mucus models, and the various techniques are differentially suited to different sources of mucus. For example, multiple particle tracking (MPT), and fluorescence recovery after photobleaching (FRAP) are two techniques, described in more detail below, applied to characterize barrier properties and microrheology of mucus and to study the diffusion of drug carriers and molecules through mucus. In conducting MPT and FRAP, one advantage of using collected native mucus or purified mucin gels is the assurance that the particle or molecule being analyzed is located within the mucus/mucin gel. In contrast, employing these techniques on excised tissue or on cell cultures presents the challenge of ensuring the particles or molecules analyzed are within the mucus layer, and not in the fluid above or cellular material below the mucus layer. This can be particularly challenging with collected tissue since it is inherently not flat but possesses macroscopic and microscopic folds. Thus, some care is required to find pockets of mucus on intestinal tissue within which to conduct tracking. Here, we focus on utility of collected native mucus and excised tissues to study drug carrier and particle transport through mucus.

3.2 Collecting and Storing Mucus and Intestinal Tissue for Native Mucus Analysis

Native gastrointestinal mucus can be collected from excised gastrointestinal tissues and utilized for studying the transport of drug carriers and particles. A common method of mucus isolation is to directly scrape the mucus from the mucosal surface, for example from a porcine stomach or intestine obtained from a local abattoir [2, 27]. Collected native mucus is commonly stored at −80°C until experimentation with maintenance of rheological

properties [12]. It was recently shown that varied methods of porcine intestinal mucus collection and storage result in rheological and barrier properties similar to those of native mucus. Specifically, properties of mucus preparations stored at −20 °C, −80 °C (with or without prior snap-freezing), or at room temperature after freeze-drying and reconstitution in water were found to be similar to those of freshly isolated porcine intestinal mucus [76]. Scraped mucosal mixtures can either be used for experimentation as is, or can be used for protein purification, including purification of mucins for later reconstituting mucin gels. The ability of both collected native mucus and purified mucins to be stored is a significant advantage with respect to convenience as well as the ability to conduct multiple experiments with a single “batch” of material. However, directly scraping the mucosal surface inevitably results in a sample that includes cellular and luminal debris that contribute to complex, undefined, and highly variable compositions, as well as possible cytotoxicity for *in vitro* studies [77]. To overcome this challenge, a protocol was recently developed for scalable isolation of intestinal mucus at 70% of the hypothetical mucus yield via solubilization with mild alkaline solution and pH adjustment for gelation followed by centrifugation, dialysis, and finally lyophilization for long term storage and use [78]. The resulting extracted material demonstrated biologically relevant viscoelastic properties, including reversible gelation at low pH and high cation concentrations. This high throughput, low-cost procedure for mucus isolation was leveraged for further *in vitro* studies investigating interactions between mucus and bacteria [79].

Utilizing intestinal tissues for analysis of mucus properties is dependent on the availability of a suitable animal model or human tissue, for example obtained after a partial resection of the intestine. As noted above, fluorescently labeled particles, molecules, or microbes can be added to the mucosal surface, and their motion within mucus can be studied via microscopy. One challenge, in addition to ensuring location of the species being studied within mucus rather than in fluid above or cells below, is removal of bulk lumen content that may impede access to the mucus surface on the tissue. Tissues are commonly gently rinsed to achieve this removal, for example with ice-cold oxygenated Kreb’s solution [80] to support tissue preservation, but the rinsing process itself has the potential to disrupt and remove mucus. Another challenge is preservation of tissue viability, as cell and tissue death may result in the release of material into the mucus layer. One approach is to dissect tissue in ice cold buffer (e.g., oxygenated Kreb’s solution) and use it as soon as possible [81]. To prevent tissue dehydration during analysis, tissue can be maintained within a humidified chamber, for example in a petri dish with wet cloths adjacent to the tissue [41, 72]. Alternatively, tissue can be maintained in a culture system where provision of nutrients and oxygen can aid in maintaining tissue integrity. For example, intestinal tissues from different segments of rodent as well as human (collected via endoscopy) gastrointestinal tract were mounted in an Ussing-type chamber where fluid was recirculated on the stromal side of the tissue but not at the mucosal surface, to help maintain the mucus integrity [81]. This technique was successful in preserving the mucus layer, such that its thickness was measured via microscopy after allowing charcoal particles to settle on the mucus surface, and the penetration of fluorescent particles was also analyzed. Further, carbachol and prostaglandin E2 (PGE2) were used to stimulate mucus production, demonstrating that the tissue was viable.

4. Applications of Fresh Intestinal Mucus Models

4.1 Analysis of Mucus as a Barrier to Drug Delivery

The efficiency of drug and drug carrier transport through intestinal mucus to the underlying mucosal tissues is controlled by the microrheological properties of mucus, including viscosity and elasticity [17], as well as pore size and potential intermolecular interactions. Drug carriers trapped in mucus *in vivo* are removed from the delivery site due to natural mucus clearance, as mucus turnover within the gastrointestinal tract occurs over 1–3 hours [82], potentially resulting in a reduced therapeutic effect. An effective orally delivered drug or carrier system intended for cellular uptake must be able to overcome potential mucoadhesive interactions or size-exclusion effects within gastrointestinal mucus so that it can efficiently penetrate the mucus and be endocytosed by epithelial cells [83]. Alternatively, if a particulate drug carrier is intended for release of drug at the epithelial barrier, two approaches have been considered in particle design: i. design of mucoadhesive particles that adhere to the mucus layer and release drug, creating a local high drug concentration [84], or ii. design of mucus-penetrating particles that efficiently transit through mucus and release drug in close proximity to the epithelium [85].

Multiple approaches have been taken to study mucus as a barrier to drug delivery, and some of the most common are summarized below.

4.1.1 MPT—MPT has been extensively used to both explore penetration of particulate materials, including fresh mucus on excised tissues [2, 3, 41, 42, 56, 64–68, 71, 72] (Table 1), and also to probe the microrheological properties of mucus [17, 86]. MPT involves introduction of particles that serve as model drug carriers and/or microrheological probes to mucus, followed by collection of short (typically on the order of 10–30s) videos of their diffusive motion, and analysis of collected video frames using an algorithm that enables extraction of x- and y-coordinates of trajectories of diffusing species over time (Fig. 4). The trajectory data are then used to calculate quantitative parameters that reflect the properties of the mucus, including diffusion coefficients and microrheological properties. It can be challenging to compare results across MPT studies due to differences in experimental parameters (e.g., the time scale on which a diffusion coefficient is measured), and thus a summary of studies highlighting these parameters and results obtained is presented here to aid in such comparisons (Table 1). One experimental challenge is that MPT inherently requires that particles are visible for tracking. For nano-scale and micro-scale materials, this is typically addressed by incorporation of a fluorescent molecule within the particles being tracked. If a fluorescent molecule is incorporated within the particles, the centroid of the fluorescent signal can be visualized via fluorescence microscopy and tracked even if the particles are on the order of 20 nm in diameter [3]. Commercially available polymeric fluorescent particles of well-defined sizes are available to serve as model probes in MPT studies. Tracking particles in native intestinal mucus presents some inherent challenges relative to reconstituted mucus gels given the highly heterogeneous nature of the mucus composition and opacity.

MPT has been utilized to gain considerable insight into barrier properties of native intestinal mucus. For example, probe particles diffused through porcine mucus from an infant more

freely than through mucus from an adult, and this was attributed to the higher DNA content in the adult mucus [68]. MPT using muco-inert PEG-coated particles in mucus collected from mouse intestine or directly on intestinal tissue demonstrated that much larger particles could penetrate through small intestinal mucus compared to colonic mucus [64]. MPT on intact porcine intestinal tissue demonstrated that 500 nm negatively charged particles diffused more freely in the inter-villus space than in the mucus covering the villus tips [87]. MPT has also been used to demonstrate differences in intestinal mucus barrier properties in animal models of disease including Hirschsprung's disease and necrotizing enterocolitis, relative to healthy controls [41, 72]. In addition, MPT has demonstrated that intestinal mucus barrier properties are modulated by stimuli presented by intestinal lumen contents [3, 42, 66]. MPT was used to explore the impact of nanoparticle size and surface charge/chemistry on diffusivity in intestinal mucus, with particle neutrality determined to be an important factor in governing efficient nanoparticle transport [65]. Diffusion coefficients of near-neutral nanoparticles synthesized from polyelectrolytes were comparable to those of particles with PEG coating, which has been shown to enable relatively rapid diffusion through mucus [59].

4.1.2 FRAP—For studying transport of molecular species and some colloidal species in intestinal mucus, FRAP has been utilized [3, 88, 89]. FRAP, unlike MPT, does not require the ability to image and track individual species, and thus is more suitable for studying transport of smaller colloidal and molecular species. FRAP requires fluorescently tagged molecules and involves bleaching fluorescence in a portion of a sample, followed by analysis of the recovery of the fluorescence in the bleached area over time, as well as fitting of the resulting intensity profile to a diffusion equation to allow extraction of quantitative parameters including diffusion coefficients. FRAP has been used to demonstrate that the diffusion coefficient of bile micelles in mucus is consistent with that for colloidal particles rather than molecular species, suggesting that bile micelles stay intact within mucus [3]. Similarly, fluorescence resonance energy transfer (FRET) was used to demonstrate that lipid nanocapsules (LNCs) loaded with lipophilic fluorescent dye stay intact within mucus, as there was only a moderate decrease in FRET efficiency associated with mixing LNCs in mucus, which was attributed to LNC swelling [70].

4.1.3 Transport Through Bulk Mucus on a Membrane—Transport through gastrointestinal mucus can also be studied by application of a bulk amount of drug or particles to one side of a mucus layer, followed by measurement of the amount that appears on the other side. To quantify the diffusing substance, it is either fluorescently or radioactively labeled or measured using chromatography. For example, an Ussing-like diffusion chamber was used with reconstituted gastric mucins between Nucleopore polycarbonate filters to examine the role of hydrophobicity, size, and surface charge on microsphere diffusion [90]. In another study, porcine colonic mucus was sandwiched between cellulose nitrate filters to demonstrate that mucus provides a diffusion barrier for butyrate, and to evaluate the diffusion coefficient of butyrate across the mucus gel relative to other polymers [91]. Porcine intestinal mucus was also used in a cell culture insert to demonstrate enhanced transport of paclitaxel across mucus when it is formulated in lipid nanocapsules vs. as Taxol® [70]. In a similar setup, it was demonstrated that smaller (12

nm) self-nanoemulsifying drug delivery systems (SNEDDS) diffuse more efficiently across intestinal mucus than larger SNEDDS (456 nm) [71]. In another study, the enhanced ability of smaller (50 nm vs. 100 nm and above) and neutral or negatively charged (vs. positively charged) particles to penetrate through intestinal mucus was demonstrated using polystyrene particles of varying size and surface chemistry in rabbit intestinal mucus [92].

4.1.4 Bulk Rheological Studies—At the macro scale, mucus is viewed as a viscoelastic material that responds to shear stress non-linearly, with high resistance to deformation under low shear rates and weak resistance to deformation under high shear rates [17]. The viscoelasticity of mucus is often characterized by studying changes in viscosity over a range of shear rates [93]. Mucus collected from the pig gastrointestinal tract is a commonly used model to study gastrointestinal mucus macrorheology [94, 95]. It has been shown that bulk rheological properties vary along the gastrointestinal tract. Gastric and colonic mucus demonstrate similar high viscoelasticity with two rheologically distinct types of mucus: an inner mucus layer having high resistance to shear stress and an outer mucus layer having low resistance to shear stress, in contrast, small intestinal mucus has relatively low viscoelasticity with closer elastic modulus (G') and viscous modulus (G") values.

4.1.5 Tensile Studies—Studies of tensile strength are used to assess the strength of adhesion between mucus and different materials. Tensile strength is often evaluated by calculating the peak detachment force between mucus and the mucoadhesive substance. One study used this approach to compare the mucoadhesion of cholestyramine and other materials including resin particulates, hydrogels, and polymers to collected human and porcine gastric mucus samples [96]. This was done by gluing fresh tissue samples, mucus side up, to a plate attached to a tensiometer and bringing the mucus into contact with the component of interest on a disc probe, followed by measurement of the detachment force. Cholestyramine exhibited relatively high muco-adhesion, but not to the same extent as Carbopol, a known bioadhesive. This technique continues to be employed in the development of new polymeric materials, for example in recent studies exploring the mucoadhesive properties of a novel thiolated acrylic acid/acrylamide-methyl propanesulfonic acid copolymer, a sprayable excipient [97]. In this study, the polymer was placed in a petri dish on a movable plate and was raised into contact with a steel plate containing a fresh porcine mucus sample. Measurement of the detachment force confirmed that preactivated thiomer provided 2.43 fold greater work of adhesion as compared to the unmodified polymer.

4.1.6 AFM—Atomic force microscopy (AFM) is commonly utilized to study the interactions between mucin molecules and drug delivery systems [98, 99]. AFM can provide high-resolution 3-dimensional images of a specimen's surface topography without destructive and complicated sample preparation processes by using a mechanical probe to sense the specimen's surface [11]. AFM has been used to study bioadhesive properties of polymers, drugs, and drug delivery systems using purified mucin [100–102]. For example, the interactions between purified porcine gastric mucin and chitosan, a commonly used mucoadhesive polymer, were studied using AFM, showing that electrostatic forces contribute to the interactions [101]. Investigation of the interactions between polyether-

modified poly(acrylic acid) (PAA) and purified gastric mucin under various pH and ionic strength conditions revealed that mucoadhesive forces decreased with increasing pH values and ionic strength [100]. AFM has also been utilized to study the mucoadhesive interactions between particles and mucus covering excised tissues [103, 104]. Sotres et al. investigated the mechanical and adhesive properties of mucus blankets on excised porcine ileum at pH=7.4 and 2 using AFM, demonstrating increased stiffness and decreased adhesiveness of the mucus blankets at the lower pH [103]. To study the bioadhesive properties of model pectin microspheres to excised mucus tissues from different sections of the porcine gastrointestinal tract, Ghori et al. developed an AFM-based technique combined with a flow-through muco-dissolution cell. This system allowed continuous perfusion of tissue while AFM imaging, enabling studies of mucosal surface topography, mucoadhesion interactions, and drug releasing with a single excised tissue [104].

4.1.7 Residence of Drug Carriers on Mucus—

One major challenge in assessing the impact of intestinal mucus on drugs and drug carriers is testing in a physiologically relevant context. If systems are designed to be mucoadhesive, one question that arises is how much of a given drug carrier adheres to and potentially diffuses through mucus as fluid transits through the GI tract [83, 84, 99, 105]. To study this experimentally, a solution containing the drug or drug carrier is typically brought into contact with the mucus and then removed, followed by measurement of drug or drug carrier remaining on the mucus. For example, the perfusion wash technique involves placing test materials onto an *ex vivo* mucus model such as excised stomach or intestinal tissues fixed on an inclined platform, followed by rinsing the tissue with simulated gastric or intestinal fluid containing test materials. The percentage of test materials adhering to mucus is then determined, for example by high performance liquid chromatography or polarized light microscopy [106, 107].

4.2 Analysis of Mucosal Response to Chemical Stimuli

It is of interest to consider how mucus properties may change in the presence of stimuli presented by intestinal lumen contents or underlying tissues, and whether or not these effects are captured in experiments exploring mucus properties. Mucin gel formation and intestinal mucus barrier properties are sensitive to pH [108]. Intestinal lumen pH can change with ingestion of food and has been suggested to change with some diseases including Crohn’s disease [109]. A steep gradient in pH is maintained across gastric mucus, suggesting that mucus plays an important role in protecting underlying tissues from highly acidic gastric lumen contents [110–112]. Multiple studies have demonstrated that exposure to food-associated stimuli can alter mucus structure and barrier properties [42, 113]. An oat fiber-rich diet was found to enhance mucus barrier properties [113]. Mackie et al. demonstrated that intestinal mucus from pigs fed with enriched β-glucan diet became more viscous and had reduced permeability to 100 nm carboxylate-modified latex beads and digested lipids. As noted above, exogenous lipids as well as calcium ions can strengthen mucus barrier properties, as indicated by the reduced transport rates of 200 nm nanoparticles and microbes through porcine intestinal mucus [42]. It is plausible that lipids interact with the hydrophobic portions of mucins [114], and calcium ions impact interactions between mucin molecules by reducing the electrostatic repulsion between negatively charged mucins to strengthen the mucus network. Interactions with lipids have been demonstrated to protect

mucins from oxidative damage [114], and mucins have in fact been shown to be able to function as reactive oxygen species scavengers at the expense of mucin solution viscoelastic properties [115].

Given the significant role of mucus in maintaining intestinal homeostasis, it is of value to consider what effects ingested materials associated with health problems may have on mucus properties. CMC and polysorbate-80 (Tween 80) are surfactant-like molecules approved for use in certain foods at concentrations up to 2% wt/vol and 1% vol/vol, respectively, by the US Food and Drug Administration. While they are generally recognized as safe (GRAS) food additives, there is evidence that their consumption may contribute to the development of obesity, metabolic syndrome, inflammatory bowel disease (IBD), and even liver dysfunction [116, 117]. Studies have suggested multiple potential effects of orally ingested emulsifiers, including increasing translocation of bacteria across Microfold (M) cells [118], shifts in microbiota composition [57], as well as changes in the mucus barrier. Chassaing et al. administrated 1% w/v CMC or 1% v/v Tween 80 via drinking water to mice for 12 weeks and discovered increased bacterial adherence to the colonic epithelium and reduced mucus thickness in mice treated with emulsifiers [116]. Lock et al. used MPT as well as tracking of GFP-expressing *E. coli* to demonstrate that acute exposure to CMC altered the structure and barrier properties of native porcine intestinal mucus, hindering diffusion of 200-nm nanoparticles with various surface modifications and decreasing bacterial swimming speed (Fig. 4) [66]. It was found that acute exposure to 1% wt/vol CMC resulted in compacted and clumped native porcine mucus with a smaller pore size, as revealed via electron microscopy. Interestingly, Tween 80 did not impact particle motion in native porcine mucus, but increased bacterial swimming speed.

Alcohol use is endemic and has been associated with significant organ damage, increased risk of disease, and increased morbidity and mortality rate after mechanical and/or thermal trauma [119]. Alcohol use has also been associated with increased intestinal permeability. Rat intestine injected with alcohol was used to demonstrate that exposure to alcohol results in increased levels of lipid within the intestinal lumen, suggesting possible extraction of lipids from mucus and associated impact on mucus barrier properties [119].

High intake of sodium chloride has been associated with multiple disorders, including metabolic syndrome, autoimmune diseases, cardiovascular diseases, and gastric cancer [120–123]. Dietary salt altered mucus production in the stomach, also promoting gastric carcinogenesis in *Helicobacter pylori*-infected Mongolian gerbils in a dose-dependent manner [124]. Increased MUC5AC and reduced MUC6 were found among *H. pylori*-infected Mongolian gerbils fed with food containing 10% NaCl compared to low-salt controls. Saline has been used as a treatment for cystic fibrosis (CF) patients intended to improve mucociliary clearance [125]. CF is a genetic disorder caused by the mutations in the cystic fibrosis conductance regulator (CFTR), a chloride channel responsible for bicarbonate ion transport [126, 127]. Defects in CFTR result in insufficient release of bicarbonate ions, rendering mucus more compact and viscous in the intestine, pancreas, and lung [128]. Gustafsson et al. showed that mucus covering the small intestine tissues from a mouse model of cystic fibrosis was more compact and firmly attached to the epithelial cells compared to normal small intestinal mucus, but exposing the cystic fibrosis model mucus to 115

mM sodium bicarbonate for 15 minutes restored the natural barrier properties [128]. In a more recent study by Ermund et al., exposing small intestinal explants from a mouse model of cystic fibrosis to hypertonic saline (1.75% ~ 5%) resulted in detachment of the mucus from the underlying epithelium, decreased viscosity, and increased permeability to 200 nm fluorescent particles [129]. Taken together, these studies highlight the significance of considering the impact of stimuli introduced by lumen contents or underlying tissues when employing native intestinal mucus models. These stimuli can alter mucus production as well as mucus barrier properties. Thus, these findings suggest that their absence in *ex vivo* native mucus models may impact experimental results.

4.3 Analysis of Interactions Between Mucin and Bacteria

The intestinal mucus layer has an intricate relationship with the host microbiota that influences mucus barrier properties, microbiota function and host health. Interactions between microbes and mucus are diverse and complex: The oligosaccharide chains on heavily O-glycosylated mucins, as well as the protein backbones themselves, provide sources of nutrients for intestinal bacteria. Rampant bacterial metabolism, however, can result in barrier degradation and subsequent inflammation. Additionally, both commensal and pathogenic bacteria express lectins and other mucus-binding proteins to enhance adherence and colonization. Adding to this complexity is the fact that glycan composition varies in different regions of the GI tract, as well as in some diseases [130]. Maintaining the delicate balance between providing carbohydrate niches for commensal microbes, while mitigating barrier erosion, penetration, and inflammation, is critical for host health [131]. The wide range of interactions between mucosal components and bacteria has been recently reviewed [131]. Here we focus on examples where intestinal fresh mucus models have provided important additional insights.

4.3.1 Adhesion—Intestinal bacteria leverage diverse biological attributes to translocate and adhere to mucus components, including adhesins, pili/fimbriae, flagella, mucus-binding proteins and lectins [132]. One example of a pathogen whose interactions with mucus are significant in invasiveness is *Campylobacter jejuni*. This microbe has been shown to utilize mucins as an energy source and even multiply on mucus alone [133], and also to express a range of mucin-binding proteins [134–136]. Studies using fresh mucus-supplemented media have elucidated some insight into pathogenicity. For example: in chickens, *C. jejuni* is a commensal microbe but becomes pathogenic in mammals. This is suspected to be because of differences in mucus and mucus glycosylation patterns [137]. One group discovered differentially expressed genes important for colonization, including some associated with iron acquisition and oxidative stress when the same strain was cultured with avian mucus compared to mammalian mucus [138], suggesting mucus domains and glycosylation patterns impact commensal and pathological functions. This study involved directly supplementing fresh, collected mucus into bacterial media, and highlights how studies of bacteria-mucus interactions can provide insight into the role of commensal and pathogenic microbes in health and disease.

A common method of studying adhesion of bacteria to mucus is to apply a suspension of the bacteria or carriers to a mucus surface for a certain period of time, remove the

suspension, and observe the amount of adherent material. This may be achieved by labeling the bacteria with a fluorescent marker [139] or radioactive tracer [140]. The technique may also be extended to bacteriophage: In one such study, adhesion of bacteriophage to fish mucus was demonstrated by exposure of fish to phage-containing fluid followed by mucus collection via scraping and quantification of bacteriophage [141]. Consistent with the notion that bacteriophages residing in mucus layers aid in prevention of pathogenic infection [142], fish pre-exposed to bacteriophage were less susceptible to infection by *Flavobacterium columnare* [141]. One challenge with this type of experiment is making sure that the mucus is not deteriorating with rinsing steps and is appropriately anchored to the underlying surface.

Atomic force microscopy (AFM) is another method used to study bacterial adhesion to mucus, providing quantitative measure of the adhesive forces between bacteria or other particles and mucus blankets on collected intestinal tissue. AFM studies of the interaction between *Lactococcus lactis* and porcine gastric mucin revealed a major role of the *O*-glycans in binding [143]. Application of this technique to study the mucosal binding of two fish pathogens, *A. dolmonicida* and *Y. ruckeri*, showed that both microbes bound to immobilized mucins, but there were no observable adhesive forces when retracting bacteria from *intact* mucus [144]. The same technique was also used to demonstrate that the mucus blanket could attract and bind particles as large as 15 μm in diameter, and further than 100 μm from the mucus surface [103].

Surface plasmon resonance (SPR) is another method that has been applied to quantitatively measure binding interactions between bacterial factors and mucins. SPR involves immobilizing ligands onto a sensor chip (commonly BIACORE) and flowing potential binding materials over the chip surface. For example, in one study, human colonic mucus samples were collected via scraping tissue, and mucins were extracted and immobilized onto a flow chip in order to explore potential binding of *Lactobacillus* species and their factors to human A-antigen [145]. Human A-antigen content in the mucins was measured, and human A-antigen itself was also bound to sensor chips. Bacteria as well as surface layer proteins (SLPs) extracted from the bacteria were then flowed over the sensor chip surface, followed by washing to remove unbound material, and quantification of mass of remaining bound reagent via measurement of resonance units (RU) [145]. Similar experiments utilizing mucus from different states of disease and health could be used to provide insight into how alterations in the mucus barrier may promote colonization by certain species. SPR has also been used to show that *Bifidobacterium longum*, one of the “early colonizers” of infant intestinal tracts commonly used as a probiotic due to multiple beneficial functions, produces extracellular vesicles containing mucin-binding proteins [146]. One advantage of SPR is that it does not require pre-labeling of the mucus or binding entity and can be considered reasonably high throughput, for example enabling screening of multiple bacterial species with a reusable sensor chip. However, one disadvantage is the need to at least partially purify and then immobilize the ligand or mucin to the sensor surface, which may result in loss of some binding entities within mucus, and possibly alter the nature of interactions relative to those that may occur with native mucus *in vivo*. BIACORE based analysis for mucin binding can also be translated to synthetic polymers being developed as drug carriers [147].

4.3.2 Motility—As mucus is essentially a polymer network with reported pore sizes ranging from considerably less than to comparable with dimensions of bacteria, and bacteria undergo interactions with mucus components, it is expected that motility and associated trajectories of bacteria are highly impacted by mucus. The association of bacteria penetration of the inner “bacteria-free” mucus layer with onset of inflammation and colitis supports the significance of mucus providing an essential barrier to bacteria, while still allowing beneficial nutrients and microbial byproducts to diffuse for use by the epithelium. Mucus viscoelastic properties influence bacterial motility. As noted above, divalent cation concentrations and pH both affect mucus barrier properties: low pH and high Ca^{2+} increase mucin aggregation and crosslinking. It has been proposed that increased interactions between mucin molecules can create channels for enhanced molecular (nutrients and signaling molecules) diffusion while simultaneously reducing the permeability of microbes through entrapment [78]. Interaction of bacteria with components of mucus gels can hinder motility. Integral to these interactions are embedded bacteriophages and immunoglobulins [142]. Interactions of bacteria with bacteriophage and immunoglobulins in the mucus gel have been studied both experimentally and theoretically. For example, theoretical modeling suggests that the run-and-tumble motion of *E. coli* creates local fluid flow within mucus that enhances phage encounter rate [148]. Antibodies secreted by immune cells into the mucus layer such as IgG and IgA can trap potential pathogens at the outer (luminal) mucosal surface. A mathematical model of bacteria-immunoglobulin and immunoglobulin-mucin interactions suggests that both IgA and IgG accumulate on individual bacteria sufficiently to prevent penetration through the mucus layer, and that agglutination, or clumping of bacteria by IgA, is only modestly important in preventing bacteria penetration [149]. While movement through mucus is necessary for sustenance of some beneficial microbes, motility restriction is a vital mechanism of preventing pathogen invasion.

Experimental analysis of bacterial motility through mucus often involves videomicroscopy and tracking of microbe trajectories. This technique has been used to study bacterial swimming in intestinal mucus on tissue collected from animal models. One approach is to carefully apply a drop of bacteria-containing solution to the exposed mucosal surface after collecting an intestinal segment and cutting it lengthwise so it can lay flat. For example, analysis of trajectories of swimming *E. coli* revealed a reduced velocity in proximal colon mucus from a rodent model of Hirschsprung’s disease relative to wild type animals [41]. This was hypothesized to potentially be due to increased amounts of sialomucins in Hirschsprung’s disease, and associated impact on bacteria-mucin interactions. Bacteria trajectory tracking on intestinal tissue was also conducted to demonstrate increased *E. coli* swimming velocity in a rodent model of prematurity, as well as hindered *E. coli* motility in a rodent model of necrotizing enterocolitis when animals were dosed with lysozyme relative to control animals [72]. Alternatively, as with particles, tracking can be conducted in collected mucus. For example, videomicroscopy and trajectory tracking in collected porcine intestinal mucus was used to demonstrate that lipids representative of the fed state reduced *E. coli* velocity as well as trajectory linearity (a measure of the degree to which the bacteria were changing direction while swimming) [72]. As noted above, it is important to consider how handling of mucus may impact its properties. Interestingly, it has been demonstrated that shearing of mucus aligns bacteria trajectories [67]. Droplets containing *B. subtilis*

and *E. coli* in medium were placed alongside droplets of cervical mucus and sandwiched between glass slides, or, alternatively, next to a “string” of mucus created using a needle, and the resultant trajectories of the bacteria were recorded [150]. Bacteria followed parallel line trajectories radially towards the center of the mucus droplet in the first case, and along the length of the mucus string in the second. It was proposed that mechanical stress on the droplet creates alignment of pores, which was supported by electron microscopy analysis. Interestingly, bacteria penetrating into the mucus were observed to swim forward and backwards without making a “U-turn” in most cases, which was attributed to disassembly of the flagella followed by reorganization on the other side of the bacteria. It would be interesting to study if similar phenomena occurred in fresh gastrointestinal mucus, or in mucus covering the intestinal epithelium, which exhibits inherent anisotropy from the apical cell surface to the lumen.

It has been demonstrated that some bacteria have the ability to alter local fluid properties to aid mucus penetration. *Helicobacter pylori*, an ulcer-producing pathogen capable of surviving the acidic conditions of the stomach, has been shown to modify the pH of its microenvironment through hydrolysis of urea to bicarbonate and ammonia, thus decreasing mucin viscoelasticity [151]. Viscoelastic moduli of gastric mucin gels with and without the pathogen present were measured using a stress-controlled rheometer, and *H. pylori* trajectories were tracked using videomicroscopy. Initial high elasticity of the mucus confined trajectories and trapped bacteria. Incubation with *H. pylori* resulted in a neutralization of the mucin from pH 4 to a pH of about 7, and an associated reduction in viscoelastic moduli and increase in bacteria motility.

4.3.3 Mucus Metabolism and Degradation—Active degradation of mucins relies on a combination of enzyme families: glycosidases, proteases including *O*-glycoproteases, and sulfatases which are found across the human gut microbiota [152, 153]. Enzymatic activity on porcine mucus has been demonstrated by prominent gut bacteria *B. thetaiotaomicron* and *B. fragilis*, among many others [154]. Microbial degradation of mucins can be detrimental to the host, for example aiding pathogen invasion. However, degradation of mucins also provides sustenance for the beneficial inhabitants of the outer mucus layer. *Akkermensia mucinophila*, a well-studied mucin degrader, is usually associated with a healthy gut. Higher *A. mucinophila* stimulates intestinal stem cell-mediated epithelial development [155]. Fermentation of mucin-derived glycans produces short chain fatty acids (SCFA) that have important effects on intestinal epithelial and immune cells [156]. SCFAs like *n*-butyrate stimulate mucin production by goblet cells as well as antibody and cytokine production by immune cells. Interestingly, levels of n-butyrate are lower in the stools of IBD patients, and data suggests this is tied to decreased utilization of mucin *O*-glycans in patients with ulcerative colitis (UC) [157]. However, mucin-degrading bacteria are generally elevated in patients with IBD [9, 158]. Bacterial degradation of mucins constitutes a delicate balance as it produces products beneficial to the host and microbiome, yet over-degradation can significantly weaken mucus barrier properties and initiate an inflammatory immune cascade. With different glycoprotein moieties serving as substrates for different bacteria, collective degradation occurs via cooperative cross-feeding: enzymatic activity from one microbe may make other mucin moieties accessible to another microbe. While many bacteria can

metabolize constituents of mucins, few can grow on mucin alone [159]. By adding fresh mucin to growth medium, it was shown that *R. turques*, *B. bifidum*, *R. gnavus*, and *A. mucinophila* can degrade between 50 and 80% of available mucins [158]. Elevated numbers of these mucin degraders may result in increased levels of other bacteria that can use released glycans. Collected native mucus and isolated mucus components (e.g., purified mucins and *O*-glycans from mucins) have been useful in analysis of what bacteria use mucus as an energy source and can degrade mucins.

In addition to enzyme-mediated breakdown, other microbially derived molecules may contribute to degradation of the mucus layer. Sulfate reducing bacteria (SRB) produce hydrogen sulfide (H_2S) and have been found in higher numbers in patients with IBD [160]. While SRB cannot metabolize mucins alone, degradation of sulfomucins by the aforementioned mucin degraders increases the availability of sulfate, promoting SRB metabolism. Recent evidence suggests that H_2S may reduce the disulfide bonds in mucus, expanding the network structure through loss of crosslinking [161, 162]. The resultant mucus may therefore be more permeable to bacteria and their metabolites. These findings suggest that the relationship between H_2S and mucus may contribute to inflammatory dysbiosis, but further work to elucidate this mechanism is needed [163].

5. Conclusion

Native intestinal mucus models can be used to provide useful insight into the role that mucus plays in interacting with drugs, drug carriers, and bacteria. Native intestinal mucus is complex in composition and structure, presenting tradeoffs in its use relative to purified mucin systems with respect to replicating certain aspects of the *in vivo* environment yet being somewhat undefined, rendering definitive conclusions regarding underlying molecular mechanisms difficult to draw. Native intestinal mucus can be used to conduct numerous analyses for understanding the role of mucus in drug delivery and interactions with intestinal lumen contents, including the microbiota. Given the advantages and limitations of using different native mucus and purified mucin preparations in conducting experiments, it is recommended that investigators interpret results in light of the experimental system employed and consider integrating complementary information from different experimental systems (e.g., collected mucus vs. intact mucus on tissues) and types of analyses (e.g., particle tracking and bulk particle transport studies to analyze mucus barrier properties). Further, advancements in *in vitro* tissue models enable analyses of mucus on intestinal microphysiological systems as another option for scientists exploring the significance of the mucus barrier in drug delivery.

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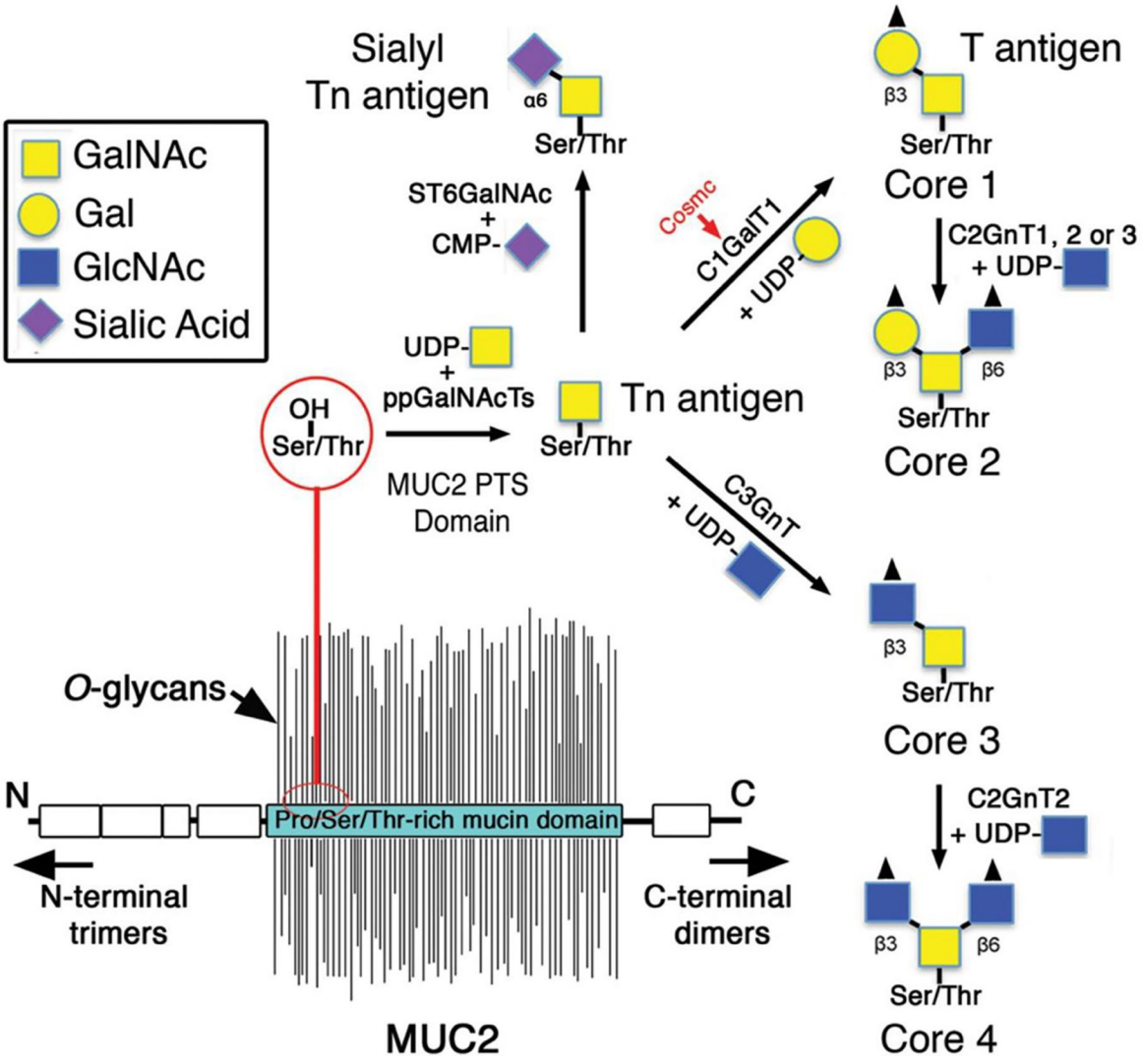

Figure 1.

MUC2 mucins are heavily glycosylated in PTS domains, imparting a "bottle brush" type structure. *O*-glycosylation starts with the addition of GalNAc from uridine diphospho (UDP)-GalNAc to a Serine (Ser) or a Threonine (Thr), creating the Tn antigen. Tn antigen can be modified with Gal, GlcNAc, or sialic acid to create the core 1 and 3 structure and sialyl Tn antigen, respectively. Core 1 and core 3 structures can be further extended to create core 2 and core 4 structures, whereas sialyl Tn antigen cannot be extended. Core 1 to 4 structures are usually further modified and/or branched by glycosyltransferases. Terminal ends of the glycan chains are often capped with fucose or sialic acid, forming significant antigen structures such as ABO blood group determinants and Lewis antigens. ppGalNAcTs = polypeptidyl GalNAc transferases; C1GalT1 = core 1 β1,3-galactosyltransferase; C2GnTs = core 2 β1,6 *N*-acetylglucosaminyltransferases; C2GnT2 = core 3 β1,3 *N*-acetylglucosaminyltransferase; ST6GalNAc = α2,6 sialyltransferase. Reproduced from Bergstrom and Xia [44].

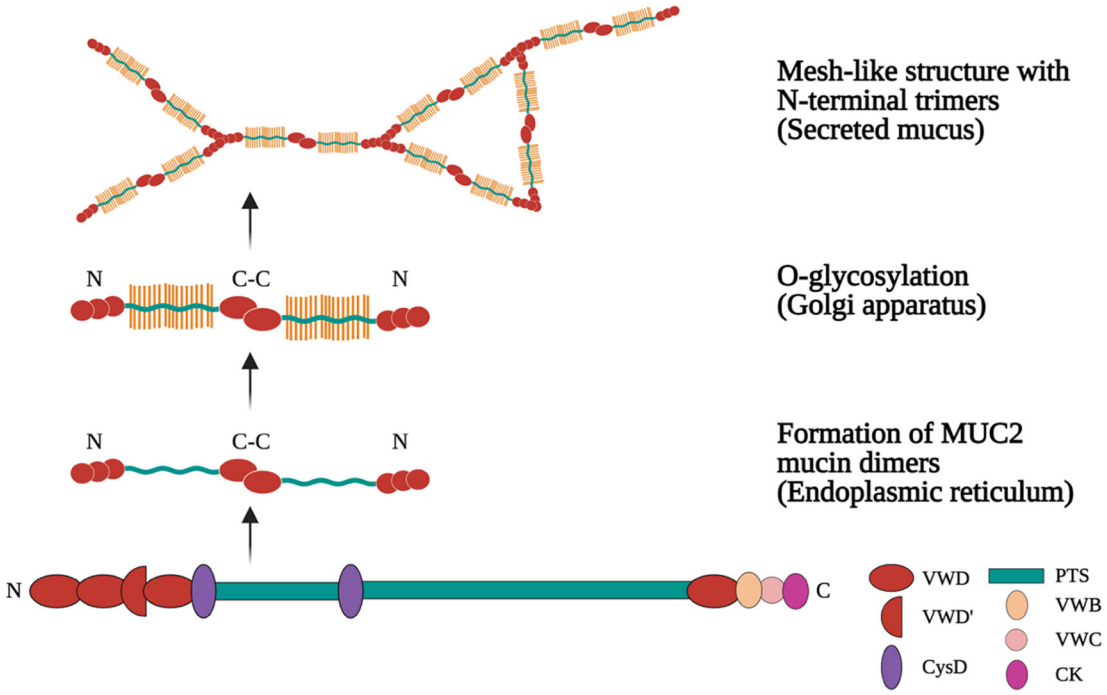

Figure 2.
Schematic diagram illustrating the domain structure of MUC2 and the process of forming the mesh-like MUC2 network. Adapted from Johansson et al. [53].

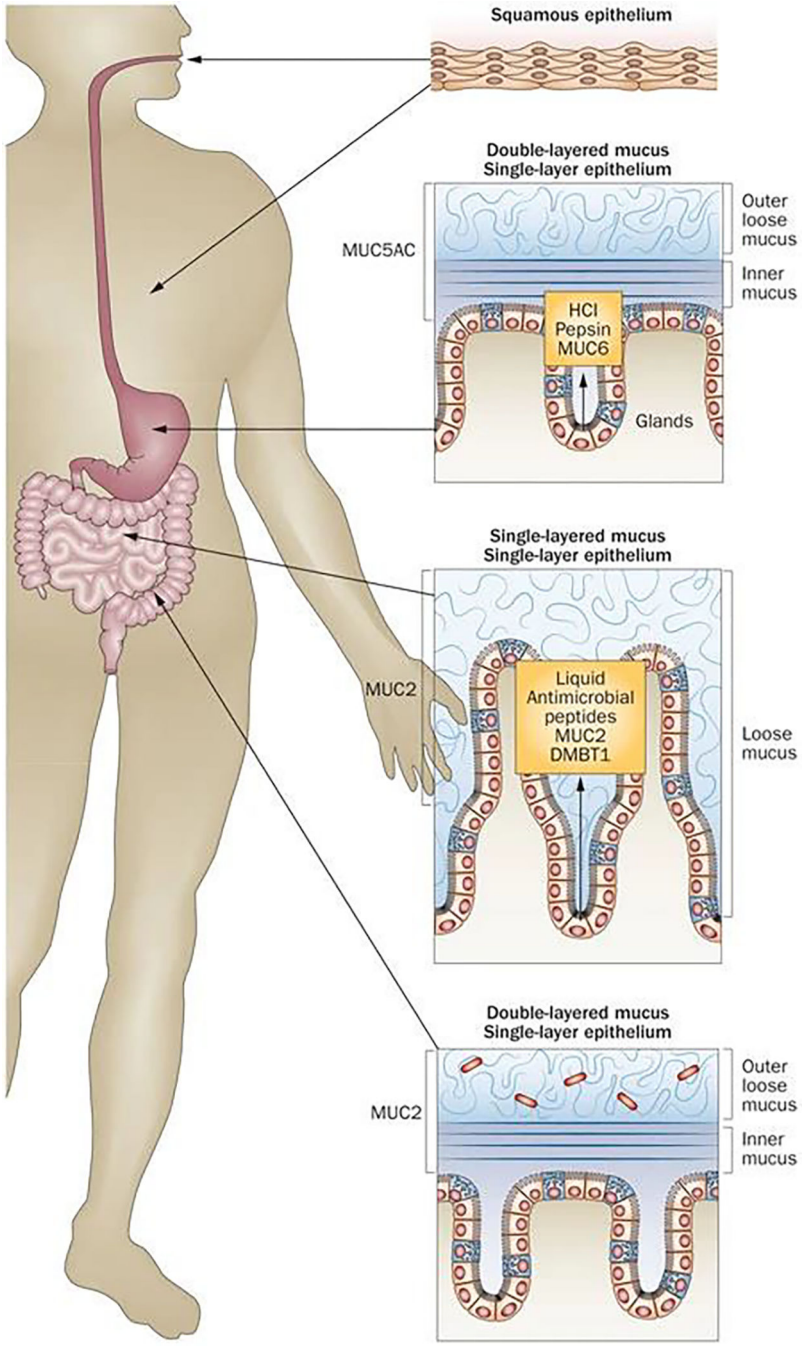

Figure 3.

Mucus organization along the gastrointestinal tract. The stomach has two layers of mucus comprised of MUC5AC mucins. The small intestinal mucus is a single-layer hydrogel consisting of MUC2 mucins, whereas mucus in the colon has two layers: an outer loose layer and an inner dense layer. Both layers of the colonic mucus are comprised of MUC2 mucins. Cells secrete factors such as antimicrobial peptides and deleted in malignant brain tumors 1 (DMBT1) into overlying mucus. Reproduced from Johansson et al. [16]

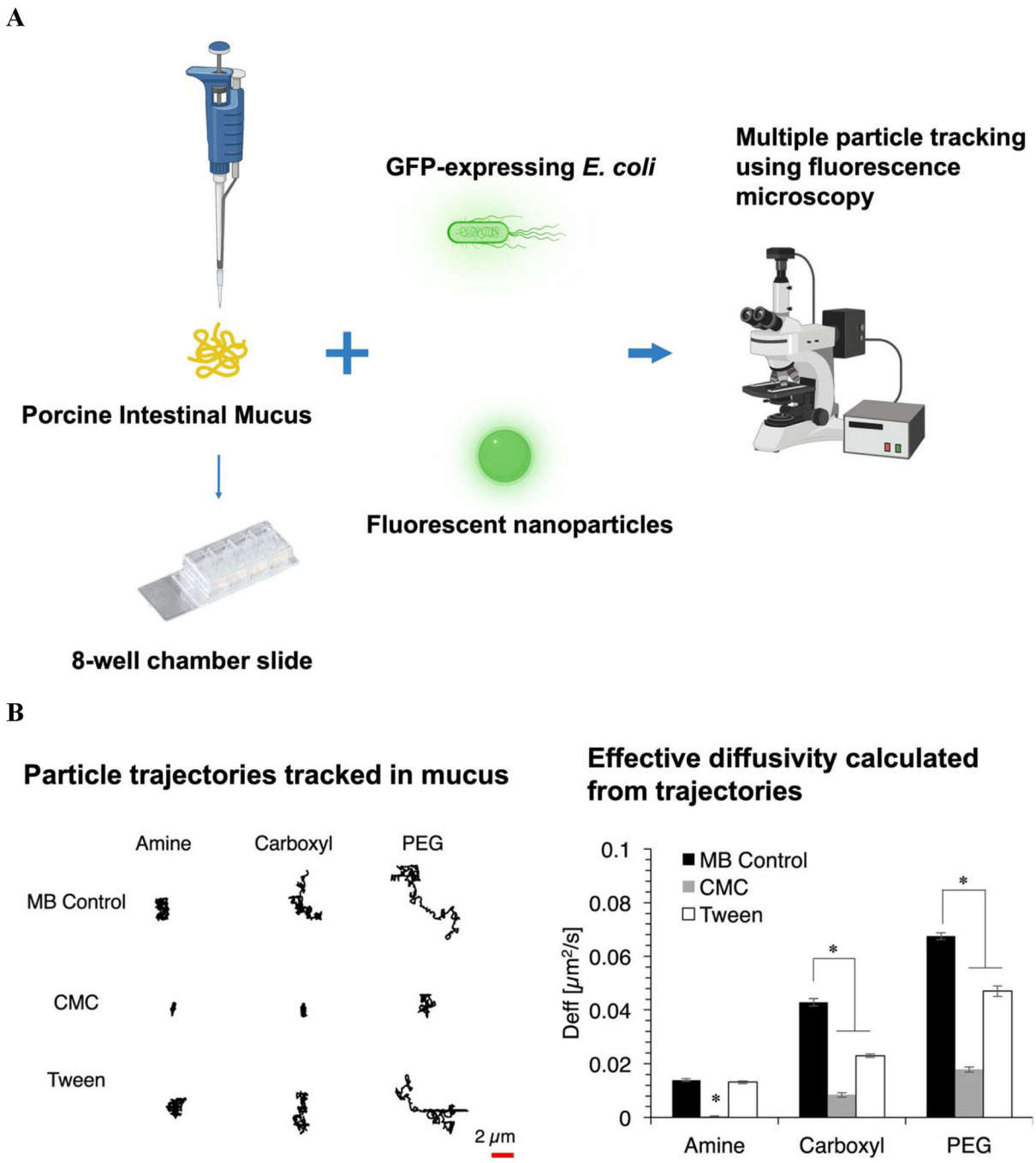

Figure 4.

(A) Simplified schematic showing the procedure of probing native porcine intestinal mucus barrier properties using MPT. (B) Representative trajectories of amine-, carboxylate-, and polyethylene glycol (PEG)-modified fluorescent polystyrene nanoparticles were used to probe the barrier properties of native pig intestinal mucus upon exposure to surfactants (carboxymethylcellulose (CMC) and Tween 80) relative to exposure to maleate buffer (MB)

as control. The trajectory data can be used to calculate effective diffusivity as an indicator of mucus barrier properties. Adapted from Lock et al. [66].

Table 1.

Parameters and findings from studies utilizing videomicroscopy and particle tracking techniques to assess transport of particles or bacteria through native intestinal mucus

Reference	Mucus Model	Particle Type	Particle Size	Particle/Bacteria Concentration*	Video Time Length (s)	Resolution (s)**	Diffusion Coefficient (μm²/s) (or MSD/bacteria velocity as noted)
[2]	Mucus scraped from pig jejunum	Amine-, carboxylate-, sulfate-modified polystyrene nanoparticles	200 nm	0.0025 w/v %	20	10	• Amine: 0.0167 • Carboxylate: 0.0632 • Sulfate: 0.0950
	Purified type II pig gastric mucin	Amine-, carboxylate-, sulfate-modified polystyrene nanoparticles	200 nm	0.0025 w/v %	20	10	• Amine: 0.0143 • Carboxylate: 0.1709 • Sulfate: 0.1751
[3]	Mucus scraped from pig jejunum	Carboxylate-, PEG-modified polystyrene nanoparticles	20–500 nm	0.0025 w/v %	20	10	• Carboxylate-100nm: 0.06 • Carboxylate-200nm: 0.026
[41]	Excised mouse proximal colon tissues from wild type and Hirschsprung's Disease model	Carboxylate-modified polystyrene nanoparticles	200 nm	0.0025 w/v %	20	10	• Average MSD for Wild type mucus : 0.25 μm² • Average MSD for Ednrb −/− mucus: 0.035 μm²
		GFP-expressing *E. coli*	n/a		20		• Average speed in WT mucus: 6.41 μm/s • Average speed in Ednrb−/− mucus: 1.78 μm/s
[42]	Mucus scraped from pig jejunum	Amine-, carboxylate-, sulfate-modified polystyrene nanoparticles	200 nm	0.0025 w/v %	20	1	• Amine: 0.00575 • Carboxylate: 0.04342 • Sulfate: 0.06205
[56]	Mucus scraped from pig jejunum	Carboxylate-modified latex beads	100, 500 nm	0.025 w/v %	9 for 100 nm, 35 for 500 nm		• 100 nm: 0.203 • 500 nm: 0.0165
[64]	Mucus scraped from mouse colon	Carboxylate-, PEG-modified polystyrene nanoparticles	40, 100, 200, 500 nm	0.02–0.08 w/v %	20	1	• Average MSD for carboxylate-modified particles: 0.0001 μm² • Average MSD for PEG-modified particles: 0.1 μm²
	Excised mouse small intestine and colon tissues	Carboxylate-, PEG-modified polystyrene nanoparticles	40, 100, 200, 500 nm	0.02–0.08 w/v %	20	1	• Average MSD for 100 nm PEG-modified particles in small intestine mucus: 0.1 μm² • Average MSD for 100 nm PEG-modified particles in colon mucus: 0.01 μm²
[65]	Mucus scraped from pig ileum	Polyelectrolyte nanoparticles prepared from chitosan and polyacrylic acid	100–350 nm	0.002% w/v %	10	1	• Negatively charged polyelectrolyte (−30.6 mV, 149 nm): 0.000366 • Neutral polyelectrolyte (+6 mV, 144 nm): 0.003182 • Positively charged polyelectrolyte (+19.2 mV, 180 nm): 0.000104

Reference	Mucus Model	Particle Type	Particle Size	Particle/Bacteria Concentration*	Video Time Length (s)	Resolution (s)**	Diffusion Coefficient (μm²/s) (or MSD/bacteria velocity as noted)
[66]	Mucus scraped from pig small intestine	Amine-, carboxylate-, PEG-modified polystyrene nanoparticles	200 nm	0.0025 w/v %	20	3	• Amine: 0.016 • Carboxylate: 0.044 • PEG: 0.068
		GFP-expressing *E. coli* (MG1655 strain)	n/a	10^6 cells/ml	20		• Average speed in mucus dosing in buffer: 5.34 μm/s • Average speed in mucus dosing in CMC: 4.33 μm/s • Average speed in mucus dosing Tween 80: 5.91μm/s
[67]	Mucus scraped from pig small intestine	GFP-expressing *E. coli* (MG1655 strain)	n/a	10^6 cells/ml	20		• Average speed in mucus dosing in buffer: 7.76 μm/s • Average speed in mucus dosing in fed state medium: 5.63 μm/s
[68]	Mucus scraped from the jejunum of adult pigs or piglets	Carboxylate-modified latex beads	500 nm, 1 μm	0.125 w/v % or 0.25 w/v %	50	1	• 500 nm: 0.084±0.012 • 1 μm: 0.039±0.003 (In *ex vivo* piglet mucus)
[72]	Excised rat ileum tissues from wild type or necrotizing enterocolitis (NEC) model	Amine-, carboxylate-, PEG-modified polystyrene nanoparticles	200 nm	0.0025 w/v %	10		• PEG-modified particles in 5-day old mucus: 0.013 • PEG-modified particles in 21-day old mucus: 0.005
		GFP-expressing *E. coli*	n/a	10^5 cells/ml	10	1	• Average speed in 5-day old mucus: 5.75 ± 0.03 μm/s • Average speed in 21-day old mucus: 5.54 ± 0.04 μm/s
[71]	Mucus scraped from pig small intestine placed on a 24-well Transwell insert	Self-nanoemulsifying drug delivery systems (SNEDD) prepared with various excipients	5–450 nm	1:100 dilution of SNEDD formulations			• The permeation behavior of SNEDDs was controlled by the particle size and excipient used

*Particle/Bacteria concentration defined as final concentration of suspension added to mucus (w/v %= weight of solids (g)/volume of suspension solution (ml)*100)

**Resolution is defined as the time scale selected for calculating mean square displacement (MSD) and diffusion coefficient