FOOD-ASSOCIATED STIMULI ENHANCE BARRIER PROPERTIES OF MUCUS

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ABSTRACT

Mucus, a complex network consisting of micro- and nano-scale fluid-filled domains formed via mucin glycoprotein interactions, provides a barrier through which nutrients and orally delivered drugs must penetrate before entering the circulatory system. Mucus provides a significant, yet poorly characterized barrier to particulate, pathogen, and small molecule transport (e.g., nutrient, toxin) to epithelial surfaces. It is important to understand mucus barrier properties as they are significant to drug delivery and potentially disease. The main objective of this project was to examine the impact of physicochemical changes occurring upon food ingestion on gastrointestinal (GI) mucus barrier properties. The motivation for studying these phenomena includes understanding how drug delivery and microbe transport through mucus may change upon food exposure, and how these effects may be exploited to enable more efficient drug delivery or block pathogen transport.

Lipid content associated with fed state intestinal contents significantly enhanced mucus barrier properties, as indicated by $10^{-140}$-fold reduction in the transport rate of 200 nm microspheres through mucus, depending on surface chemistry. Physiologically relevant increases in $[Ca^{2+}]$ resulted in 3-fold, 4-fold and 2-fold reduction of amine-, carboxylate- and sulfate modified particle transport rates, respectively, likely due to binding of $[Ca^{2+}]$ to mucin glycoproteins and thus enhanced cross-linking of the mucus gel network. Reduction of pH from 6.5 to 3.5 also affected mucus viscoelasticity, reducing particle transport rates approximately 5-fold for amine-modified particles and 10-fold for carboxylate- and sulfate modified microspheres.
Macroscopic visual observation and micro-scale lectin staining patterns indicated mucus gel structural changes, including clumping into regions impenetrable by microspheres, correlating with altered transport properties. Histological examination of intestinal tissue indicated food ingestion can prevent microsphere contact with and endocytosis by intestinal epithelium. Furthermore, exposure of mucus to lipids associated with fed state intestinal contents reduced *E.Coli* motility over 6 -fold in GI mucus.

Impact of food-associated lipids on transport of molecular species, specifically model drug carriers (<500 Da, 5 Å), in gastrointestinal (GI) mucus was also studied utilizing electron paramagnetic resonance (EPR). Lipid contents associated with fed intestinal state reduced transport rates of nitroxide spin probes nearly 1.5 fold in mucus. Molecular properties had a considerable influence on small molecule transport in mucus. Positively charged 4-amino tempo was 10- fold slower than negatively charged 3-carboxy proxyl and 4-hydroxy tempo. Additionally, hydrophobic interactions are likely important, as diffusion of hydrophobic 4-hydroxy tempo benzoate spin probe in mucus was slower than negatively charged 3-carboxy proxyl (3–fold) and 4-hydroxy tempo (2-fold).

In summary, these findings could provide meaningful guidance on fundamental understanding of mechanisms behind the “food effect” on intestinal barrier properties. Additionally, these results could guide strategies relevant to oral delivery of drugs, enhancement in nutrient uptake, or modulation of pathogen invasion in diseases such as inflammatory bowel disease, or exposure to bioterrorism agents. A new, relatively facile model for controlling intestinal epithelial exposure could be introduced to the biomedical community.
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1.0 INTRODUCTION

Among the various drug delivery routes, oral administration is frequently used due to numerous advantages such as painless, convenient and cost-effective delivery. Although solid-dose drug formulations, such as tablets and capsules, have a high degree of drug stability and provide accurate dosing, the unpredictable nature of gastrointestinal (GI) tract absorption reduces drug efficacy. Effective delivery of orally administered drug carriers to target cells is often precluded by GI tract which forms a formidable barrier against many pharmaceutical components. For instance, food ingestion results in significant physicochemical changes in the environmental pH, gastric mobility and emptying time. These changes in GI tract impact the rate and extent of drug absorption.

Drug carriers delivered to the GI, respiratory, and cervicovaginal tracts must be able to penetrate through mucus barriers; these barriers prevent the transport of foreign microorganisms to the underlying epithelium [4-6]. Additionally, the mucus barrier is a significant barrier to nanocarrier systems as well as small molecule transport to epithelial surfaces [7-13]. Mucus consists of micro- and nano-scale fluid-filled domains in a network of entangled fibers, the major structural component of which is mucin glycoproteins. Complex network of mucus gel also includes lipids, salts, cellular serum macromolecules, cells and other cellular debris [1, 8, 13]. Numerous intermolecular interactions between mucus constituents results in the high viscoelasticity and effective porosity of the complex mucus network, which retards the transport of molecules or drug carriers [5].
Lipids originating from food or delivery vehicles have a substantial yet poorly understood impact on absorption and bioavailability of orally delivered compounds which includes drugs and nutrients. Most of the nutrients as well as vitamins and cholesterol are highly solubilized in mixed micelles that are formed from food-associated bile salts and phospholipids [14]. The influence of higher bile salt concentrations in the lipid digestion is incorporated into colloidal structures including micelles. As bile salt micelles significantly expand the solubilization capacity of the small intestine for orally delivered drugs, transport capability of these small particles (~ 10 nm) has not been studied across the mucus barrier. Lipid contents associated with food arrival impacts drug absorption to a certain degree depending on time of dosing in relation to eating [15-20]. The influence of lipids on oral compound absorption originates from colloidal structures they form and effects on transport through the intestinal mucosa. Whilst not fully understood and yet potentially highly important, lipids likely alter mucus barrier properties in the GI tract. Changes in ionic strength can alter mucus viscoelasticity and affects effective drug delivery in mucus. Inorganic salts present in mucus represent up to 1 % of the dry weight [1]. Mucus gel network consists of weak non-covalent cross-linked bonds and abundance of intermolecular interactions, including hydrogen bonding; electrostatic and hydrophobic interactions govern mucus viscoelasticity. Mucus rheology is affected by various factors including ionic content. After food arrival, calcium ion concentration in the small intestine changes significantly. Despite many strides that have been made in characterizing the effect of calcium ion on mucus rheology, the impact of calcium on effective drug transport through GI mucus remains poorly defined.
Mucin composed of crosslinked, bundled and entangled fibers that have a high sialic acid and sulfate content which makes the mucus highly negatively charged. These sialic acids are likely correlated to high viscous mucus elastic gel [21]. Meanwhile, high acidic environments (lower pH) interacts with carboxyl groups on sialic acids results in increase the mucus viscoelasticity [22]. As it was mentioned gastrointestinal pH plays an important role in modulating drug delivery and nutrient absorption[23]. After food arrival, median small intestinal pH is around 5.4, whereas median fasting pH is close to 6.1. However, pH values fluctuate and vary between individuals from a minimum as low as pH 3.1 to a maximum of pH 6.7[24]. Acidity is known to alter mucus barrier properties[25]. Decreasing pH has been demonstrated to induce mucin aggregation and increase gastric mucin solution viscosity[26, 27]. It has been suggested that this is due to neutralization of glycosidic residues, however analysis of native and deglycosylated gastric mucin aggregation at low pH using atomic force microscopy indicated that oligosaccharide side chains are not directly involved[28]. It is unclear if similar pH effects are observed in intestinal mucus.

Mucosal membranes form a finely tuned hydrogel barrier that control pathogen invasion in the GI, respiratory and cervicovaginal tracts. Mucus protects underlying epithelia from pathogen entry, regulates food and drug absorption in the gastrointestinal tract and self modulates barrier properties with the menstrual cycle to control sperm penetration[29]. Natural defense mechanisms native to mucosal surfaces prevent pathogen intrusion and can break down in disease.
For example, Ca\textsuperscript{2+} and pH-modulated permeability are important in disruption of stomach resistance to pathogen entry with gastric ulcers and menstrual cycle variation of sperm mobility. However, these defense mechanism and associated barrier properties are not well understood.

The dense mucus network can efficiently trap most micro-environmental particles through steric hindrance and/or adhesion forces via hydrophobic, electrostatic or hydrogen bonding interaction [8]. Similarly, conventional model drug carriers that are highly mucoadhesive are likely to be trapped by the same forces. Along with all physicochemical properties of mucus gel network, mesh spacing (“pore” size) play significant role on the barrier properties of mucus. Mucin fibers can sterically preclude particulates that are too large to pass the mucus mesh spacing. The thickness of the mucus structure varies for different mucosal surfaces. In the GI tract, the thickest mucus layer is the colon however the interfiber spacing exhibits significant variation from 10 nm to 500 nm [30-37]. Foreign particles are also trapped and removed from the GI tract by the help of protective mucus coatings. However, viruses can diffuse through mucus and penetrate to the epithelial cells. The main reason behind this penetration is due to a smaller size of the mucus mesh spacing than viruses. Whilst not fully understood, the analysis of intestinal mucus surface would enable to understand diffusion mechanism across GI tract layer to the underlying epithelium.
Various food-associated stimuli to be studied include lipids, pH and [Ca^{2+}], which are naturally present in the gastrointestinal environment. From a drug delivery standpoint, these food-associated stimuli offer feasible strategies (e.g. incorporation into a tablet, capsule, or other drug carriers) to enhance small molecule/drug carrier transport as well as controlling pathogen invasion. In summary, understanding the mechanism behind the food effect can improve lipid-based delivery of drugs, enhancement in nutrient exchange within the mucus barrier, minimize possible pathogen invasion such as inflammatory bowel disease, and minimize exposure to bioterrorism agents.
2.0 CRITICAL LITERATURE REVIEW

As mentioned above, mucus provides a permeable barrier for nutrient absorption while preventing foreign pathogens, environmental particulates, and toxins from diffusing through to the underlying epithelia layer. In addition to affording protection, mucus layer acts as a barrier to drug absorption. The presence of mucus also plays a significant role on sustained and prolonged drug delivery via the development of mucoadhesive dosage formulations [38-40]. The interactions between mucus constituents and drug delivery systems are important to explore the barrier properties of mucus against transmucosal drug absorption in order to control pathogen invasion.

2.1 Composition of Mucus

The main constituents of mucus are primarily crosslinked and entangled mucin fibers produced by goblet cells and submucosal glands [41, 42], lipids, salts, electrolytes, serum and macromolecules, bacteria and water. Although the exact composition may vary depending on its secretion location and the presence of disease conditions, overall mucus contains ~ 95% water, 0.5-5% proteins and lipids [43, 44], 0.5-1% salts by mass [43, 44].

2.1.1 Mucins and Sugars

Mucin is the major component of mucus and could be found in two forms: soluble secretory mucin and membrane bound mucin [45, 46]. Membrane-bound mucins contain hydrophobic regions keeping the molecules in the plasma membrane. The absence of disulfide bonds in membrane-bound mucins result in a lack of intermolecular interactions through disulfide bridges compared to secretory mucins [47].
High viscoelastic mucus structure comes from large secretory mucin molecules that are 0.5-50 MDa in size [7] formed by the individual mucin monomers linkage that are around 0.3-0.5 MDa in size [48, 49].

There are twenty proteins assigned to the MUC gene database from which MUC2, MUC5AC and MUC6 are the mucin proteins found in the GI tract [50]. Mucin protein backbone contains high levels of serine, threonine, alanine, glycine, proline residues linked with oligosaccharide components. Oligosaccharide chains are speculated to play an important role in protecting internal tissues against proteolytic degradation by digestive proteases [51]. Sugar residues consist of a series of Gal β [52, 53] and GlcNAc β [52-54] units that are often terminated by α glycosidic-linked galactose, sialic acid and sulfate. The sialic acid and sulfate components contribute high density negative charge to the glycoproteins that could modulate nutrient and drug passage through mucus layer. Sialomucin and sulfate contents are also correlated to mucus viscosity and elasticity [21].

Figure 1. Interstructural overview of mucins. Adopted from Cone et.al [1]
Heavily glycosylated regions of mucin proteins are separated with hydrophobic, covalently linked lipids that facilitate adhesive mucus-environment interactions [55]. These lipid coated domains are correlated to the viscoelastic properties of the mucus gel. Even though mucins significantly contribute to the gel properties of mucus, elastic and viscous features are governed by water, lipid, and ion content [1].

2.1.2 Lipids

In addition to glycoprotein components, mucus contains significant amounts of lipids with a mass ratio up to 2 % [43, 44]. Most of the lipids are covalently associated with the hydrophobic mucin protein domains [56]. The influence of lipids on orally delivered drug compound absorption across intestinal mucosa originates in part from colloidal structures the lipids form [57]. Lipids present in mucus include cholesterol, ceramide, palmitic acid, stearic acid, oleic acid, linoleic acid, and other free fatty acids [58]. It has been demonstrated that removal of lipids from mucus significantly alters its viscoelastic properties [59, 60]. Extraction of lipids from gastric mucus glycoprotein leads to 80-85 % decrease in the viscosity [61]. In particular, lipid content and composition affect the viscoelasticity in expectorated airway secretions from cystic fibrosis patients [59]. Overall mucus viscoelasticity highly correlated upon changes on the amount of lipids. However, in spite of the tremendous physiological relevance of exposure of intestinal mucus to lipid mixtures originating from food or drug delivery systems, the potential impact of exogenous lipids on barrier properties of intestinal mucus has not been explored.
2.1.3 Salts

Mineral salts in mucus comprise a mass percentage up to 1% [1]. Mucus properties depend in part on ionic interactions between mucins, and salts can thus directly alter mucus structure [62-64]. Monomers of the main intestinal mucin, MUC2, form intermolecular links with [Ca$^{2+}$] that increase mucus viscoelasticity [64, 65]. After food arrival, [Ca$^{2+}$] concentration varies in the small intestine. Reported calcium ion concentration prior to eating is between 4 to 5 mM, and it could go up to 20 mM after eating [66]. While multiple reports have demonstrated the impact of [Ca$^{2+}$] on physical properties of mucin solutions [63, 64], the impact of [Ca$^{2+}$] on drug or drug carrier transport through intestinal mucus has not been characterized.

2.1.3.1 GI pH

Gastrointestinal pH plays an important role in modulating drug delivery and nutrient absorption [67]. Mucus pH in the stomach varies from 1 to where it is nearly neutral at the epithelial surface. The pH is partially neutralized in the duodenum where it is around 7-8 in the colon and rectum [68]. After food arrival, median small intestinal pH is around 5.4, whereas median fasting pH is close to 6.1. However, pH values fluctuate and vary between individuals from a minimum as low as pH 3.1 to a maximum of pH 6.7 [69]. Acidity is known to alter mucus barrier properties [70]. Decreasing pH has been demonstrated to induce mucin aggregation and increase gastric mucin solution viscosity [71, 72].
It has been suggested that this is due to neutralization of glycosidic residues; however analysis of native and deglycosylated gastric mucin aggregation at low pH using atomic force microscopy indicated that oligosaccharide side chains are not directly involved. It is unclear if similar pH effects are observed in intestinal mucus.

2.2 Mucus Thickness

Continuous secretion of mucus is essential to keep the protective and lubricating properties. Adherent mucus layer functioning depends on its thickness and stability as well as the physical and chemical properties of the gel. At the diseased states mucus layer can be compromised during which mucus thickness is one the major target [73, 74]. There is also a constant replenishment of the adherent mucus layer and the collective glycolipids-glycoproteins that make up the glycocalyx in the GI tract that functions to remove microorganisms trapped by mucus [50]. This constant turnover mechanism directly affects the thickness of the mucus layer. Nutrient absorption rate is another factor that could alter the mucus thickness in the GI tract [2].

According to Atuma et al [2] mucus layer consist of two components, a loosely adherent gel that can be sucked out and a firmly adherent gel that remains on top of membrane. The relative thickness of these two components varies depending on location (Figure 2). In the GI tract, the mucus is thickest in stomach ranging between 40-450 µm and the colon is around ~ 150 µm [75, 76] which potentially increase protection mechanism of mucus against pathogens and acidic environment [77]. The thickness also varies in the small intestine depending on fasted state; food arrival often associated with an increase of loosely adherent layer [55] as well as increased mucus turnover time [78].
2.3 Mucoadhesivity

As it was stated before mucins are the main component responsible for the gel-like properties of the mucus. When glycoprotein concentrations reach to a threshold value the hydrodynamic volumes of the molecules start overlapping and gel formation occurs. Mucoadhesion term defined as the concept of the attachment of natural or synthetic polymers to a mucus surface. There is an urgent need to develop and/or improve mucoadhesive materials in order to increase the efficacy of drug and gene delivery.
To achieve this, one must understand the mechanism of bonding between a polymer and the mucus barrier however this phenomenon is still not completely clear yet.

In general, the mechanism of mucoadhesion is separated into two categories: The contact stage that can be described as the initial intimate contact between the mucoadhesive and the mucus membrane. In the consolidation stage, with the help of moisture the mucoadhesive material break free and entangle with mucin chains so that the formation of weak chemical bonds occur [79, 80].

2.3.1 Factors that can Change Mucoadhesive Properties

Mucoadhesion is a complex process and several factors significantly impact mucoadhesive properties. Some of the characteristics that have altered polymeric hydrogel mucoadhesive properties include hydrogen bonding capacity, concentration, charge, high polymer molecular weight, high polymer chain flexibility and cross-linking density.

2.3.1.1 Hydrogen Bonding Capacity and Flexibility

Polymers with a high amount of hydrogen-bonding chemical groups such as hydroxyls and carboxyls increase hydrogen bonding potential [81]. Polymers containing considerable amount of flexibility increase the desired entanglement with the mucus. Flexibility of the polymer also improves hydrogen bonding capacity. Overall, mobile and flexible polymers correlated to their viscosities and diffusivities, thus higher flexibility of a polymer results faster diffusion in the mucus gel [81].
2.3.1.2 Molecular Weight and Cross Linking Density

Mucus mesh pore size, average molecular weight of polymers and cross-linking density are important factors on polymer-mucus network interactions. Increase in molecular weight directly correlated with increase in mucoadhesive strength of polymeric materials [82]. Dense crosslinked polymers absorbed less amount of water into the polymer network results in insufficient swelling of polymer, thus decrease interpenetration rate of polymers across mucin fibers [81].

2.3.1.3 Concentration

Development of a strong mucoadhesive polymer can be correlated by the polymer chain length that penetrates into the mucus surface. If the polymer concentration is low the number of penetrated polymer chains into the mucus would be small, thus the inter-molecular reaction is unstable. Overall, in order to produce better mucoadhesivity polymer concentration should be higher. Surface chemistry of polymer network also plays an important role on penetration rate; this phenomenon will be discussed later.

In summary, delivery of mucoadhesive drug systems, specifically to GI tract is an important context. Mucoadhesive polymers have been used as model drug carriers for a long time to modulate the drug delivery in different locations of the GI tract. Understanding these factors mentioned above would help us to design more efficient delivery to the epithelia underlying the mucus barrier.
2.4 Mucoadhesive Nanocarrier Systems

There has been many works carrier out on the design and preparation of polymeric nanoparticles [83-86]. Their functions as oral delivery system mainly was the main focus however the behavior of these particulate drug carriers in the gastrointestinal tract has not been characterized clearly. The intestinal mucus is the major barrier to overcome for oral drug delivery. Orally delivered nanocarriers will be affected by physico-chemical environment of the GI tract. Intestinal contents will influence the stability of drug carriers. These polymeric nanocarriers would be degraded due to physical changes such as pH levels, presence of bile salts, phospholipids and enzymes, ionic contents and lipids. Thus, it is extremely important to investigate gastric and intestinal simulated fluids in order to understand how and where the active drug carriers will be released.

2.4.1 Role of Surface Chemistry and Particle Size

Surface of mucosal tissues is protected by a highly viscoelastic layer [7]. Even though protective mucus coatings trap and remove external particles from the GI tract [8] viruses could diffuse through mucus and reach to the epithelium. This is related to the smaller size of viruses than mucus mesh spacing and non-mucoadhesive surfaces. Recent strategies have shown how to overcome the mucus barrier by mimicking viruses to develop nanoparticles [7, 11]. Prepared particles were smaller sizes (≤ 200 nm) to diffuse through mucus and established longer retention time by avoiding elimination from mucilliary clearance [87]. There have been done numerous studies on mucoadhesive nanoparticles. Strong interactions between chains on particles surfaces and mucus could affect particle
diffusivities and retention time at mucosal surface. Mucus containing a high density of negatively charged glycoproteins attracts positively charged nanoparticles. Cationic polymers or cationic coated nanoparticles bind to mucin to form particles will increase residence time in GI tract and decrease diffusivities. Former coworker Crater and Carrier [88] demonstrated that anionic particles were 20-30 times faster in diffusivity in comparison with cationic particles. Transport rates were inversely related to particle surface potentials where negatively charged carboxylate- and sulfate-modified particles showed significantly higher transport rates than positively charged amine-modified particles [88]. On the other hand, covalently modified with carboxyl modified particles in diameter 59 nm were significantly hindered in human cervical mucus [89]. Dawson et al. found that relative diffusivities of surface modified either with carboxyl or amine groups were about 300-fold slower compared to same particles in water [5]. Similarly Norris et al. studied surface modified carboxyl, amine and sulfate particle transport and found slow transport of nanoparticles through gastrointestinal mucus [90]. To reduce the effect of surface chemistry and particle interactions with mucus, a hydrophilic and uncharged polymer, PEG, was used to coat particle surface. Particles coated with PEG make their surfaces neutral and increase their diffusion through mucus [12, 91-93]. In conclusion, for efficient oral delivery it is important to establish a balance between mucoadhesion and mucus penetration. Particle size is significant in order to avoid steric inhibition by the fiber mesh as well as surface chemistry to reduce adhesivity to mucin fibers.
2.5 Mechanical Properties of Mucus

Mucus is a thick substance and complex network that cover the luminal surface of many organs in humans and animals. The function of mucus varies for each type of organs. Complex gel structures such as mucus show physical behaviors that are between those of viscous liquid and elastic solid. In order to understand its physical function rheological measurements including viscosity and elasticity are used.

A rheological measurement indicates how “soft” or “hard” material is. Commonly used rheological characterization performed by using controlled stress or controlled strain rheometer. Measurements that are used to characterize internal structure of complex materials are stress relaxation, creep and sinusoidal oscillations. Even though these conventional rheology measurements used to detect mechanical response of a broad range of materials they would not be applied to biological samples where these samples require small quantities. Microrheology, rheology on a micro length scale, can be applied to determine the relation between stress and deformation in biological materials.

2.5.1 Microrheology of Mucus

The efficient delivery of drug and gene carriers to mucosal tissues is often hindered by highly viscoelastic and adhesive mucus barrier. It is crucial to engineer nanoparticles capable of penetrating through mucus barrier by understanding the viscous and elastic features of mucus at length scales relevant to nanoscale drug delivery systems. Microrheology has recently gained popularity especially exploring complementary information on mechanical properties mucus in small sample volumes inaccessible to bulk rheology.
In the recent study, Norwalk (38 nm) and human papilloma (HPV; 55 nm) were able to penetrate mucus indicating particulates smaller than the average mucus mesh pore size do not adhere to mucus barrier [89]. Estimated mucus pore size shows that viruses are capable of infect mucosal tissues when their sizes are smaller such as polio (28 nm), hepatitis B (43 nm), adenoviruses (60-90 nm) and rotavirus (75 nm) [55]. These results suggest that mucus microviscosity remains similar to pure media (water) viscosity at length scales up to 60 nm. Larger 180 nm herpes simplex virus (HSV) transport in mucus was reduced more than 100 –fold compared to transport in water indicate that effective viscosity for length scale of ~ 200 nm significantly increased due to approximate 100 nm interfiber spacing in mucus [94]. As the diameter of particles increase microviscosity increment becomes more evident. Transport of 1 µm particles exhibit significantly reduced diffusivities corresponding to higher microviscosity values [95]. Despite big differences in the relevant length scales, viscous and elastic moduli can be reflected in part by macrorheological characterization. For instance, Sanders et.al. found variations in the bulk elastic modulus of mucus samples that the percentage of nanoparticles transported through mucus increased with increasing bulk elasticity. This finding was interpreted by the change of heterogenic mucus mesh structure resulted in larger pores for particle diffusion as elasticity increases [96].

Mucus microrheology would also help us to better understand pathogen-mucus environment interactions, specifically how mucus efficiently removes particular infectious pathogens via adhesive interactions. With an improved microrheological characterization of diseased states of mucus we will be able to explore more on pathology and mucus physiology.
2.6 Transport of Pathogens in Mucus

Gastrointestinal epithelial cells have evolved to have robust barrier mechanism which is resistant to microbial passage. However, enteric pathogens have developed various strategies to circumvent the protective barrier of mucus and penetrate to epithelial cells. Intelligently, enteric microbial developed mechanism to allow effective penetration through mucus, mucus degradation and disruption of cells which secrets and produce barriers. Most of the pathogens are chemo attracted to mucus. Presences of flagella propel most pathogens through mucus environment. In addition, some pathogens such as Helicobacter Pylori increase the pH of its environment result in decreasing viscoelasticity of mucus environment [97]. Due to mucus aggregation, microbial can be removed from the body; in this case microorganisms have evolved mechanism to degrade mucus by secreting enzymes. These enzymes degrade the mucin oligosaccharides leading the mucin peptide backbone to proteases. This proteolytic activity results in disassembly of mucin chains and decrease mucus viscosity leading pathogens to diffuse through weak mucus barrier [98]. Physicochemical properties such as size and surface chemistry of viruses provide some insight on rapid transport across mucosal tissues. Virus particles that infect mucus vary in sizes (Capsid viruses: polio, 28 nm; hepatitis B, 43 nm; adenoviruses, 60–90 nm; rotavirus, 75 nm. Enveloped viruses: HIV, 120 nm; HSV, 180 nm) and are able to penetrate through mucus barrier without interacting with hydrophobic patches on mucin fibers.
2.7 Techniques to study Drug and Gene Carrier Delivery Systems

Quantitative investigations of mucus gel barriers have focused mainly on drug or gene carrier model of particle transport properties. Particle interactions with its biological environment have been poorly characterized. Dynamic relations of drug carrier vectors within the mucus barrier have long been investigated by measuring rate of permeability through a thin layer of mucus in a diffusion chamber [47, 99]. Diffusion of various sizes of polymeric particles in reconstituted porcine gastric mucus gel has been first studied by Norris and Sinko using sandwich type of a Transwell-Snapwell diffusion chamber. 300 nm particles sizes showed significant decrease in translocation permeability [100]. By using the same experimental setup, Sanders et al. found that the larger particles (560 nm) with carboxylate modifies surfaces showed extremely low percentage penetration through 220 µm thick CF sputum layer after 150 min [96]. Diffusion-chamber technique was used recently to analyze actins’ role on mucolytics agents to enhance the diffusion rate of particles in mucus [101]. Although diffusion-chamber technique is easy to setup there are certain shortcomings such as estimating precise thickness of mucus gel layer, homogeneously distribution of mucus throughout the filters and most importantly adhesive function of mucus that blocks the filter pores [102].

To prevent the shortcomings of diffusion chamber technique a number of studies have been done to investigate dynamic transport of nanoparticles using microscopic techniques including fluorescence recovery after photobleaching (FRAP) and real-time high resolution multiple particle tracking (MPT). FRAP has been extensively used to study protein and virus diffusion in human cervical mucus [89, 102].
Impact of liposomes on the diffusion of plasmid DNA in reconstituted bovine cervical mucus was also studied using FRAP technique [103]. Diffusion of macromolecules in CF sputum was analyzed using confocal scanning laser microscopes in 3D using FRAP technique [104]. Although this procedure provided some insight into diffusion of particulate systems, FRAP grants us only average diffusion rates over many particles which most likely provides lack of information on individual particle level and transport characterization of individual particle diffusivities. To circumvent this issue Hanes and coworkers have established a new technique named real-time high resolution multiple particle tracking (MPT) for drug and gene delivery applications [4, 6, 12, 105-107].

2.7.1 Multiple Particle Tracking

MPT allows tracking the microscopic motion of individual particles that are adherent to mucus at any given time with high spatiotemporal resolution. MPT can also be used to simulate quantitative and qualitative transport mechanism of diffusing particles such as diffusivity, viscoelasticity, pore size, velocity, and direction of particle vectors and transport mode in complex biological fluids [108, 109]. MPT was first used to measure transport rates of carboxylate- and amine-modified nanoparticles sized 100- and 500 nm in diameter in fresh human sputum [5]. In other work of same group, MPT was applied to measure the diffusion of biodegradable hydrophobic PLGA nanoparticles coated with anionic DNA using cationic surfactant to study the impact of surface chemistry [6]. Similarly, multiple particle tracking was used to examine the barrier properties of gastrointestinal mucus and its dependence on surface chemistry by using cationic and anionic modified particles.
It was found that transport rates were inversely related to particle surface potentials with anionic particles displayed higher transport rates than cationic particles [110]. Various sized particle transport rates were also quantified using MPT in fresh undiluted human cervicovaginal mucus [105].

2.7.2 Electron Paramagnetic Resonance

Electron paramagnetic resonance (EPR) spectroscopy is a powerful tool that can provide important structural and dynamic information on different biological environments. EPR spectroscopy requires the introduction of a stable free-radical group such as nitroxide into the system. The EPR system consists of a single unpaired electron spin exist in a molecular orbital. The electron exist in one of two magnetic components, \( m_s = + \frac{1}{2} \) and \( m_s = -\frac{1}{2} \) and they are stable when there is no magnetic field. However when there is absorption or emission of a photon of energy the \( -\frac{1}{2} \) state decreases and the energy of the \( +\frac{1}{2} \) increases.

![EPR Transition](image)

**Figure 3.** EPR transition happens when the energy contained in the microwave photons matches the splitting between two electron spin states. Image adopted from wiki.
This increment between the two states widened the energy states gap until it matches the energy of the microwaves as shown by the double-arrow in the plot (Figure 3). The unpaired electron starts spinning at this point results in a net absorption of energy which is then converted into an EPR spectrum. The upper spectrum indicates the simulated absorption for free electrons in a varying magnetic field. The lower spectrum is a representation of the first derivative of the absorption spectrum (Figure 3).

The potential of using EPR could be used nearly any biological system with the help of spin labeled probes. By placing stable radicals at specific locations on biological macromolecules EPR usage became a powerful method to obtain important information on structural and dynamics of small size peptides, proteins, macromolecules and nucleic acids [111-115]. EPR spectroscopy is not limited by the size of the sample or the optical features of the sample. Measurements can be done on samples using proteins in solution to densely prepared membrane suspensions, tissues or samples frozen and kept at cryogenic temperatures [116]. EPR spectroscopy of spin labeled probes also reveals the polarity of medium surrounding, intra and intermolecular distances between two nitroxide molecules [117]. Analysis of EPR spectra allows us to model protein structure with a spatial resolution at the level of the backbone [118].

As it was stated before the mucus layer protecting epithelial surfaces of tissues is a critical barrier in drug delivery and pathogen transport. However interestingly, diffusion rates of small molecular weight compounds, especially lipophilic compounds, can be significantly reduced in mucus in spite of the reported pore size on the order of 100 nm [119, 120].
EPR spectra to mucus can be utilized to characterize diffusion coefficients [which can be used to approximate concentration profiles and time for transport across mucus] of small molecular weight (<500 Da) compounds dosed in test media. EPR affords the ability to noninvasively study the micro-viscosity and micro-polarity of the microenvironment of a paramagnetic probe (model drug), and thus how microenvironment changes with exposure to food-associated stimuli.

2.7.3 Fluorescence Recovery After Photobleaching (FRAP)

As the name implies, the function behind FRAP is that the fluorophores undergo bleaching when exposed to intense light. An intense laser is used to bombard a region of the sample where there are fluorescently labeled molecules. Right after the bleaching, a low intensity laser light is used to recover bleached area by the inward diffusion of non-bleached molecules into the bleached region and outward diffusion of bleached molecules. As shown in figure 4, kinetic behaviors of fluorescently labeled molecules using FRAP determined in three steps. In the first step, a defined area (ROI) selected for bleaching and pre-bleaching applied by low laser power. \( F_i \) indicates the initial values of fluorescence induced by laser power. In the second phase, high laser power bleaches the ROI. Fluorescent intensity is shown as \( F_0 \), which reflects depth of bleach. Right after bleaching, low laser power is used for recovery in fluorescence. When the intensity reaches to its steady state \( (F_\infty) \) one can estimate the mobile fraction of molecules.
Figure 4. Schematic representation of fluorescence recovery after photobleaching measurements. Figure adopted from [3]

While FRAP has been used since 80s to study the dynamic cells and cell membranes in complex environment [121, 122], the method has been utilized a lot since then on the mobility of lipids and protein membranes. For instance, FRAP can be used to evaluate the in-vivo protein membrane or lipid rafts on the motility of various proteins [123].
Bile salt micelles increase in concentration in the fed state gastrointestinal (GI) environment and are known to be important to the solubilization and absorption of orally delivered compounds. It is not clear if these colloidal structures stay intact within mucus, and thus if bile micelles interact with the intestinal epithelial surface. Elucidation of bile micelle transport phenomena in mucus would facilitate mechanistic understanding and prediction of the impact of food-associated bile micelles on orally delivered compound absorption. FRAP will be utilized to explore bile salt micelles transport in mucus as well as to observe if bile salt micelles stay intact or dissociate within the mucus layer.
3.0 EXPERIMENTAL

The influence of lipids on oral absorption derives from colloidal structures they make and hence effects on drug transport through the intestinal mucus. Function of lipids in the GI tract mucus has particularly understudied yet potentially highly important. While many studies up to date have focused on sputum or gastric mucus, recent study indicated that phospholipids and phosphatidylcholine content in intestinal mucus govern in controlling pathogen colonization [124]. However, there is very little information related to the influence of lipid colloidal phases and associated physiological changes such as pH, [Ca\(^{2+}\)] on transport of drug or gene carriers through intestinal mucus. Proposed project also study the impact of bile salts and phospholipids at elevated concentrations. After food arrival, bile salts and phospholipids form endogenous micelles, and ingested lipids form microemulsion droplets which produce fatty acids and monoglycerides that can partition into bile salt micelles and/or form vesicles [11, 125]. However it is not clear if these colloidal structures stay intact across the intestinal mucus layer. This will be studied in the proposed study. The comprehensive transport studies, including variably sized and functionalized particulates, model microbes, and small molecular weight compounds, as well as nano- to macro-scale structural analyses proposed will provide novel insight into the mechanisms of lipid impact on intestinal mucus essential for exploitation of these phenomena for therapeutic purposes. As the proposed stimuli to be studied: lipids, pH, and [Ca\(+2\)], are inherently present in the normal GI environment, they offer feasible and attractive, from regulatory and manufacturing viewpoints, strategies for enhancing small molecule/drug carrier transport, and/or controlling pathogen invasion.
If these agents significantly impact mucus transport, they or excipients that may control their action (e.g., a buffering agent) could be easily incorporated into a tablet, capsule, or other drug carrier. In addition, proposed study results can be used to provide meaningful guidance on dosing in the presence of food, and fundamental understanding of mechanisms behind the “food effect.”

In summary, insight provided from the proposed studies may enable lipid-based delivery of drugs, enhancement in nutrient uptake, or control of pathogen invasion in diseases such as necrotizing enterocolitis or inflammatory bowel disease, or exposure to bioterrorism agents. A new, relatively facile model for controlling intestinal epithelial exposure will be introduced to the medical community.

3.1 Native Porcine Mucus Collection and Preparation

Pig intestines (Research 87, Boylston, MA) were purchased from a local abattoir. Native porcine intestinal mucus was extracted from pig jejunum within 2 h of slaughter. Mucus was scraped with a spatula, placed in 2 ml mucus specimen and kept at -80°C until experimentation. Each specimen was thawed for about 30 minutes at room temperature before experiments were conducted.

3.2 Preparation of Biorelevant Media

Simulated intestinal contents were prepared using maleate buffer at physiologically relevant pH levels (5.5, 6.5), as well as lower pH values representing extremes experienced in the stomach and possibly upper duodenum (3.5), and physiological Ca++ concentrations (0, 5, 10, 20 mM), fed state levels of bile salt (sodium taurodeoxycholate, NaTDC) and phospholipids (L-alpha-phosphatidylcholine from egg yolk, PC). Model
bile components were mixed with maleate buffer on a stirring plate at 37°C. To simulate the food-associated lipid intake soybean oil, sodium oleate and monoglycerol was added to bile/phospholipid and maleate buffer solution.

<table>
<thead>
<tr>
<th>Maleate Buffer</th>
<th>Triz-ma</th>
<th>100 mM</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>NaCl</td>
<td>65 mM</td>
</tr>
<tr>
<td></td>
<td>CaCl₂</td>
<td>0-20 mM</td>
</tr>
<tr>
<td></td>
<td>NaN₃</td>
<td>3 mM</td>
</tr>
<tr>
<td></td>
<td>NaOH</td>
<td>0-40 mM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model Bile/Phospholipid</th>
<th>NaTDC</th>
<th>12 mM</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Lecithin</td>
<td>4 mM</td>
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</table>

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Soybean Oil</th>
<th>35 mM</th>
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<tbody>
<tr>
<td></td>
<td>Sodium Oleate</td>
<td>30 mM</td>
</tr>
<tr>
<td></td>
<td>1-Oleoyl-rac-glycerol</td>
<td>15 mM</td>
</tr>
</tbody>
</table>

Table 1. Bio-relevant media [126].

3.3 Preparation and Characterization of Microspheres

Fluorescently labeled yellow-green FluoSpheres (Invitrogen Molecular Probes, Carlsbad, CA) were used to prepare particle suspensions. 200 nm in diameter amine-, carboxylate-, and sulfate-modified (2% solids in distilled water with 2 mM azide) microspheres were used as model carriers. 20 nm, 40 nm, 100 nm and 500 nm carboxylate-modified particles were used to investigate particle size effect on particle transport in mucus. 200 nm and 500 nm polyethylene glycol (PEG) particles were used to understand surface chemistry effect on particle transport. The particle sizes and zeta potentials (ζ-potentials) of the polystyrene particles was determined at room temperature by dynamic light scattering in various biorelevant test media using a 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY).
3.4 Ex-Vivo Tissue Preparation

Small intestinal tissue was extracted from 3 weeks old wild type FVB/N mice. Animals were placed into CO$_2$ chamber and introduced 100% carbon dioxide. Chamber was filled with carbon dioxide with a rate of 20% of the chamber volume per minute to fulfill the objective of rapid unconsciousness with minimal distress to the animals. They were kept in the chamber for about 2 minutes.

![Figure 5. Schematic representation of CO$_2$ euthanasia and intestinal tissue dissection](image)

Lack of respiration and faded eye color was observed on rodents after 2 minutes. Animals then were laid down on a sterile blanket. Intestine fragments were dissected, cut open to expose the intestinal lumen, and placed into a chamber on a microscope slide.

3.5 In-Vivo Tissue Preparation

Rats (Sprague-Dawley), male, 70-90 days old, 300-375 g in weight were dosed with or without test lipid system (soybean oil). Rats were fasted 18 h prior to procedure and they were housed on raised wire floors. The purpose of the raised wire floors is to prevent coprophagy. 2 ml of food solution was administered by oral gavage.
After 30 min, rats were anesthetized using 2.5-3 % isoflurane vapor. The abdominal cavity was entered by way of a 2 cm midline abdominal skin incision positioned 1 cm below the diaphragm along the linea alba. Using a 22G needle syringe 0.05 ml fluorescent polystyrene carboxylate-modified microspheres 200 nm in diameter was injected into the duodenal lumen. Due to high acidic environment in gastric fluid model drug solutions could be denatured in stomach, in order to prevent any damage on drug solutions particles were injected through small intestine where pH is nearly neutral. Rats were kept anesthetized under 2.5 % isoflurane on a warm water blanket for 30 min to let particles dispersed throughout the lumen. Rats were sacrificed under anesthesia so that the pain or discomfort associated with this procedure is anticipated to be bare minimum. Intestinal contents were collected and microsphere solutions were imaged using fluorescence microscope.

### 3.6 Multiple Particle Tracking of Microspheres

Fluorescently labeled particle trajectories were captured using a 12.5 megapixel cooled Olympus DP70 digital color camera (Olympus, Center Valley, PA) mounted on an inverted Olympus IX51 microscope attached with X-Cite 120 fluorescence system (EXFO, Mississauga, Ontario, Canada). Particle tracking experiments in native porcine mucus were carried out on non-fluorescent 8-well polystyrene medium chambers (Thermo Fisher Scientific, Rochester, NY). Particle trajectories in excised tissue were tracked on non-fluorescent glass microscope slides. 10 µl of diluted particle solutions (0.0025% wt/vol) were added onto 200 µl of native pig gastrointestinal mucus. 5 µl of diluted particle suspensions were added on 1 mm cut excised tissue. Samples were covered and equilibrated for 2 h at room temperature prior to microscopy.
Particle trajectories were captured by Olympus DP imaging software with a frame rate of 30 fps for 20 s. Trajectories of n~100 particles were analyzed for each experiment and three experiments were performed from three different mucus specimens for each experimental setup to account for mucus variability. Each particle trajectories were generated using the feature point detection and tracking algorithm of the ParticleTracker ImageJ plugin developed by Sbalzarini et al [127]. Particle coordinates were converted into time-averaged mean squared displacement (MSD) and effective diffusivities ($D_{\text{eff}}$):

$$\text{MSD} = [x(t+\tau)-x(t)]^2 + [y(t+\tau)-y(t)]^2$$

$$D_{\text{eff}} = \text{MSD}/(4\tau)$$

Where; $x(t)$ and $y(t)$ are the nanoparticle coordinates at a given time, $\tau$ is the time scale [4, 106-108]. Two-dimensional (2D) time-independent diffusion coefficients ($D_0$) were determined by fitting MSD versus time scale plots to the anomalous subdiffusive transport relation:

$$\text{MSD} = 4D_0\tau^\alpha$$

where $\alpha$ is the anomalous exponent, which indicates the degree of particle transport obstruction ($\alpha = 1$ represents unobstructed Brownian diffusion). For 2D diffusion coefficients to be equivalent to 3D diffusion coefficients it was assumed that mucus is locally isotropic; hence particle displacements in the x, y, and z directions are uncorrelated [4, 5]. Diffusion coefficients of particles in mucus will be compared with the theoretical diffusion coefficients of the same size particles diffusing in water using the Stokes-Einstein equation [4]:
$D_0 = \frac{k_B T}{6 \pi \eta a}$ \hspace{1cm} (4)

Where:

- $k_B$ = Boltzmann constant,
- $T$ = temperature,
- $\eta$ = fluid viscosity,
- $a$ = particle radius

### 3.6.1 Characterization of individual particle transport modes

Individual particle transport modes (diffusive, subdiffusive, or immobile) were characterized using anomalous exponents determined from the slopes of MSD versus time scale log-log plots. Slopes ($\alpha$) greater than 0.9 represented diffusive particle transport, between 0.2 and 0.9 represented subdiffusive particle transport, and less than 0.2 represented particle immobilization.

### 3.6.2 Modeling of Particle Penetration in Mucus

Particle diffusion coefficients were used to estimate the fraction of microspheres expected to penetrate intestinal mucus layers using a numerical integration of Fick’s second law:

$$\frac{dC}{dt} = D_{\text{eff}} \frac{d^2C}{dx^2}$$ \hspace{1cm} (5)

Where:

- $C$ is the concentration of particles,
- $t$ = time
- $x$ = position.

Boundary conditions included assumption of a constant concentration at the mucus surface and initial absence of particles within the mucus layer [11, 125]
3.7 Microrheological Characterization of Mucus via PTM

Microrheological characterization of mucus is important to understand the local mechanical properties of mucus environment. Mucus has low viscosity fluid dispersed in micro-structural fiber domains. Dynamic motions of nano-scale entities diffusing through mucus are hence controlled by microrheology of mucus environment. The motion of these small size entities which do not interact with mucus elements are explained by Stokes-Einstein relation for diffusion [128]. Mechanical properties of gastrointestinal mucus could be revealed from analysis of time scale dependent mean square displacements of particles, as previously described [129, 130].

The viscoelastic spectrum:

\[ G(s) = \frac{2k_BT}{\pi a_s} \langle \Delta r(s) \rangle \]  

(6)

can be calculated using MSD values,

Where: \( G(s) \) = viscoelastic spectrum,

\( k_B \) =Boltzmann’s constant,

\( T \) = temperature,

\( s \) = Laplace frequency,

\( a \) = particle radius, and

\( \langle \Delta r(s) \rangle \) is the Laplace transform of the mean square displacement
The Fourier transformation of $G(s)$ denotes the complex viscoelastic modulus:

$$G^*(\omega) = G'(\omega) + iG''(\omega)$$  \hspace{1cm} (7)

Where: $G'(\omega) =$ elastic or storage modulus, which is the tendency to recover shape following stress-induced deformation, and

$G'': = $ viscous or loss modulus, which represents resistance to flow.

### 3.8 Mucus Imaging

Confocal imaging of lectin stained mucus exposed to food-associated stimuli will provide important insight on barrier properties of mucus. 80 µl of mucus was stained with 4 µl of 10 µg/ml lectin from Ulex europaeus agglutinin (UEA-1) conjugated with TRITC (Sigma Aldrich- L4889). After 20 min incubation in a dark humid chamber, 4 µl of yellow-green fluorescently labeled carboxylate-modified particles (diameter: 0.2 µm) diluted in test medium was added as a model particulate system and allowed to diffuse across the mucus barrier for 2 h. Structure indicated via lectin staining and relative position of mucus and particles were assessed using a Zeiss LSM 700 confocal microscope. Macro-scale changes in mucus structure upon addition of food-associated stimuli were visually observed and imaged for each type of preparation.

#### 3.8.1 Histology

Rat small intestine tissues were fixed in Carnoy’s solution to have better preserved mucus layer in paraffin blocks [131, 132]. Carnoy's solution is a fixative composed of 60% ethanol, 30% chloroform and 10% glacial acetic acid. 5 µm thick sections were prepared and stained using Lectin (25 µg/ml) to detect mucins and Hoescht 33342 (10 µg/ml) to pick up nuclei.
3.9 Bile Salt Micelle Preparation for FRAP

Bile Salt micelles were prepared similarly for FRAP and MPT. The biorelevant medium simulating the intestinal fluid contents consisted of 100 mM Trizma maleate, 65 mM NaCl, 10 mM CaCl$_2$ and 3 mM NaN$_3$ at pH 6.5 was prepared. Model bile was comprised of sodium taurodeoxycholate (NaTDC) and fluorescently labeled α-BODIPY® FL C$_{12}$-HPC as a model of the human bile, prepared in maleate buffer. Since it is unknown if bile salt micelles stay intact within mucus, bile salt micelles were prepared above and below their critical micelle concentration (CMC) to ascertain if micelles remain intact in mucus exposed to simulated intestinal fluids. Bile salt micelles above CMC were prepared by adding 12 mM/ 4 mM BS/PL ratio and bile salt micelles below CMC were prepared by adding 3 mM / 1 mM BS/PL ratio on top of maleate buffer. Native porcine mucus was collected from pig jejunum as explained before. 100 µl of mucus was placed on a microscope slide attached with a frame sealing chamber (Bio-Rad, Hercules, CA), and 5 µl of micelles solution was added on top of the mucus with minimal perturbation of the mucus structure. Samples were kept in a humid chamber for 2 h prior to confocal microscopy.

3.9.1 Confocal Microscopy for FRAP

Imaging for FRAP experiments was performed on a Zeiss LSM 700 laser scanning microscope (LSM) using a 63X Plan-Apochromat, NA 1.4 objective. Fluorescently labeled phospholipids were excited with 488-nm light at 50 % output.
512 x 512 pixel resolution images with a nominal speed 9 (pixel dwell time 3.15 µsec) was captured with 5 % laser transmission to avoid photobleaching the entire image. Each experiment consists of total 500 scans from the ROI and each scan takes 200 ms with a 1.5 s delay between scans. The circular bleached ROIs are 3.1 µm in diameter.

### 3.9.2 Data Analysis for FRAP imaging

Mathematical modeling of hankel transform was applied to calculate experimental micelle diffusion coefficients using matlab code developed by Jönsson et al [133]. This procedure is based on circular averaging of each image, followed by spatial frequency analysis of averaged circular bleached data. Further matlab coding details can be found online: [http://www.mathworks.com/matlabcentral/fileexchange/29388-frapanalysis/content/frap_analysis]({http://www.mathworks.com/matlabcentral/fileexchange/29388-frapanalysis/content/frap_analysis%202p5/frap_analysis.m})

Theoretically, the fluorescently labeled phospholipids stay as single lipid molecules below their CMC. In order to estimate the diffusion coefficient of single lipid molecule, Wilke - Chang equation was used [134]:

\[
D_{AB} = 7.4 \times 10^{-8} \frac{\varphi_B M W_B}{\eta V_A^2 T} \quad (8)
\]

Where, \(D_{AB}\) diffusivity of compound A in solvent B (cm\(^2\)/s), \(\varphi_B\) is the constant which accounts for solvent/ solvent interactions (2.6 for water), \(T\) is temperature (K), \(\eta\) is the viscosity of solvent B (cp), \(M W_B\) is molecular weight of solvent B (g/mol), \(V_A\) is the molar volume of compound A (cm\(^3\)/mol).

Estimated micelle diffusion coefficients in water was calculated using Stokes-Einstein equation as stated in equation 4 where this time ‘a’ stands for micelle radius.
3.10 Model Microbe Preparation for Pathogen Transport

Mucus, a biological hydrogel barrier, controls pathogen entry into the gastrointestinal and respiratory tracts. Self-modulation of mucus barrier regulates food and drug absorption in the gastrointestinal tract [71] however the defense mechanism and associated barrier properties of mucus are not well understood. ATCC strain 700926 *Escherichia coli* (*E.Coli*) was used as a model pathogen. *E.Coli* from frozen glycerol stocks (-70 °C) was streaked onto agar plates three times to obtain single colonies to be inoculated into 10 ml Luria-Bertani (LB) medium. Single colonies were grown at 37 °C for ~12 h with vigorous shaking (300 rpm). E. coli was dosed into test media (25 µl test medium: 5 µl cells in LB). Trajectories of single cells were captured using video microscopy.

3.10.1 Video Microscopy and Bacteria Tracking Algorithm

Diffusion of individual bacteria were obtained using an Olympus DP70 digital color camera (Olympus, Center Valley, PA) mounted on an inverted Olympus IX51 microscope with attached X-Cite 120 fluorescence illumination system (EXFO, Mississauga, Ontario, Canada). Bacteria diffusion videos were captured at 50X magnification with 512x512 pixel resolution (with 2x2 binning) with a frame rate of approximately 30 fps for 20 s. 220x200 pixel region of interest (ROI) selection was used to reduce the field of view and facilitate bacteria trajectory analysis. Individual bacteria trajectories and velocities were obtained using a modified version of the IDL colloid-tracking from Crocker and Grier [135, 136]. The algorithm was edited to account for elongated particles instead of spherical ones since the *E.Coli* are rod-shaped [136]. Three runs were performed for each particle and microbe experiments and a one tailed, unequal variance Student’s t-test was used to evaluate significance.
Individual bacteria mobility was categorized depending on bacteria trajectories. Track linearity (TL) was expressed as the ratio between the distance bacteria covers, DIS, and the length, LEN, of the bacteria track [137]. This ratio was also arbitrary classified by Lemichez et al [138] that when the ratio is greater than 70 % it corresponds to linear track (LT) where bacteria supposedly move freely. When the ratio is between 30–70% it indicates curvilinear tracks and when it falls below 30 % it corresponds to rotating tracks (RT).

Figure 6. Classification of the linearity of bacterial trajectories

\[
\text{Track Linearity Percentage (TL\%) = (DIS/LEN) \times 100}
\]

- $> 70 \text{\%} = LT$ (Linear Track)
- $70 - 30 \text{\%} > = CT$ (Curvilinear Track)
- $< 30 \text{\%} = RT$ (Rotating Track)
3.11 Small Molecule Transport in Mucus using EPR

EPR, a novel approach to biogel transport analysis was utilized to analyze the microenvironment of small molecular weight (<500 Da) compounds in mucus. EPR enabled elucidation of microenvironment (i.e. micro-viscosity and micro-polarity), and quantitative assessment of probe diffusion within mucus gel. As mentioned previously, EPR is able to detect unpaired electrons and allows for identification of the molecular structure near an unpaired electron. By assessing the lineshape changes it is possible to gain insight into the molecular motions of a molecule, and the fluidity or microviscosity of its local environment can be calculated.

3.11.1 Sample Preparation for EPR

Influence of lipids on small molecule transport in porcine intestinal mucus as well as the effects of surface chemistry on small molecule diffusion through gastrointestinal mucus was investigated using nitroxide spin probe systems with various surface moieties. The four model probes used in this study, included, positively charged 4-amino-tempo, negatively charged 3-carboxy- proxyl, hydrophobic 4-hydroxy-tempo benzoate and 4-hydroxy-tempo (Figure 7). Model drug compounds were dissolved in maleate buffer, in maleate buffer + bile components + soybean oil (50 mM final soybean oil concentration, no other lipid components) and in the FED State (table 1) at a concentration of roughly 1.5 mM. Probe solutions were left at room temperature for approximately 2 h to dissolve in the solution.

Mucus was added to 8-well polystyrene medium chamber slides (Thermo Fisher Scientific, Rochester, NY) and probe solution was added 1/10 (v/v) ratio on top of the
mucus and samples were incubated for 1 h in a humid chamber prior to EPR spectroscopy.

![Molecular structure of model spin probes. A) 4-Amino-Tempo, B) 3-Carboxy-Proxyl, C) 4-Hydroxy-Tempo, D) 4-Hydroxy-Tempo Benzoate](image)

After the 1 h incubation, 30 μL of sample was loaded into a capillary (Wilmad, for Eg. CV1012Q, 1mm inner diameter) using a pipette with the tip cut off. Bee’s wax was then used to seal both ends of the capillary and a small amount of Teflon tape was put on the top end to help stabilize the small capillary inside the larger capillary.

3.11.2 EPR set-up and Computer Settings

A rectangular cavity was used in order to run samples in capillaries. EPR spectra were obtained on a 9 GHz X band Bruker EMX spectrometer with temperature control (Bruker ER 4111 VT).

The experimental parameters that were used are as follows: attenuation: 15 dB, microwave bridge frequency ~9 GHz, modulation frequency: 100 kHz, microwave
power: \(\sim 6.3\) mW, sweep width 70 G, sweep time 83.89 s, time constant 327.68, modulation amplitude: 1 G, and 5 cycles were run for each sample and spectra were then averaged. Spectra were then fitted and analyzed using the Multicomponent EPR Labview module of Altenback to perform Simplex fitting. The fitting procedure contained variations in parameters including \(\log_{10}\) of the isotropic rotational diffusion constant (R).

Diffusion coefficients of spin probes and the microviscosities were calculated using Stokes-Einstein (SE) and Stokes-Einstein-Debye (SED) relations. These relations were derived from classical hydrodynamics (Stokes Law) and simple kinetic theory (The Einstein relation). Briefly, the Stokes-Einstein relation:

\[
D = \frac{k_B T}{6\pi \eta r_{\text{eff}}} 
\]  
(9)

Where, \(D\) is the translational diffusion constant, \(k_B\) is the Boltzmann constant, \(T\) is the temperature, \(\eta\) is the coefficient of viscosity, \(r_{\text{eff}}\) is the hydrodynamic radius of a molecule.

In the Stokes-Einstein-Debye relation:

\[
\tau_I = \frac{6V_{\text{eff}}\eta}{k_B T I(1+1)} 
\]  
(10)

where, \(\tau_I\) is the reorientation diffusion correlation time, which indicates the relaxation of spherical harmonics order I, and \(V_{\text{eff}}\) is the effective molecular volume which was calculated using sphere volume equation:

\[
V_{\text{eff}} = \frac{4}{3} \pi r_{\text{eff}}^3 
\]  
(11)

where, \(r_{\text{eff}}\) is calculated by taking the longest distance between the furthest atoms using 3D molecular shape of the spin probes.
Diffusion correlation time could also be related to average rotational rate constant, \( R \), determined via EPR spectra:

\[
\tau_I = 1/(6 \times 10^R) \tag{12}
\]

Thus, environment viscosity can be calculated combining equation (9) and (11):

\[
\eta = \frac{k_B T}{6 \times 10^R \nu_{eff}} \tag{13}
\]
4.0 RESULTS AND DISCUSSION

4.1 Impact of Lipids on Transport of Microspheres in Native Porcine Intestinal Mucus

Intestinal contents characteristic of the fed state, including model bile and partially digested triglyceride, markedly hindered particle transport through gastrointestinal mucus (Fig 1.a-f). Coordinates of fluorescently labeled polystyrene microspheres with differing surface chemistry were captured using real-time multiple particle tracking and transformed into time-averaged MSDs at various time scales, which were used to calculate effective diffusivities and local microviscosities, and to estimate particle penetration percentages. The ensemble-average particle transport rates decreased with time, as expected in a heterogeneous medium, such as mucus (Fig 1.d-f). Relative to diffusion in water (calculated using SE equation), the ensemble average effective diffusivities ($D_{\text{eff}}$) of amine-, carboxylate- and sulfate- modified microspheres in GI mucus at $\tau = 10$ s reduced 340-, 80-, and 35- fold when dosed in maleate buffer, whereas 3300-, 10800-, and 340- fold reductions in $D_{\text{eff}}$ were observed when dosed in fed state medium (Fig 8.a). Average $\alpha$ values were reduced from 0.46 to 0.018, 0.74 to 0.09, and 0.87 to 0.36 for amine-, carboxylate-, and sulfate-modified particles, respectively, when dosed in fed state compared to maleate buffer. These results indicated the high degree of transport hindrance by the presence of lipids.
Figure 8. Bile components and food-associated lipids hinder particulate transport in intestinal mucus. Ensemble $<\text{MSD}>$ versus time scale plots for a) amine- b) carboxylate-, c) sulfate-modified particles in porcine intestinal mucus. Corresponding ensemble $<\text{D}_{\text{eff}}>_{\text{V}}$ versus time scale plots for d) amine- e) carboxylate-, f) sulfate-modified particles. MB = maleate buffer, BS/PL = bile salt/phospholipid mixture, FED = model fed state intestinal contents.

In an attempt to uncouple the effects of lipids in fed state from effects of bile components (NaTDC and phosphatidylcholine), transport of particles in maleate buffer containing model bile components (NaTDC and phosphatidylcholine), and no other lipids, was also studied. In the fasted state, bile salt and phospholipid concentrations are approximately 5 mM and 1.25 mM, respectively, rising to 12 mM and 4 mM upon eating, when food-associated lipids (mainly triglycerides) are introduced. Transport analyses indicate that both food-associated triglycerides and elevated bile levels play an important role in modulating the properties of the gastrointestinal mucus barrier. Particles dosed to mucus in model bile were less mobile compared to those dosed in maleate buffer solution.
At a time scale of 10 s, amine-, carboxylate- and sulfate-modified particle diffusion coefficients were reduced 2.5, 4, and 5.5-fold, respectively, in the presence of BS/PL as compared to MB. However, comparison of transport in maleate buffer containing BS/PL versus fed state reveals significant impact of lipids in addition to BS/PL. Particle transport rates at $\tau = 10$ s (as reflected in $D_{\text{eff}}$) were hindered 4, 35, and 2-fold in fed state medium relative to maleate buffer with BS/PL.

![Graph](image)

**Figure 9. Estimated particle penetration through various thicknesses of mucus layer in small intestine. Lipids are predicted to decrease penetration of microspheres across an intestinal mucus layer**

Ultimately, the practical implication of changes in mucus barrier properties lies on the ability of various entities, including drug carrier systems, microbes, etc., to reach the underlying epithelium. Percent penetration of a dosed particle population across a mucus barrier of a given thickness can be estimated using a mathematical model based on Fick’s second law and effective diffusivities of microspheres (Figure 9).
In the absence of lipids, approximately 35% and 45% of a given dose of carboxylate- and sulfate-modified particles are predicted to cross a 10 μm thick layer of mucus (similar to the mucus layer in the ileum [8, 139]) in about an hour, a time frame on the same order as the mucus turnover rate, reported to be 50-270 min, whereas ~3% of amine modified particles can be expected to penetrate [140]. The fraction of particle penetration when particles are dosed in the FED state decreases to 1%, 5%, and 2% for carboxylate-, sulfate-, and amine- modified particles, respectively. The increased thickness of mucus in other portions of the GI tract (e.g., cecum mucus thickness of 37 μm [141]) would further reduce the estimated fraction of particle penetration (Figure 9).

Figure 10. The average diffusion coefficients for amine-, carboxylate-, and sulfate-modified particles normalized with theoretical diffusivity of 200 nm diameter particles in water.

Particles were dosed to mucus in maleate buffer, model bile, or fed state lipids.
The extent of particle transport (lipids vs buffer) and particle-mucus interactions are further evident through the normalized average diffusivities (see Figure 10), which are 340- to 3200-fold lower ($\tau = 10$ s) than the theoretical diffusivity of the same particles diffusing in water calculated from the Stokes-Einstein relation (Eq. 4) for amine-modified particles in MB and FED state, respectively. Similarly, the normalized diffusivities of carboxylate- and sulfate-modified particles are only 70- to 35-fold in MB and over 10000 to 3350–fold in FED state reduced ($\tau = 10$ s) compared to the theoretical diffusivity of the same size particles in water.

Figure 11. A) Particle size measurements of microspheres suspended in maleate buffer, bile salts/phospholipids in maleate buffer and fed state lipids. B) Zeta potential values of microspheres diluted in maleate buffer, bile salts/phospholipids with maleate buffer and fed intestinal state contents including lipids.

Examining particle size and surface properties provided some insight into the influence of lipid on the kinetic profiles of amine-, carboxylate-, and sulfate-modified nanoparticles in gastrointestinal mucus.
Analysis of particle diameters confirmed the formation of particle aggregates for amine-modified particles, which was visually observed using microscopy. Particle aggregate diameters ranged from 200 nm to 2500 nm, with an average diameter in the range of between 631 to 700 nm for particles in maleate buffer and fed state lipids respectively (Figure 11A). Analysis of larger particle aggregates over a micron in diameter was avoided by utilizing the kernel and cut-off radii using the feature point detection feature of the ParticleTracker ImageJ plugin; however, some of tracked amine-modified nanoparticles may include particle aggregates with effective diameters less than a micron. Conversely, even though there was a slight increase in the effective diameters of carboxylate- and sulfate- modified particles their diameters were close to the manufacturer’s specifications, 221 nm - 228 nm in maleate buffer and 281 nm - 293 nm in fed state for carboxylate- and sulfate-modified particles respectively (Figure 11A).

Decreases in diffusivities of 10 % for amine-, and nearly 20 % for carboxylate and sulfate- modified particles are expected based on Stokes-Einstein equation due to moderately increased particle sizes upon incubation in lipid-containing media. However, actual diffusivities when dosed in fed state medium relative to maleate buffer were reduced 70 % for amine- and sulfate and 95 % for carboxylate- modified particles at a time scale of 10 s. The fact that the measured particle sizes are close to the reported mesh size of mucus (~100 nm [142, 143]) may contribute to some extent to the greater reduction in diffusivity than that expected purely due to increased size, but there may also be a change in the intermolecular interactions governing mucus barrier properties, as further supported by observed structural changes.
Most mucus glycoproteins contain significant levels of sialic acid and sulfated oligosaccharides, which result in a highly negatively charged mucus surface. In addition to charged interactions, mucin fibers likely interact via hydrophobic or hydrogen bonding interactions. An enhancement in diffusivity may have been anticipated due to minimized particle-mucin interactions, as a result of both negatively charged surfaces. Alternatively an increase in particle positive surface potential for amine-modified particles (Figure 11B) lead to a decrease in diffusivity, which was expected due to enhanced interactions.

While increases in particle surface potential only affected the amine-modified particles, changes to the physiological environment and the mucus surface, affected transport of all surface modified particles. Elevation of calcium ion concentration may have reduced anionic functionalities of the mucus network or the particles, contributing to reduced particle mobilities. Furthermore, acidity likely disrupts and reduces the negative charges of the carboxyl groups on sialic acid within the mucin fibers which also hindered particle mobility.

**Figure 12.** Lipids alter the viscoelastic properties of intestinal mucus. (A-C) Local elastic and viscous moduli as a function of frequency for A) amine- B) carboxylate- C) sulfate-modified particles.
To probe structural changes in the mucus gel network upon addition of lipids, the time scale-dependent displacements were used to quantify the frequency-dependent viscous or loss modulus, $G''$, and elastic or storage modulus $G'$ (Figure 12). For all microspheres studied, GI mucus poses higher elastic modulus than viscous modulus, indicating a gel-like network structure. Furthermore, the increase in elastic modulus with addition of lipids is greater than that of viscous modulus, indicating strengthening of the mucus gel. Gel strength can be quantified using the phase angle $\delta$, calculated from the inverse tangent of $G''/G'$ (Figure 13). $\delta > 45^\circ$ represents a viscoelastic liquid, while $\delta < 45^\circ$ indicates a viscoelastic solid $[1]$. According to this criterion, fed state intestinal lipid contents significantly strengthen the mucus gel, rendering it more elastic relative to buffer solution.

![Figure 13. Lipids strengthen intestinal mucus gels. Phase angle, $\delta$, at a frequency of $2\pi$ rad/s is decreased for microspheres dosed in fed state lipid mixture relative to maleate buffer. Error bars denote standard errors based on three independent experiments, with $n \geq 100$ microspheres for each experiment, * $P < 0.05$]
4.2 Ex-Vivo and In-Vivo Analysis of Lipid Impact on Intestinal Mucosa Barrier

To determine if lipids would have a similar effect on mucus that was intact upon intestinal tissue, the mucosal surface of an explant of mouse intestinal tissue collected immediately after sacrifice was exposed to particles diluted in fed state medium or maleate buffer. Intestinal contents characteristic of the fed state reduced transport rates of microspheres over 5-fold compared to buffer at a time scale of 10 s (Figure 14A). To further test if similar effects would be seen with partially digested oil in vivo, microspheres were injected into the duodenum of rats 1 hour after orally dosing soybean oil or water used as a control. Oral dosing of soybean oil reduced microsphere effective diffusivities 10-fold (Figure 14B).

Figure 14. Ensemble <MSD> versus time scale plots for carboxylate-modified microspheres in A) mouse intestinal explants, B) rat intestinal explants after in vivo oral soybean oil dosing. Data represent the ensemble average of three separate experiments. Error bars denote standard errors based on three independent experiments, with n≥100 microspheres for each experiment, * P < 0.05.
4.3 Influence of [Ca++] and pH on Particle Transport

Exposure of intestinal mucus to physiologically relevant increases in [Ca\textsuperscript{2+}] and decreases in pH provided additional evidence that food-associated stimuli enhance mucus barrier properties. With increasing [Ca\textsuperscript{2+}], the mobility of all types of particles in intestinal mucus decreased.

![Graphs showing MSD and D_eff versus time scale plots for different types of microspheres at different Ca++ concentrations.]

**Figure 15.** Increasing [Ca\textsuperscript{2+}] (i.e., 5, 10, 20 mM) hindered particulate transport in intestinal mucus. Ensemble <MSD> versus time scale plots for A) amine- B) carboxylate-, C) sulfate-modified microspheres in porcine intestinal mucus. Corresponding ensemble <D\textsubscript{eff}> versus time scale plots for D) amine- E) carboxylate-, F) sulfate-modified microspheres.

At a time scale of 10 s, the relative transport rates of carboxylate-, sulfate-, and amine-modified microspheres dosed at [Ca\textsuperscript{2+}] of 20 mM were 4, 2, and 3-fold lower, respectively, than those of the same particles dosed at [Ca\textsuperscript{2+}] of 5 mM (Figure 14).
The decrease in diffusivity with an increase in [Ca^{2+}] from 10 mM to 20 mM translated to an estimated decrease in particle penetration. At a mucus layer thickness of 50 µm, carboxylate- and sulfate- modified particle penetration decreased from approximately 30% to 15% and 45% to nearly 30% respectively. It was again predicted that very few amine-modified particles would penetrate a 50 µm thick mucus layer within 1 h of dosing at both [Ca^{2+}] = 10 mM and 20 mM (Figure 15).

![Graph showing estimated particle penetration through various thicknesses of mucus after 1 hour. Calcium ion has a slight influence on particle penetration through mucus.](image)

The impact of calcium ion concentration on particle-mucus interaction for amine-modified particles is further evident through the normalized average diffusivities (Figure 16), which are 20- to 40-fold lower (τ = 10 s) than the theoretical diffusivity of the same particles diffusing in water at [Ca^{2+}] 10 mM and 20 mM, respectively.
FIGURE 17. Average diffusivities for amine-, carboxylate-, and sulfate-modified particles in native porcine mucus normalized with the calculated diffusivity of the same size particles in water at various time scales. Decreasing diffusivities with increasing time scale shows a slight dependence on time. $D_{\text{water}}$ was calculated using the Stokes-Einstein equation.

Influence of calcium on carboxylate- and sulfate-modified particles are evident that the normalized diffusivities of carboxylate- and sulfate-modified particles are 35- to 20- fold reduced ($\tau = 10$ s) compared to the theoretical diffusivity of the same particles in water at $[\text{Ca}^{++}]$ 20 mM.
Figure 18. Decreasing pH hinders particle transport in intestinal mucus. Ensemble <MSD> versus time scale plots for A) amine- C) carboxylate-, E) sulfate-modified microspheres in porcine intestinal mucus. Corresponding ensemble $<D_{\text{eff}}>$ versus time scale plots for B) amine-, D) carboxylate-, F) sulfate-modified microspheres.

As the pH of the dosing solution decreases, particle mobility was hindered (Figure. 17 A, C, E). At a time scale of 10 s, carboxylate-, sulfate-, and amine-modified particle diffusivities were reduced 6.5-, 7- and 3- fold, respectively, with a decrease of pH from 6.5 to 3.5.

Estimations of particle penetration again indicate that acidic conditions strongly impact the significance of a physiologically relevant mucus barrier thickness to particle translocation (Fig. 18). At pH 6.5, carboxylate-, and sulfate-modified particles would be able to penetrate a 100 μm mucus layer, whereas at pH 3.5 all particles are not able to penetrate over 30 μm mucus layer.
Figure 19. Estimated particle penetration through various thicknesses of mucus after 1 hour. pH of the mucus environment has a significant influence on particle penetration through mucus.

Amine-modified microsphere diffusivities in mucus were 340- and 1800- fold lower when dosed at pH 6.5 and pH 3.5, respectively, compared with their theoretical diffusivities in water. Carboxylate-and sulfate-modified particle diffusivities in mucus were 750-and 350- fold lower when dosed at pH 3.5 than their diffusivities in water (Figure 19).
FIGURE 20. Average diffusivities for amine-, carboxylate-, and sulfate-modified particles in native porcine mucus normalized with the calculated diffusivity of the same size particles in water at various pH.
<table>
<thead>
<tr>
<th>Medium</th>
<th>Particle</th>
<th>Diameter,nm</th>
<th>Zeta potential, (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maleate Buffer [Ca+2]= 5 mM</td>
<td>Amine</td>
<td>615 ± 10.3</td>
<td>7.74 ± 1.65</td>
</tr>
<tr>
<td></td>
<td>Carboxylate</td>
<td>219 ± 1.1</td>
<td>-16.2 ± 1.12</td>
</tr>
<tr>
<td></td>
<td>Sulfate</td>
<td>226 ± 1.2</td>
<td>-24.6 ± 1.5</td>
</tr>
<tr>
<td>Maleate Buffer [Ca+2]= 10 mM</td>
<td>Amine</td>
<td>631 ± 11.2</td>
<td>9.19 ± 1.98</td>
</tr>
<tr>
<td></td>
<td>Carboxylate</td>
<td>221 ± 0.8</td>
<td>-13.3 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Sulfate</td>
<td>228 ± 1.1</td>
<td>-21.2 ± 2.1</td>
</tr>
<tr>
<td>Maleate Buffer [Ca+2]= 20 mM</td>
<td>Amine</td>
<td>665 ± 16.5</td>
<td>14.56 ± 2.19</td>
</tr>
<tr>
<td></td>
<td>Carboxylate</td>
<td>246 ± 2.7</td>
<td>-5.13 ± 0.96</td>
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<tr>
<td></td>
<td>Sulfate</td>
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<tr>
<td>Maleate Buffer, pH 3.5</td>
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<td>642 ± 14.4</td>
<td>16.65 ± 2.02</td>
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<tr>
<td></td>
<td>Carboxylate</td>
<td>228 ± 2.6</td>
<td>-2.12 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Sulfate</td>
<td>235 ± 2.1</td>
<td>-6.15 ± 1.6</td>
</tr>
<tr>
<td>Maleate Buffer, pH 5.5</td>
<td>Amine</td>
<td>635 ± 8.4</td>
<td>14.4 ± 1.88</td>
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<td></td>
<td>Carboxylate</td>
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<td>-12.5 ± 1.2</td>
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<td></td>
<td>Sulfate</td>
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<tr>
<td>Maleate Buffer, pH 6.5</td>
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<td>9.19 ± 1.98</td>
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<tr>
<td></td>
<td>Carboxylate</td>
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<td>-13.3 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Sulfate</td>
<td>228 ± 1.1</td>
<td>-21.2 ± 2.1</td>
</tr>
<tr>
<td>Maleate Buffer+ BS/PL</td>
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<td>Sulfate</td>
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</tr>
<tr>
<td></td>
<td>Sulfate</td>
<td>293 ± 4.8</td>
<td>-24.4 ± 1.6</td>
</tr>
</tbody>
</table>

Table 2. Particle size and Surface Potentials of amine-, carboxylate-, and sulfate-modified particles in various test media
Investigating surface charge and size of particles in the various media tested provided some insight into particle transport profiles. Zeta potential measurements were dependent upon the $[Ca^{2+}]$ and pH of the medium. Increasing calcium ion concentration or decreasing pH resulted in a less negative (carboxylate- and sulfate- particles) or more positive (amine- particles) zeta potential, and also reduced particle diffusivities. Calcium ions interacting with the ions on the particle surface could change effective particle charge. Also, the pKa of the chemical groups on the particles (2.34, 2 and 9.6 for carboxylate-, sulfate- and amine- modified particles, respectively, as reported by the manufacturer) relative to the pH of the medium indicates that the carboxylate- and sulfate- surface groups could become partially neutral (approximately 5-10%) at pH 3.5.

### 4.4 Mucus Structural Changes Upon Exposure to Stimuli

The effects of food-associated stimuli on mucus structure and associated particle location within gastrointestinal mucus were also investigated. Particles dosed in maleate buffer alone (Fig. 20a) and maleate buffer with increased calcium concentration, $[Ca^{2+}] = 20$ mM (Fig. 20b) were fairly uniformly distributed throughout mucus. Particles were generally located in regions of minimal or no lectin staining, separated by regions on the order of 10 µm or less which stained positively with the lectin Ulex europaeus agglutinin I (UEA1). Particles dosed in the fed state medium (Fig. 20d) as well as in highly acidic (pH 3.5) medium (Fig. 20c) were generally not uniformly distributed across mucus structure, but rather accumulated at outer edges of regions on the order of 100 µm in size and diffusely staining with lectin.
Figure 21. Lipids and pH changes alter mucus structure and microparticle distribution within mucus. Particles dosed to mucus were diluted in A) Maleate buffer at pH 6.5 and [Ca^{2+}] 10 mM B) Maleate Buffer at [Ca^{2+}] 20 mM, C) Maleate buffer at pH 3.5 and [Ca^{2+}] 10 mM, D) Fed state lipids, including model bile components phospholipid and bile salt. Mucus appears more uniform and particles were dispersed more homogeneously for cases A and B. White arrows represent particles. Scale bars = 50 µm.
Changes in mucus upon exposure to microspheres dosed in various media were also macroscopically visible (Fig. 21). Exposure to media containing Ca\(^{2+}\) appeared to result in moderate clumping of mucus, creating a less homogenous medium (Fig. 21D,F) relative to untreated mucus (Fig. 21A,C,E) or mucus exposed to buffer not containing Ca\(^{2+}\) (Fig. 21B). Particles dosed in maleate buffer at pH 3.5 (Fig. 21J) and in fed state medium (Fig. 21L) induced a dramatic clumping of mucus into dense regions with clear boundaries from surrounding transparent fluid. The observed clumping phenomenon correlated with hindered particle transport in mucus.

![Figure 22](image.png)

**Figure 22.** Macroscopic changes in mucus structure are visible after exposure to \([\text{Ca}^{2+}]\), low pH medium or food-associated lipids and bile components. Mucus samples are shown before and after exposure (2 hr) to microspheres diluted in various media: A, C, E, G, I, K) Plain mucus prior to microspheres exposure, B) untreated mucus after 2 hr, D) maleate buffer medium without CaCl\(_2\), F) maleate buffer with a [CaCl\(_2\)] = 10 mM, H) maleate buffer with a [CaCl\(_2\)] = 20 mM, J) maleate buffer at pH 3.5 and L) fed state medium.
In order to verify the predicted penetration, rats were orally dosed soybean oil or water (control) 1 hr prior to dosing carboxylate-modified nanoparticles. The duodenum segments were collected 1 h after dosing nanoparticles, fixed, and processed for histology. The nanoparticles dosed post-soybean oil ingestion were not able to reach the epithelial surface, while the rats dosed post-water ingestion reached the epithelial surface, as evidenced by visualization of numerous particles endocytosed within the epithelium (Fig. 22).
4.5 Impact of Food-Associated Lipids on Various Size Microspheres

Impact of fed intestinal state of lipids investigated on different size polymeric microsphere diffusion through gastrointestinal mucus. 100 nm, 200 nm, and 500 nm carboxylate-modified particles were used in this system.

Interestingly, fed state lipids have a greater impact on particle transport than the mucus mesh pore size effect. Diffusion of 100 nm particles diluted in fed state buffer is slower than diffusion of 500 nm particles diluted in maleate buffer (Figure 23B). Expectedly, lipids reduced the transport rate of same size particles significantly. There is a 30–fold reduction in diffusivities of 100 nm particles in fed state compared to buffer whereas this is more significant for particles at 200 nm and 500 nm. Transport of 200 and 500 nm particles were over 400–fold slower than same size particles in mucus.
Influence of lipids on particle transport is further evident through estimated penetration rates in mucus. 100 nm, 200 nm and 500 nm particles were capable of penetrating 50 µm mucus layer where particles in fed states were completely immobilized and not able to penetrate through 50 µm thick mucus layer (Figure 23C).

Particle diffusion is highly dependent on pore size: A highly heterogeneous porosity would result in either much higher than the ensemble average (pores with diameters greater than 200 nm) or much lower than the ensemble average (pores with diameters close to or less than 200 nm). However, the mucus components interactions with lipids likely increased possible particle-environment interactions, which could change porosity and hence reduce diffusivities.

4.6 Influence of Surface Chemistry on Particle Transport

To investigate impact of surface chemistries on particle transport upon addition of food-associated lipids PEG –modified particles were used along with carboxylate –modified particles with various sizes. Lipids significantly impact the carboxylate-modified particle transport while they have minimal impact on the transport of PEG-modified nanoparticles. PEGylation greatly increased particle transport rates, as is evident by the 500-, and 30- fold higher ensemble MSDs (1s) of 200 and 500 nm PEGylated particles compared with corresponding carboxylate-modified particles of the same size in fed state at t = 1 s.
In maleate buffer, more than 30% of 200 nm carboxylate-modified particles could cross 50 μm mucus layer within 1 h, while in the presence of lipids, particles would penetrate at very low efficiencies (<1% after 1h). In contrast, 200 nm PEG modified particles would penetrate more freely, as more than 50% of PEG-modified particles are predicted to cross a 50 μm mucus layer within 1h in buffer and fed state, respectively.

These results indicate that the impact of food-associated lipids on barrier properties of GI mucus is likely due mainly to changes in particle-mucus interaction. The impact of lipids was dependent on surface chemistry of particles, and was minimal for PEGylated particles which have been demonstrated to diffuse freely through mucus, likely due to lack of significant interactions.
4.7 Microscopic Motion of Bile Salt Micelles in Gastrointestinal Mucus

Bile salt micelles in the fed state gastrointestinal environment play an important role on absorption of orally delivered compounds. However, it is not clear if these micelles stay intact or disassociate within the mucus layer. Here, microscopic transport of micelles in intestinal mucus was studied using FRAP method.

![Representative images of GI mucus before bleaching (Pre) and at 0, 60 seconds after photobleaching. Scale bar: 10 µm.](image)

To examine diffusivity of bile salt micelles specific region of interests were chosen and bombarded with high power laser. Several images were captured before bleaching (Pre), right after bleaching, and after the bleached samples recovered within 60 seconds and reached steady state conditions (Figure 25). Bile salt micelles over CMC have recovery efficiency nearly 80% whereas micelles below CMC have 60% recovery efficiency. Three separate experiments were run for micelles above and below CMC values (Figure 26 B). Normalized intensities were used to calculate diffusion coefficients of bile salt micelles in mucus.
Figure 27. A) Normalized, averaged experimental recovery curve is shown in intensity at each time point, B) Bleaching efficiency of micelles with error bars representing standard deviation.

Estimated diffusivities of micelles in water ($D_{eff} = 9.6 \times 10^{-7}$ cm$^2$/s) were over 3-fold higher than experimental diffusivities of micelles in mucus; suggesting that micelles are hindered by the heterogeneous mucus structure (Figure 27).

Figure 28. Measured and Calculated Diffusion Coefficients
Additionally, micelle (dosed above CMC) diffusivities were more than 13- and 20-fold lower than both the estimated and experimental diffusivities of lipid molecules (dosed below CMC), which supports that micelles are likely present in mucus (Figure 27). These results strongly support the presence of intact micelles within mucus. Understanding the transport phenomena of micelles would help us predict their function in solubilization and absorption of orally delivered compounds.

In this study, it has been demonstrated that food-associated lipids and physicochemical changes can significantly impact particle transport through mucus. Our results suggest that intestinal mucus becomes more elastic upon the addition of exogenous lipids associated with food and certain lipid-based drug delivery systems. While hydrophobic entities in mucus likely adsorb significant amounts of lipids to alter mucus gel properties, the precise mechanism is not clear. Bile salts and phospholipids form colloidal structures, endogenous micelles, which may also impact structure and barrier properties of the intestinal mucus layer.

4.8 Impact of Food-Associated Lipids on Microbe Transport in GI Mucus

Mucus plays an important role in selectively binding pathogens to prevent their penetration to the epithelium line. In this section, the barrier properties of gastrointestinal mucus was investigated using ATCC strain 700926 Escherichia Coli microbe by real time tracking method. Bacteria transport was studied in relation to food-associated lipids.
Figure 29. Transport behaviors of *E. Coli* in GI mucus. Representative 20 s trajectories in A) Maleate buffer, B) Fed state intestinal lipids. Scale bar: 5 µm

Heterogeneous structure of mucus highly entangle with model microbes result in different type of trajectory shapes from linear to curve (Figure 28). Mathematical track linearity was determined as explained before (Figure 6) [137]. Briefly, three videos were taken from different preparations with approximately 25-30 bacteria in each field of view for a total of n ≥80 bacteria trajectories for each test media.
Assessments of track linearity of E. coli in mucus shows that microbes associated with fed lipids have superior rotating movements in comparison to buffer indicating a stronger mucosal barrier against bacteria. In contrast, straight motion of E. coli microbes was confirmed in buffer that it has significantly higher Linear Type (LT) motion compared to fed state. Average speed of bacteria in maleate buffer was found to be 19.32 µm/sec ranging from 37.39 µm/sec to 1.25 µm/sec. On the other hand bacteria diluted in fed state were significantly slower where its average speed was 3.3 µm/sec ranging from 5.8 µm/sec to 0.63 µm/sec (Figure 30). Physiological stimuli associated with eating likely impact microbial transport, as many bacteria appeared to be trapped by mucus in the presence of lipids.
Figure 31. Average speed of *E.Coli* diluted in maleate buffer and Fed State buffer.

Data represent mean ± standard error of three independent experiments, with *n* ≥80 cells for each experiment.

Pathogenic organisms must penetrate the protective mucus biolayer that overlies the gastrointestinal epithelium before they can adhere to or invade mucosal cells and cause diseases. To date it has been shown that some components of mucus such as sugars and oligosaccharides in glycoproteins act as ligands for bacteria and viruses, blocking binding of pathogenic microorganisms to the intestinal mucosa and protect humans and animals against gastrointestinal infection [144]. Other studies have also shown that glycosphingolipids may act as receptors for pathogenic microorganisms, including viruses, bacteria and fungi [145].
Furthermore, phospholipids in mucus has been described as putative receptors for *Helicobacter pylori* [145]. It is speculated that pathogens are able to adhere to the glycolipids and phospholipids in mucus [146]. In this study we have shown that exogenous lipids from food-associated stimuli influenced microbe transport in GI mucus. It is highly possible that in addition to potential interactions between mucus phospholipids and microbes, additional lipids may enhance the adherence potential of mucus to microbes resulting in a more complex environment and thus leads slower microbe transport. These results may enable us to design lipid-based delivery of drugs and control pathogen invasion in diseases such as necrotizing enterocolitis or inflammatory bowel disease, or exposure to bioterrorism agents.

4.9 Characterize impact of lipids on small molecule transport in mucus

In an effort to perform non-invasive tracking of nitroxide spin probes (model drug), electron paramagnetic resonance (EPR) was used. Properties of the probe’s microenvironment, such as microviscosity [147] and kinetic profile was determined using the obtained EPR spectrum.
Figure 32. EPR spectra of different surface moieties of nitroxide spin probes diluted in various test media. 4-Amino Tempo in A) Maleate buffer, B) soybean oil in maleate buffer, C) fed state. 3-Carboxy-Proxyl in D) Maleate buffer, E) soybean oil in maleate buffer, F) fed state. 4-Hydroxy Tempo in G) Maleate buffer, H) soybean oil in maleate buffer, I) fed state. 4-Hydroxy Tempo Benzoate in J) Maleate buffer, K) soybean oil in maleate buffer, L) fed state.

The amplitude and the line width of the EPR lines are close to each other for spin probes diluted in maleate buffer (Figure 31. A-D-G-J) suggesting probes are able to rotate without any barrier; a phenomenon that is usually seen in less complex environment. Upon addition of soybean oils or food-associated lipids EPR spectra lines show a
decreasing trend indicating an anisotropic motion of spin probes where the molecules do not have a free mobility in all directions (Figure 31.B through L). The elongated shape of the probe molecule is likely constrained to some extent by the polar chains of the lipid phase or the hydrophobic domains of mucus constituents leading to greater rotation around its long axis.

![Graph showing diffusion coefficients of nitroxide spin probes diluted in various test media](image)

**Figure 33.** Diffusion coefficients of nitroxide spin probes diluted in various test media (i.e., maleate buffer, soybean oil, fed state) in mucus. Data represent mean ± standard error of three independent experiments.

Rotational diffusion constant parameter, R, was found for each spin probes diluted in test media exposed to mucus using Multicomponent EPR Labview module of Altenbach. R values were then used to calculate diffusion coefficients and micro-viscosities by using equations (8) and (12).
Expectedly, rotational diffusion coefficients of spin probes were decreased nearly 1.5-fold for each probe in soybean oil and fed intestinal lipids compared to buffer exposed to mucus (Figure 32). Even though there was a slight change in diffusivities between soybean oil and more complex medium, i.e., fed state, the differences were not significant as compared with maleate buffer. Potential hydrogen bonding, electrostatic and hydrophobic interactions in mucus coupled with the lipids-probe entanglement could result in decreased diffusivities of spin probes. Slow transport of model drugs were also supported by their effective local viscosities (Figure 33). Upon addition of fed intestinal lipids, complex mucus environment became more viscous and prevent free rotational diffusion of spin probes.

![Graph showing effective micro-viscosity of model drugs](image)

**Figure 34.** Effective micro-viscosity of the microenvironment of model drugs. Data represent mean ± standard error of three independent experiments.
4.9.1 Influence of Surface Chemistry on Probe Diffusion

The effects of surface chemistry on small molecule diffusion through gastrointestinal mucus were investigated using spin probe systems with various surface moieties. Diffusion coefficients of probes show that mobility is correlated with surface charge. Negatively charged 3-Carboxy-Proxyl has the highest diffusion coefficient whereas positively charged 4-Amino Tempo has the slowest diffusivities. The relatively high degree of probe-environment interactions between 4-Amino Tempo and mucin fibers greatly reduced spin probe diffusion coefficients. Mucus contains a high density of negatively charged glycoproteins along with hydrophobic proteins and lipid domains. The correlation between surface charge and spin probe mobility is found to be consistent. Transport rates for 4-Amino Tempo probes were slightly over 10 -fold lower than those of 3-Carboxy-Proxyl and 4-Hydroxy Tempo and nearly 4 -fold lower than 4-Hydroxy Tempo Benzoate in maleate buffer. Additionally, hydrophobic interactions represent important mechanism on nanoscale drug carriers or bacteria penetration in mucus. Mucin fibers tend to form moderate interactions with surfaces that are hydrophobic. Diffusion of hydrophobic 4-Hydroxy Tempo Benzoate spin probe was 3 -fold and 2 –fold slower than negatively charged 3-Carboxy-Proxyl and 4-Hydroxy Tempo in maleate buffer.

Mucus possess the dual capacity to form polyvalent adhesive interactions using both hydrophobic and electrostatic forces especially with nano and micro scale polymeric nanoparticles designed to deliver drugs. It has been an issue to design such particulate systems that overcome mucosal adhesive gel barrier. In this investigation small molecular weight (<500Da, ~ 5 Å in diameter) compound diffusivities were tested in different test media exposed to mucus.
Our previous work on 200 nm amine-, carboxylate-, and sulfate-modified polystyrene microspheres have shown that positively charged amine microspheres diffusivities in maleate buffer were 13 –fold and 27 –fold slower than negatively charged carboxylate- and sulfate-modified microspheres in maleate buffer at a time scale 10 s respectively. Interestingly negative mucus fibers influence even small molecular transport significantly. Several researchers have also shown that the diffusion of a number of both small and large molecules including amines [148], bovine serum albumin[149] lysozyme [149] decreased due to hydrophobic interactions. Our results also suggest that hydrophobic spin probe, 4 Hydroxy Tempo Benzoate, is diffusion limited in the mucus gel. Nonetheless, small molecule diffusion is not restricted as much as larger molecules, perhaps because small molecules are not capable of forming polyvalent adhesive bonds with the mucus structure.
5.0 CONCLUSIONS

5.1 Impact of Food-Associated Stimuli on Particulate and Small Molecule Transport in Mucus

In this investigation, the impact of exposure to lipids and physicochemical stimuli, including pH and [Ca^{2+}], associated with eating on particle transport through intestinal mucus has been studied using microstructural analysis and real-time multiple particle tracking (MPT). Stimuli were directly exposed to native mucus collected (gently scraped) from porcine intestine, or intact on excised tissue from mouse. Impact of exposure to stimuli in vivo was tested by oral dosing to rat. MPT is a powerful technique that enables probing of particle-environment interactions by tracking the motion of hundreds of individual particles.

In summary, while it has been indicated that endogenous lipids impact mucus viscoelastic properties, and [Ca^{2+}] and pH changes are significant to mucin solution viscoelastic properties, the majority of relevant studies have been conducted on mucins isolated from non-intestinal (e.g., airways, stomach) anatomical sites. This is particularly important given the variations in mucins expressed at different anatomical sites. The impact of exogenous lipids and other physicochemical intestinal lumen changes associated with food intake on transport properties of intestinal mucus have not been characterized. Here, we utilize MPT and structural analysis to demonstrate that food-associated physicochemical stimuli significantly alter barrier properties of intestinal mucus, with important implications pertaining to physiological control of exposure to ingested particles and microbes, and oral drug delivery.
Lipid content associated with fed state intestinal contents significantly enhanced mucus barrier properties, as indicated by 10 – 140-fold reduction in the transport rate of 200 nm microspheres through mucus, depending on surface chemistry. Physiologically relevant increases in [Ca\(^{2+}\)] resulted in 3-fold, 4-fold and 2-fold reduction of amine-, carboxylate- and sulfate modified particle transport rates, respectively, likely due to binding of [Ca\(^{2+}\)] to mucin glycoproteins and thus enhanced cross-linking of the mucus gel network. Reduction of pH from 6.5 to 3.5 also affected mucus viscoelasticity, reducing particle transport rates approximately 5-fold for amine-modified particles and 10-fold for carboxylate- and sulfate modified microspheres. Macroscopic visual observation and micro-scale lectin staining patterns indicated mucus gel structural changes, including clumping into regions impenetrable by microspheres, correlating with altered transport properties. Histological examination of intestinal tissue indicated food ingestion can prevent microsphere contact with and endocytosis by intestinal epithelium. Influence of surface chemistries were investigated on particle transport upon addition of food-associated lipids using PEG –modified particles along with carboxylate –modified particles. Results have shown that lipids significantly impact the carboxylate-modified particle transport while they have minimal impact on the transport of PEG-modified nanoparticles.

Taken together, these results indicate that GI mucus barriers are significantly altered by stimuli associated with eating and potentially dosing of lipid-based delivery systems; these stimuli represent broadly relevant variables to consider upon designing oral dosage forms and potential safe, feasible mechanisms by which to modulate mucus barrier properties.
A novel approach to biogel transport analysis, electron paramagnetic resonance was used to explore the microenvironment of small molecular weight compounds in food associated lipids when they exposed to mucus. Results indicate that mucus barrier was altered upon addition of fed state contents compared to buffer. Microviscosity of spin probes increased in fed state resulted in lower translational diffusion coefficients in comparison to buffer in mucus. Influence of surface chemistry on gastrointestinal mucus barrier was also examined using various surface moieties of nitroxide spin probes. Results indicate that the GI mucus can also pose a significant barrier to small drug carrier transport.

Transport rates were found to be inversely related to particle surface potentials, with negatively charged 3-Carboxy-Proxyl and 4-Hydroxy Tempo spin probes displayed significantly higher transport rates than positively charged 4-Amino-Tempo spin probes most likely due to electrostatic adhesive interactions with mucin fibers. Additionally, potential hydrophobic adhesive interactions in mucus limited the rotational diffusion of hydrophobic spin probe, 4-Hydroxy tempo Benzoate. Taken together, these results highlight the importance of mucus barrier alteration in the context of eating. Understanding the mechanism behind the food effect offers feasible strategies to enhance small molecule/drug carrier transport in mucus. Furthermore, lipid based drug delivery systems as well as enhancement in nutrient uptake could be achieved. In addition, provided results could be used to design meaningful dosing in the presence of food.
5.2 Impact of Food-Associated Lipids on Bacteria Transport in Mucus

Real time video tracking has been used to assess the motility of *E.Coli* exposed to food-associated lipids and buffer. Using IDL tracking software mathematical reconstitution of trajectories in x and y plane was determined to calculate bacterial velocity and linearity. Data reported in this project show that bacteria velocity exposed to fed intestinal state was nearly 6–fold slower than bacteria diluted in maleate buffer. Heterogeneous structure of mucus possibly entangle with food-associated lipids and reduce bacteria motility as the data show that significant portion of bacteria made curvilinear track instead of straight track. These results could be used to estimate traversal times of specific microbes and to propose natural, safe methodology to inhibit invasion of pathogens.
6.0 RECOMMENDATIONS

In general, *in vivo* studies investigating oral delivery of nanoparticles have mainly focused on therapeutic effects or pharmacokinetics, instead of understanding the fate of the particles and how the results might translate in humans. To develop a successful in vivo drug delivery mechanism in human, it is worth to briefly discuss and study the relevance of similar system in animal models. In previous study, we are mainly focus on characterizing the barrier properties of rats, mice, and porcine intestinal mucus against drug delivery and nanoparticle distribution in the GI tract. More specifically, impact of fed intestinal lipids associated with eating on the barrier properties of intestinal mucus and how the food arrival affects model drug and microbe penetration in these animal models. Even though selected animal model intestines working mechanism close to humans, there are still significant differences in bacterial colonization, stomach emptying times, intestinal transit times, mucus thickness, etc. which could impact effective drug delivery. Hence, utilizing human mucus in addition to selected animal models might lead to a better comprehensive knowledge of mucosal barrier properties and ultimately, if the same phenomenon observed in human mucus too, these findings could be incorporated into a tablet or a capsule for efficient lipid based drug delivery.
6.1 Characterization of Mucus Structure in Response to Lipid Exposure using Electron Microscopy

Mucus structural changes associated with exposure to food-associated stimuli could be analyzed using microscopic and micro-/macro-rheological techniques. [In addition, lipids (bound to mucus and not bound) can be quantified and characterized before and after exposure to lipids and changes in [Ca\(^{2+}\)] and pH.] Microscopic techniques include TEM with a specialized fixation technique tailored to heavily glycosylated matrix as well as an advanced QF/DEM technique designed to enable visualization of fine matrix structure not visible with standard EM. [We anticipate a change in the effective pore size of the mucus mesh with exposure to lipids and food-associated physicochemical stimuli. The pore size change will result from changes in inter-molecular interactions, and should thus be supported by measured changes in transport and rheological properties.] Rheological characterization can provide complementary insight into changes in mucus gel interactions and structure. Microrheology provides length- and timescale-dependent micro-structural information (e.g., pore size), while macrorheology provides length- and timescale-independent information useful for understanding bulk gel structural changes (e.g., increase in mucin interactions leading to more elastic character).
6.2 Impact of Disease States on Barrier Properties of Mucus

Quantitative Analysis of Number and Size of Pores in Mucus Structure

One location where mucus is a particularly important barrier is in the gastrointestinal (GI) tract. In the GI tract, orally delivered drugs must first traverse through mucus layer before it can be absorbed across the epithelium. Effective delivery of drugs to target cells is usually constrained by highly viscoelastic mucus layer. Foreign particles are also trapped and removed from the GI tract by the help of protective mucus coatings. However, viruses can diffuse through mucus and penetrate to the epithelial cells. The main reason behind this penetration is due to a smaller size of the mucus mesh spacing than viruses. Whilst not fully understood, the analysis of intestinal mucus surface will aid to understand the structure of the first barrier entities encounter in diffusing across GI tract layer to the underlying epithelium.

The dense mucus network including proteins, carbohydrates, lipids, salts, antibodies, bacteria, serum macromolecules and cellular debris efficiently trap most micro-environmental particles through steric obstruction and/or adhesion forces via hydrophobic, electrostatic or hydrogen bonding interaction. Similarly, conventional model drug carriers that are highly mucoadhesive are likely to be trapped by the same forces. Along with all physicochemical properties of mucus gel network, mesh spacing (“pore” size) play significant role on the barrier properties of mucus. Mucin fibers can sterically preclude particulates that are too large to pass the mucus mesh spacing. The thickness of the mucus structure varies for different mucosal surfaces. In the GI tract, the thickest mucus layer is the colon however the interfiber spacing exhibits significant variation from 10 nm to 500 nm.
Structural properties of mucus are sensitive to sample preparation and analysis method. The microstructural study of GI tract has been difficult to preserve a continuous layer of mucus blanket. Wojtas et al [150] successfully addressed the issue of mucus blanket retention in intestinal samples by advancing the anhydrous fixation protocol described by Sims et al. [151] using standard electron microscopy (SEM). Multiple questions [Does magnification have an effect to obtain different pore size areas and number of pores, how does the different part of intestine affect the porosity and do the fixation techniques change the porosity or mucus concentration in intestine] could be addressed using intestinal mucus samples prepared by aqueous and solvent fixative techniques and analyzed by electron microscope.

6.3 Impact of Disease States on Barrier Properties of Mucus

Mucus, a highly complex gel composed of mucin glycoproteins, provides a barrier against biological pathogens, chemical irritants and environmental toxins. Structural changes in the mucosal lining due to disease or physiological environmental changes could possibly affect the mucosal barrier, which may lead to pathogen invasion.

6.3.1 Impact of Impact of Hirschsprung’s Disease State on Barrier Properties of Colonic Mucus

Hirschsprung’s disease is a developmental abnormality of the nervous system seen in approximately 1 in every 5000 live births, with 90% of the cases occurring in the neonatal period. Also known as intestinal aganglionosis, this disease state is characterized by a lack of ganglion cells in the distal intestine which causes absence of peristalsis, hypertrophy, and dilation of the colon.
HD is often complicated by enterocolitis (HAEC), the most common cause of mortality, resulting in the following symptoms: distension, diarrhea, fever, sepsis, and death. HAEC occurs in 18-50% of HD cases before treatment and up to 22% after treatment.

The treatment options for HD are limited, and current options involve long term complications. The most widely accepted treatments rely on surgery to remove the diseased portion and reattach the shorter colon. Treatment often results in abnormal bowel function, approximately 50% of cases, sexual dysfunction, urinary dysfunction and enterocolitis.

While HD patients are more prone to contracting enterocolitis, the mechanism is not understood. Suggested theories include changes in mucus layer properties, colonic microbiota, innate immunity and epithelial barrier properties. Although all of these theories are being investigated, current research of this disease state only focuses on the mucosal barrier properties to a small degree. Given the important role of mucus in protecting underlying epithelium from microbial invasion, we hypothesize that the inherent barrier properties of the mucus lining in the colon are altered in HD. To test this hypothesis, transport of passively diffusing entities (polystyrene microspheres), as well as *Escherichia coli*, as an actively transported microbe, through colonic mucus in an HD animal model could be used to explore barrier properties of HD colonic mucus layer.
6.3.2 Barrier Property Differences of Gastrointestinal Mucus Due to Necrotizing Enterocolitis

Mucus barriers in premature intestine do not prevent transport of microbes and/or microbial agents to the underlying epithelium to the same extent as mucus at full term, resulting in atypical infection with intestinal microbes in necrotizing enterocolitis (NEC). NEC has been the most investigated, debated, and clinically devastating gastrointestinal disease of preterm infants for the last four decades. NEC carries a mortality ranging from 10-50% which has remained essentially unchanged since the disease was first described, and children who survive surgical NEC often face severe long-term complications including neurodevelopmental delays and long-term neurological and intestinal rehabilitative needs, such as those associated with short bowel syndrome. The only proven method to protect from NEC is feeding with breastmilk as opposed to formula, while probiotic prophylaxis treatments show promising reduction of incidence of NEC but not mortality associated with NEC, and require further testing due to safety concerns associated with invasion of microorganisms and sepsis. The proven efficacy of breastmilk supports the proposed approach: analysis within mucus of multiple factors essential to mucus barrier properties which are interestingly also associated with feeding breastmilk, and experimental design of prophylactic combinations of these factors. It is anticipated that the comprehensive, statistically designed method of exploring these factors could uncover synergistic relations between them in preventing microbial invasion and NEC. The pathophysiology of NEC is still undetermined, yet the multiple observations and theories are all consistent with an altered mucus barrier.
Premature intestinal mucus barriers can be “strengthened” at the time of feeding using safe, natural, synergistic factors that modulate mucus barrier properties, including lipids, calcium, pH modification, exogenous mucins, and oligosaccharides. Interestingly, these “mucus strengthening” factors are also associated with feeding breastmilk. These hypotheses could be tested by quantitatively characterizing the barrier properties of intestinal mucus using a rat model for NEC and establishing the ability of a prophylactic solution to prevent microbe transport through mucus and development of NEC.

6.3.3 Enhancing Mucus Barrier Properties using Exogenous Strengthening Agents to Prevent Microbe Transport

Mucus presents a finely tuned biological hydrogel barrier, controlling pathogen invasion in the gastrointestinal and respiratory tracts. Mucus protects underlying epithelia from pathogen entry; self modulates barrier properties with the menstrual cycle to control sperm penetration, and regulates food and drug absorption in the gastrointestinal tract. However, these defense mechanism and associated barrier properties are not well understood.

Natural defense mechanisms built into mucosal surfaces prevent pathogen intrusion and can break down in disease. For example, Ca^{2+} and pH-modulated permeability are important in disruption of stomach resistance to pathogen entry with gastric ulcers and menstrual cycle variation of sperm mobility. Sugars, including sugars and oligosaccharides present in breastmilk glycoproteins, act as ligands for bacteria and viruses, blocking binding of pathogenic microorganisms to the intestinal mucosa and protecting animals and humans against gastrointestinal infection. Milk (human and cow) contains Muc1, a highly glycosylated mucin. Mucins are very large (MW> ~1e6) heavily glycosylated glycoproteins which are the major structural components of mucus.
Muc-1 and its associated sugars have been demonstrated to prevent pathogen adhesion to mucosa. In addition, specific proteins present in mucus, including TFF3 and RELM, are known to contribute significantly to mucosal barrier properties and prevention of pathogen invasion. In intestinal mucus, trefoil factors have been demonstrated to be important in protection of underlying epithelium from pathogens and healing of injured mucosa[152], and appears to act synergistically with mucin in these roles, potentially by strengthening the barrier properties of the mucus gel layer. Similarly, RELM-beta is important in regulating mucus barrier integrity and susceptibility of underlying epithelium to inflammation. It is secreted by goblet cells in the gut and is itself a mucin secretagogue, resulting in a more physically robust mucus barrier. The proposed strategy is to utilize these natural defense mechanisms to protect from exposure, enabling antibiotics and natural defense mechanisms to clear microorganisms prior to infection.
### NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>BS</td>
<td>Bile Salt</td>
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<td>FRAP</td>
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<tr>
<td>nm</td>
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<td>Laser Scanning Microscope</td>
</tr>
<tr>
<td>(\mu g)</td>
<td>Micro gram</td>
</tr>
<tr>
<td>ml</td>
<td>MiliLiter</td>
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<tr>
<td>CMC</td>
<td>Critical Micelle Concentration</td>
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<tr>
<td>PL</td>
<td>Phospholipid</td>
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<tr>
<td>(D_{AB})</td>
<td>Diffusivity of Compound A in Solvent B</td>
</tr>
<tr>
<td>(\varphi_B)</td>
<td>constant which accounts for solvent/solvent interactions</td>
</tr>
<tr>
<td>K</td>
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</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>(E.Coli)</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>fps</td>
<td>Frame Per Second</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
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<tr>
<td>TL</td>
<td>Track Linearity</td>
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<tr>
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<td>R</td>
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<td>SED</td>
<td>Stokes-Einstein-Debye</td>
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<td>r\text{eff}</td>
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<td>Reorientation Diffusion Correlation Time</td>
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<td>I</td>
<td>Spherical Harmonics Order</td>
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<td>Maleate Buffer</td>
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<td>\delta</td>
<td>Phase Angle</td>
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<td>\AA</td>
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8.0 REFERENCES


42. Matthews, L.W., S. Spector, J. Lemm, J.L. Potter, *Studies on pulmonary secretions. I. The over-all chemical composition of pulmonary secretions from*


