Biophysical determinants of biofilm formation in the gut
Sandra L. Arias and Ilana L. Brito

Abstract
The gastrointestinal (GI) tract harbors the most complex microbial ecosystem in the human body. The mucosal layer that covers the GI tract serves as a polymer-based defensive barrier that prevents the microbiome from reaching the epithelium and disseminating inside the body. Colonization of the mucus may result in the formation of structured polymicrobial communities or biofilms, a hallmark in pathologies such as colorectal cancer, inflammatory bowel disease, and chronic gut wounds. However, the mechanisms by which multispecies biofilms establish on the gut mucosa are unknown. Whether mucus-associated biofilms exist as part of a healthy mucosal barrier is still debated. Here, we discuss the impact that diet and microbial-derived polymers have on mucus structure and microcolony formation and highlight relevant biophysical forces that further shape nascent biofilms.

Introduction
A thick mucus lines the gut epithelium, serving as a barrier to prevent the breach of bacteria into the human body. Loss of mucosal integrity, such as through enzymatic degradation, is associated with augmented microbial colonization and increased translocation of bacterial antigens into the mucosa [1]. Colonization of the mucus layer may also result in the formation of structured polymicrobial communities or biofilms. These biofilms are a characteristic hallmark of right-sided colorectal cancer tumors and have also been implicated in gastrointestinal disorders such as ulcerative colitis and Crohn’s disease [2–4]. Both commensal and pathogenic bacteria employ multiple strategies to colonize the mucus, including adhesins and lectins that recognize specific glycans contained in the mucin glycoproteins, the major constituent of the mucus [5]; however, whether mucus-resident bacteria form biofilms as part of a healthy mucosal barrier is still debated [6,7].

Several hypotheses have been put forth to explain the mechanical forces by which biofilms are impeded from growing on and within colonic mucus. Computational and experimental studies indicate that bacterial adhesion to the mucus might be used by the host to control the position and abundance of specific microbes simply through physical restraint [8,9]. Yet, in contrast to abiotic surfaces, where bacterial adhesion is the initial step in biofilm initiation [10], bacterial binding to the mucus alone may not support the formation of large microcolonies. This is suggested by the low occurrence of biofilms in healthy biopsies. Alternative explanations include the mechanical stress exerted by intestinal fluid and stool passage, the rapid secretion and shedding of the mucus layer, and the fast turnover rate of the epithelium [11,12].

However, there are multiple putative biophysical determinants of biofilm formation unaccounted for in this list. Here, we discuss the impact that diet and microbial-derived polymers have on mucus structure and microcolony formation and highlight relevant biophysical forces contributing to nascent biofilms.

Polymer-mucin interactions shaping the mucosal microenvironment
Mucus is a complex polymer gel rich in mucin glycoproteins that are responsible for its viscoelastic and gel-like properties [13]. Polymer gels form supramolecular networks of long polymer strands held together by chemical or physical crosslinks via covalent disulfate bridges, nonmucin proteins, Ca2+-mediated links, physical entangles, and low-energy electrostatic and hydrophobic bonds [14]. At neutral pH, sialic acid and sulfate groups located on mucin glycoproteins make the mucus behave as an anionic polyelectrolyte gel [13]. Polyelectrolyte gels undergo strong volume transitions in response to the pH, ion strength, and dielectric

Addresses
Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY, 14850, USA

Corresponding author: Brito, Ilana L (ibrito@cornell.edu)

Current Opinion in Biomedical Engineering 2021, 18:100275
This review comes from a themed issue on Futures of BME: Bioengineering of the Microbiome
Edited by Ankur Singh and Ilana Brito

Received 8 December 2020, revised 28 January 2021, accepted 1 February 2021
https://doi.org/10.1016/j.cobme.2021.100275
2468-4511/© 2021 Published by Elsevier Inc.

Keywords
Biofilms, ucus, ut microbiome, Cellular aggregation.
Pseudomonas aeruginosa dehydrated mucus creates a favorable niche in which limits the diffusion of antimicrobial molecules like concentrations of quorum-sensing signaling molecules; sion, resulting in high local bacterial densities with high immune defenses. Specifically, dehydrated mucus in the collapse [19]. With an impaired clearance system, the cilia to transport the mucus and inhaled particles out the lungs. airways, densely tethered mucins in the periciliary layer ted by the prevalence of biofilms in the dehydrated thus, contributing to biofilm initiation. This is suppor-

Increasing the residence time of mucosal microbes, and diffusion properties of the mucus network, compression at the same time may affect the clearance tion from a watery gel to an elastic solid). Mucus mucus rheology, we infer that polymer-induced osmotic behavior was consistent with a Donnan mechanism, in with a similar chemical composition [18*]. This compression when compared to uncharged polymers found that charged polymers such as the food additive carboxymethyl cellulose exerted a higher mucus (Figure 1a) [17]. Notably, the gut microbiome actively modulated the compression of the colonic mucus in vivo and ex vivo and demonstrated that this behavior depended on the concentration and of these polymers (Figure 1a) [17]. Datta and colleagues recently measured the change in thickness of murine colonic mucus in response to high-molecular-weight (high-Mw) dietary fibers, including dextrin, pectin, and pullulan, and neutral polymers, such as polyethylene glycol (PEG) [17]. These authors found that both dietary fiber and PEG compressed the murine colonic mucus and demonstrated that this behavior depended on the concentration and Mw of these polymers (Figure 1a) [17]. Notably, the gut microbiome actively modulated the compression of the colonic mucus in vivo by breaking down dietary fibers into small noncompressing polymers [17]. Moreover, the authors found that charged polymers such as the food additive carboxymethyl cellulose exerted a higher mucus compression when compared to uncharged polymers with a similar chemical composition [18*]. This behavior was consistent with a Donnan mechanism, in which mobile ions are partitioned outside the mucus layer by charged polymers, driving the flux of water out of the mucus network [18*].

Although these experiments did not measure changes in mucus rheology, we infer that polymer-induced osmotic compression can reduce mucus viscosity (i.e. its transition from a watery gel to an elastic solid). Mucus compression at the same time may affect the clearance and diffusion properties of the mucus network, increasing the residence time of mucosal microbes, and thus, contributing to biofilm initiation. This is supported by the prevalence of biofilms in the dehydrated airway mucus of cystic fibrosis (CF) patients. In the airways, densely tethered mucins in the periciliary layer transport the mucus and inhaled particles out the lungs. Mucus clearance fails when dehydrated mucus osmotically compressed the periciliary layer, causing the cilia to collapse [19]. With an impaired clearance system, the dehydrated mucus creates a favorable niche in which Pseudomonas aeruginosa can thrive and evade the host immune defenses. Specifically, dehydrated mucus in the airways restricts bacterial motility and molecular diffusion, resulting in high local bacterial densities with high concentrations of quorum-sensing signaling molecules; limits the diffusion of antimicrobial molecules like lactoferrin secreted by submucosal glands; and impairs the ability of neutrophils to penetrate and eradicate the P. aeruginosa microcolonies [20].

Extracellular polymeric substances (EPS) secreted by microorganisms in biofilms can also drive bacterial surface motility and biofilm growth by generating osmotic pressure gradients in the extracellular space [21]. EPS-generated osmotic pressure may provide bacteria cells in the biofilm with the force to move through the mucus layer, coordinated simultaneously by the enzymatic degradation of mucin glycoproteins. Bacterial motility generated by EPS-driven osmotic pressure has been demonstrated in vitro in Bacillus subtilis and Vibrio cholerae biofilms on agar plates [21,22]. Importantly, this motility mechanism is independent of flagella-mediated functions and is controlled only by the content and cross-linking density of the exopolysaccharides that form the biofilm matrix (Figure 1b) [22]. Because the mucus layer possesses gel-like properties, similar osmotic effects may also influence V. cholerae colonization and biofilm expansion in the human gut [22].

In contrast to large nonpenetrating polymers, there are numerous polymers used to transport drugs across the mucus barrier that can infiltrate, aggregate, or form complexes with mucin glycoproteins [23]. The magnitude of those interactions depends on several factors, including the conformational adaptation, flexibility, and Mw of the penetrating polymer, and the magnitude of the electrostatic interactions, hydrogen bonding, hydrophobic interactions, and physical entanglement between the polymer and mucin [24,25]. For example, high-Mw chitosan, a mucoadhesive cationic polysaccharide, associates with mucin, forming complexes with sizes ranging between 200 and 900 nm. These complexes vary in size as a function of the chitosan-to-mucin mass ratio and their mutual electrostatic neutralization, indicating that the mucin-chitosan interactions are mostly electrostatic [25]; however, molecular mass and chain flexibility (determined by the charge density) also play a significant role in the complex formation (Figure 1c) [25]. The high affinity of chitosan toward mucin reduces the mucin’s relative viscosity. When combined, these macromolecules decrease in hydrodynamic volume, probably resulting in the formation of pores or the contraction of the mucus network in vivo [25].

The interaction between penetrating polymers and mucus is likely relevant to biofilm initiation in the colonic mucus because most of the exopolysaccharides secreted by EPS-producing microorganisms contain charged polysaccharides and proteins. Thus, they can exhibit the same associative and infiltrating capabilities as penetrating polymers, supporting persistence in the mucus layer and possibly biofilm formation. For example, the cationic exopolysaccharide Pel secreted...
by *P. aeruginosa* crosslinks with eDNA, forms aggregates with mucin glycoproteins, and provides structural stability to nascent biofilms in the lungs [26]. Similarly, the cationic exopolysaccharide poly-N-acetylglucosamine (PNAG) produced by *Staphylococcus epidermis* can interact with synthetic polymeric brushes made of poly(lysine)-graft-poly(ethylene glycol), which is an antiadhesive coating that resists protein adsorption, suggesting that bacterial exopolysaccharides can penetrate and interact with other polymeric systems through analogous physical mechanisms [27].

Other aspects of polymers, in addition to their charge, affect their interactions with the mucus. For example, the interaction strength between mucin and negatively charged polymers (polyanions), like pectin and alginate, depends on the degree of chain flexibility and *M*<sub>w</sub> [24]. Stiff (determined by the persistence length) and low-*M*<sub>w</sub> polyanions preferably interact with the mucin’s hydrophobic globular regions, whereas flexible and high-*M*<sub>w</sub> polyanions bridge distance sites in the mucin, reducing the mucin’s hydrodynamic volume and viscosity (Figure 1c). Neutral polysaccharides like dextran and *Streptococcus thermophilus*-derived exopolysaccharide do not interact with mucin [24]; however, dextran sodium sulfate (DSS) is more mucoadhesive than dextran due to sulfate groups that enhance its polyelectrolyte properties [24]. Interestingly, DSS is widely used in mouse models of inflammatory bowel disease because of its capacity to disrupt epithelial and mucosal barriers [28]. Histological examination of colitis induced by DSS indicates that the severity of the disease depends on the charge and *M*<sub>w</sub> of this polyelectrolyte [29]. DSS with an *M*<sub>w</sub> of 500 kDa does not produce colitis in mice, possibly because it is excluded from the mucus barrier, like large nonpenetrating polymers. In contrast, 5 kDa and 40 kDa DSS induce colitis in the upper and lower colon, respectively [29].

---

**Figure 1**

Interactions of mucin with nonpenetrating and infiltrating polymer solutions as a function of their molecular weight, concentration, flexibility, and charge. (a) Nonpenetrating high-molecular-weight (*M*<sub>w</sub>) polymers osmotically compress the mucus layer. High-*M*<sub>w</sub> polyelectrolytes achieve a higher compression due to mobilization of counterions. (b) Extracellular polymeric substances (EPS, in green) and proteins (red dots) generate osmotic forces that contribute to biofilm expansion. (c) Penetration of the mucus network by positively charged (polycationic), negatively charged (polyanionic), and neutral polymers and their effect on mucin’s relative viscosity as determined by changes in its hydrodynamic volume and as a function of mucin to polymer concentration. The dashed circles represent the changes in the mucin’s hydrodynamic volume.
Biophysical forces acting on mucus-resident bacterial communities

Biofilms have traditionally been studied as large aggregates of up to 1,200 μm forming surface-attached mushroom-like structures [30]. Except for oral plaque, in vivo biofilms are embedded in host-derived macromolecules, in which they form small aggregates or microcolonies with sizes that range between 5 and 200 μm [30]. Cystic fibrosis infections, osteomyelitis, ulcerative colitis, and chronic wound infections are examples where matrix-embedded biofilms are present [4,31–33]. Bacteria embedded in host-derived macromolecules (e.g., mucus) can be aggregated into microcolonies by two distinct physical mechanisms that do not require canonical biofilm-forming functions: depletion aggregation and bridging aggregation [34, 35**]. These forces result in the formation of spatially segregated communities of bacteria without requiring them to carry out cell division.

In depletion aggregation, nonadsorbing polymers generate mutual attraction forces between neighboring cells. This attraction results from the loss of conformational entropy of the polymer segments in the volume separating neighboring cells. As a result, the polymer segments are excluded from this intercellular gap creating an unbalanced osmotic pressure that pushes the cells together into clusters (Figure 2a) [34]. The entropically driven aggregation of P. aeruginosa by mucin is one of many examples where biofilm assembly functions are not required for microcolony formation [35**]. Moreover, depletion aggregation is enhanced when polymer concentration and cell numbers increase [34]. However, when two or more nonadsorbing polymers are mixed, depletion aggregation can occur at low cell numbers [35**]. This is the case early in the development of CF, where physiologically relevant polymer mixtures of mucin, DNA, and F-actin present in CF airway secretions can aggregate P. aeruginosa at...
concentrations as low as $10^5$ CFU/mL [35**]. Bridging aggregation, on the other hand, arises when adsorbing polymer segments act as a scaffold by connecting neighboring cells via electrostatic attraction. Since bacteria usually carry a negative charge at physiological pH due to the presence of surface structures such as lipopolysaccharides, cationic polymers, including chitosan and PNAG (an exopolysaccharide secreted by many pathogens), can promote cell—cell attachment by bridging aggregation [35**,36]. Unlike bacterial genes that play a role in biofilm formation, both of these cell aggregation processes are intrinsic to mucus physical properties.

Microcolonies embedded on the colonic mucus may also experience compression and shear forces that can prompt biofilm formation. For example, recent studies suggest that compression forces experienced by uropathogenic Escherichia coli when confined into small spaces lead to the upregulation of exopolysaccharides and the expression of biofilm-associated cell-surface structures like curli [37*]. Moreover, shear forces derived from fluid flow do not merely wash bacteria out of the body. Instead, bacterial movement is affected by the gradients in fluid velocity, which exerts additional force and torque [38], promoting different motile responses from the bacteria. For motile, rod-shaped bacteria, hydrodynamic shear rates promote a sessile and surface-attached lifestyle over free-swimming [39]. This shear-induced trapping results from the interplay between the cell aspect ratio, bacterial motility, and flow velocity gradients, which affect the swimming direction of single cells to areas with low velocity but high shear rates (i.e., nearby microchannel sidewalls) [39]. In nascent V. cholera biofilms, fluid flow also causes bacterial cells to organize perpendicular to the flow direction, resulting in three-dimensional aggregates with a vertically aligned core [40*]. In vitro assays have been instrumental in connecting the effects of mechanical forces on cells and the response of individual cells to these nanoscale and microscale forces.

The responses of pathogenic bacteria to mechanical forces within their microenvironment challenge our common-sense intuition about the action of fluid flow on the bacterial colonization dynamics. Infective endocarditis (IE) involves bacterial colonization of heart valves, a microenvironment characterized by high shear forces and turbulent blood flow. In this condition, Staphylococcus aureus uses a family of cell-wall anchored adhesins (e.g., clumping factor (Clf) A and B) that undergo dynamic force-induced extension and conformational changes in response to tensile force [41,42]. At high flow rates, these force-sensitive molecular switches increase the adhesion strength of S. aureus to platelets and host blood proteins, such as fibronectin, fibrinogen, and von Willebrand factor, allowing this pathogen to colonize both inflamed and damaged heart valves [42,43]. In contrast, low mechanical tension triggers receptor/ligand dissociation, facilitating bacterial dispersion [41]. Force-sensitive molecular switches, known as catch-bonds, are widespread among both pathogens and commensals. They also control the adhesion strength of uropathogenic E. coli to mannoside residues on epithelial cells [44], the binding force of Streptococcus pneumoniae to collagen I [45], and the adhesion resistance of Ruminococcus champanellensis to cellulose fibers in the gut [46**]. Besides mechanical interactions with extracellular macromolecules and EPS matrix components, direct cell—cell interactions can also contribute to inter and intraspecies aggregation, especially in polymicrobial communities. For example, in the oral cavity, the ability to aggregate intergenerically determines the succession of genera that colonize the teeth and gingiva, with bacterial species like Fusobacterium nucleatum serving as a bridge microorganism between early and late colonizers [47]. F. nucleatum uses a repertoire of surface-attached adhesins, including Fap2, RadD, and Cmp2, that physically links multiple species in the oral cavity, thus stabilizing the developing dental plaque [48,49]. Compared to catch bonds, direct measurement of adhesion forces between F. nucleatum and its coaggregating partners has not been performed.

**Concluding remarks**

The mechanism by which biofilms can form in the colonic mucus is far from understood. In this review, we emphasize the physical principles underlying the gel-like properties of the mucus barrier that contribute to this process. Robust structural transitions of the mucus network such as polymer-induced compression and viscosity loss may act against mechanical forces that prevent biofilms from forming, including mucus shedding and mechanical clearance by the passage of luminal contents. Moreover, intrinsic physical properties of the mucus layer, together with microenvironmental factors and microbial activities, can further promote biofilm growth, for example, through depletion aggregation, compression-induced EPS production, and EPS-driven biofilm expansion. Future studies are needed to unveil the crosstalk between polymer-intrinsic and bacteria—intrinsic properties that enable the formation of mucus-invasive biofilms. Characterizing these factors will contribute to our understanding of the host—microbial interactions at intestinal epithelium and to the development of better prophylaxis to prevent the formation of deleterious biofilms.

**Declaration of competing interest**

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

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest


This work shows that biofilm formation is linked to self-imposed mechanical stress.


In this work, the authors elucidated the mechanism by which biofilms acquired a 3D structure under hydrodynamic conditions.

