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Biophysical determinants of biofilm formation in the gut

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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 is 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 has 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].

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Declaration of interests

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.

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, which 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, non-mucin proteins, Ca^{2+} -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 properties of the solvent [15]. For example, porcine gastric mucin, which is homologous to the human gastric mucin, undergoes a dramatic change in viscosity (sol-gel transition) at low pH (~ 2), protecting the epithelium from the highly acidic gastric juice of the stomach [16].

Like synthetic polymer gels, the mucus network can also collapse or dehydrate osmotically when surrounded by solutions of large non-penetrating polymers ingested as ingredients in pharmaceutical products, food additives, and dietary fiber. Changes in mucus structure present conditions that may alter the penetration and colonization potential of organisms within the mucus layer. Datta and colleagues recently measured the change in thickness of murine colonic mucus in response to high-molecular-weight (high- M_w) 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 *in vivo* and *ex vivo* and demonstrated that this behavior depended on the concentration and M_w 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 non-compressing 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 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 crosslinking 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 non-penetrating 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 *M_w* 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-*M_w* chitosan, a mucoadhesive cationic polysaccharide, associates with mucin, forming complexes with sizes ranging between 200 and 900nm. 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 towards 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(l-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, depend on the degree of chain flexibility and M_w [24]. Stiff (determined by the persistence length) and low- M_w polyanions preferably interact with the mucin's hydrophobic globular regions, whereas flexible and high- M_w 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 severity of disease depends on the charge and M_w of this polyelectrolyte [29]. DSS with a M_w of 500 kDa does not produce colitis in mice possibly because it is excluded from the mucus barrier, like large-non penetrating polymers. In contrast, 5 kDa and 40 kDa DSS induce colitis in the upper and lower colon, respectively [29].

Biophysical forces acting on mucus-resident bacterial communities

Biofilms have traditionally been studied as large aggregates of up to 1200 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 to 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 exert 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 a 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 nano- and micro-scale 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 *Escherichia coli* to mannose 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.

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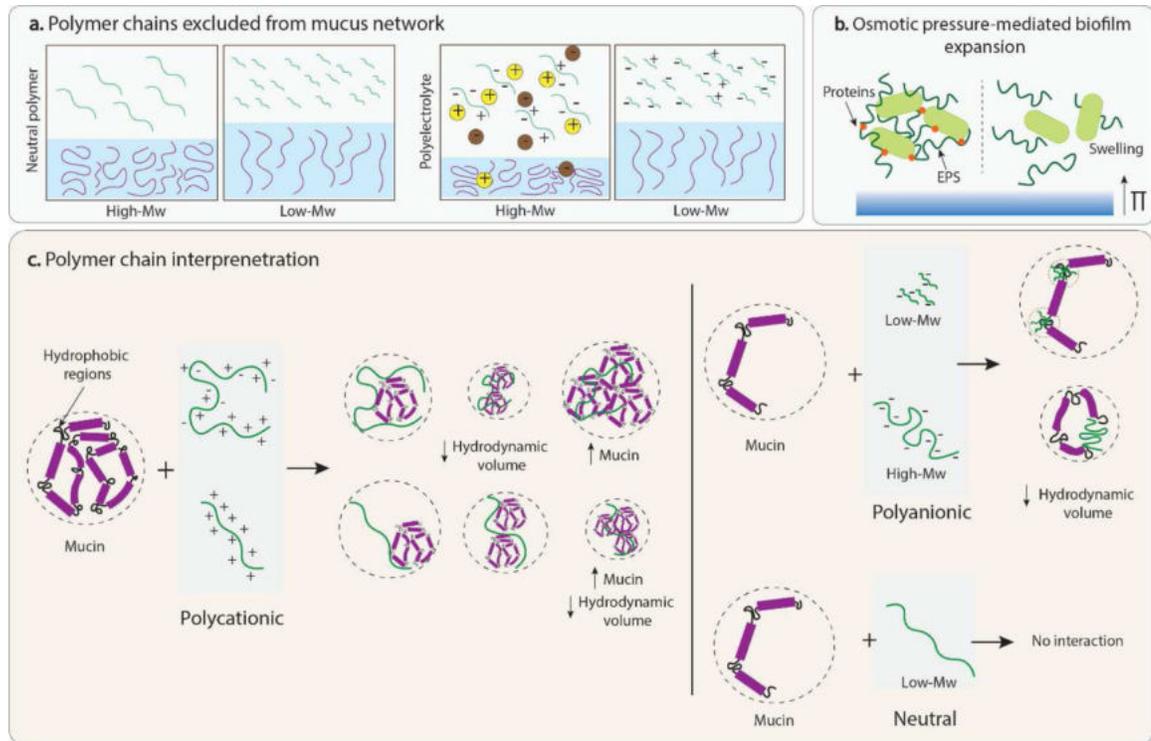


Figure 1. Interactions of mucin with non-penetrating and infiltrating polymer solutions as a function of their molecular weight, concentration, flexibility, and charge.

(a) Non-penetrating high-molecular-weight (M_w) polymers osmotically compress the mucus layer. High- M_w 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.

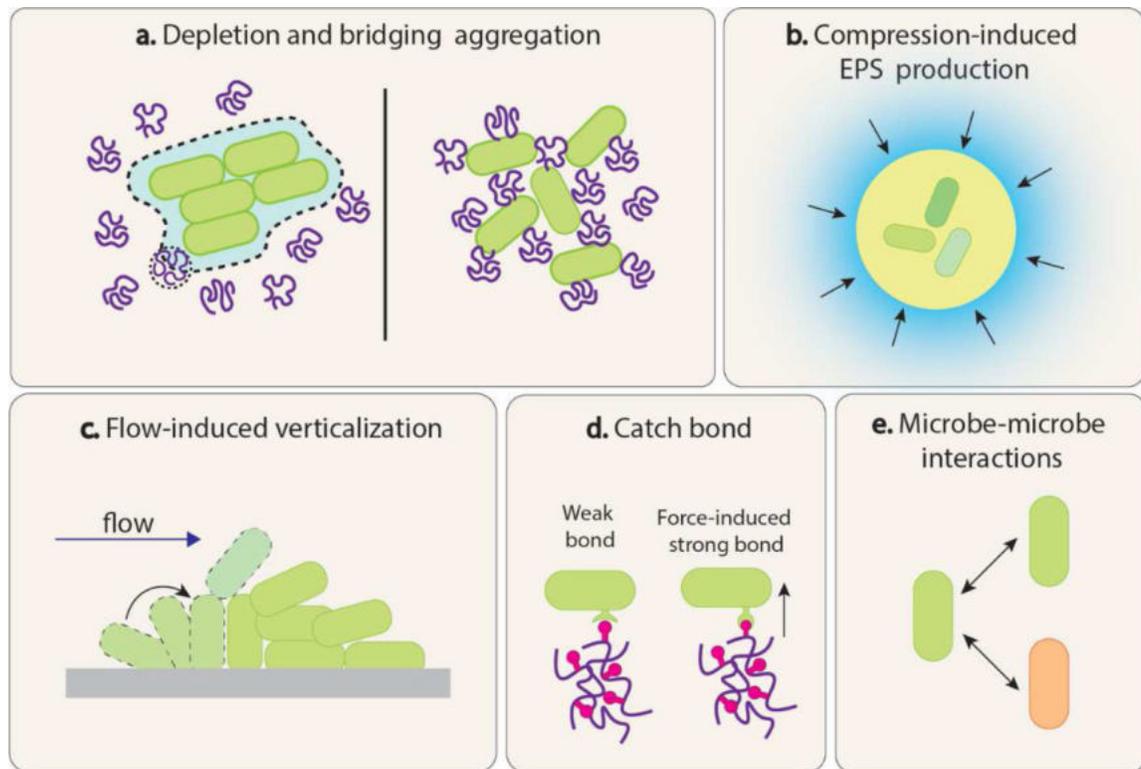


Figure 2. Biophysical forces influencing biofilm formation.

(a) Depletion and bridging aggregation of bacteria induced by mucin and bacterial exopolysaccharides. Polymer segments are illustrated as coils, whereas the depletion layer (left panel) is indicated by the dashed line around the aligned cells. Notice that in depletion aggregation, the polymer segments cannot approach the cell surface for a distance shorter than its radius of gyration. In bridging aggregation (right panel), polymer coils adsorb to the cell surface, linking together neighboring cells via electrostatic interactions. (b) Compression forces that biofilms may experience when growing on and within the mucus layer. In uropathogenic *Escherichia coli*, compression forces upregulate EPS production. (c) Fluid flow-induced verticalization of bacteria favors the formation of 3D assemblies *in vitro*. (d) Catch bonds undergo dynamic force-induced extension and conformational changes in response to tensile force and are widespread among both pathogens and commensals. (e) Cell-to-cell interactions are critical during the formation of complex, highly structured polymicrobial biofilms such as those formed on teeth's supra- and subgingival areas and may also contribute to the mechanics of *in vivo* biofilms.