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Mucosa-Mimetic Materials for the Study of Intestinal Homeostasis and Disease

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Mucus is a viscoelastic hydrogel that lines and protects the epithelial surfaces of the body that houses commensal microbiota and functions in host defense against pathogen invasion. As a first-line physical and biochemical barrier, intestinal mucus is involved in immune surveillance and spatial organization of the microbiome, while dysfunction of the gut mucus barrier is implicated in several diseases. Mucus can be collected from a variety of mammalian sources for study, however, established methods are challenging in terms of scale and efficiency, as well as with regard to rheological similarity to native human mucus. Therefore, there is a need for mucus-mimetic hydrogels that more accurately reflect the physical and chemical profile of the in vivo human epithelial environment to enable the investigation of the role of mucus in human disease and interactions with the intestinal microbiome. This review will evaluate the material properties of synthetic mucus mimics to date designed to address the above need, with a focus toward an improved understanding of the biochemical and immunological functions of these biopolymers related to utility for research and therapeutic applications.

1. Introduction

Mucus is a viscoelastic hydrogel that lines and protects the gastrointestinal (GI) tract.[1] Mucin glycoproteins, the structural component of mucus, can be categorized as either secreted, which form the functional component of free-flowing mucus, or attached to cells, where their function is primarily immunological.[1–2] Though both categories of mucus perform critical functions, gastrointestinal free-flowing mucus will be the focus of the current review. Gastrointestinal mucus is secreted and shed by goblet or gastric mucous cells that are interspersed throughout the epithelia, either continuously or following stimulation depending on the location and function of the specific goblet cell type.[3] The properties and organization of the mucus produced by these cells varies along the gastrointestinal tract; the small intestine contains one penetrable mucus layer, while the stomach and the large intestine each possess a dense lower layer that is tightly adhered to the epithelial surface and an upper layer that is more loosely attached.[4] In this review, we discuss the structure, composition, and function of intestinal mucus, including an evaluation of the biochemical properties of intestinal mucus mimics in relation to native human intestinal mucus. In addition, we discuss how synthetic analogues of mucus have been applied to replicate the functions of native mucus, with an emphasis on microbial interactions. Further, potential limitations and utility of each mucus mimic are also highlighted to advance the study of microbiome homeostasis, intestinal disease pathology, and treatment.

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2. Structure and Composition of Mucus

2.1. Components of Mucus: Types of Mucins

Mucus is a viscoelastic hydrogel that contains water (90–95%), lipids, fats, electrolytes, mucins, and additional proteins such as immunoglobulins, growth factors, and antimicrobial peptides.[1] Mucin glycoproteins, consisting of a protein backbone and complex glycans protruding outward from the protein backbone, are responsible for the properties of free-flowing mucus, specifically for the gel-forming secreted mucin glycoproteins, e.g., MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9, and MUC19 (MUC stands for mucin) which in humans are encoded by their respective genes (e.g., MUC5B protein is encoded by MUC5B gene and so on).[1] The mucin composition of mucus varies by tissue and location in the body, and an epithelial cell can secrete multiple types of mucins.[5] MUC2 is the predominant gel-forming mucin along the GI tract and provides the structural basis for intestinal mucus in both the small intestine and across both layers of the colonic mucosa, while gastric mucus is primarily composed of MUC5AC.[4,6]

2.2. Mucin Structure

Mucins are high molecular weight (MW) (0.5–20 MDa) glycoproteins consisting of 80% carbohydrate by weight, with

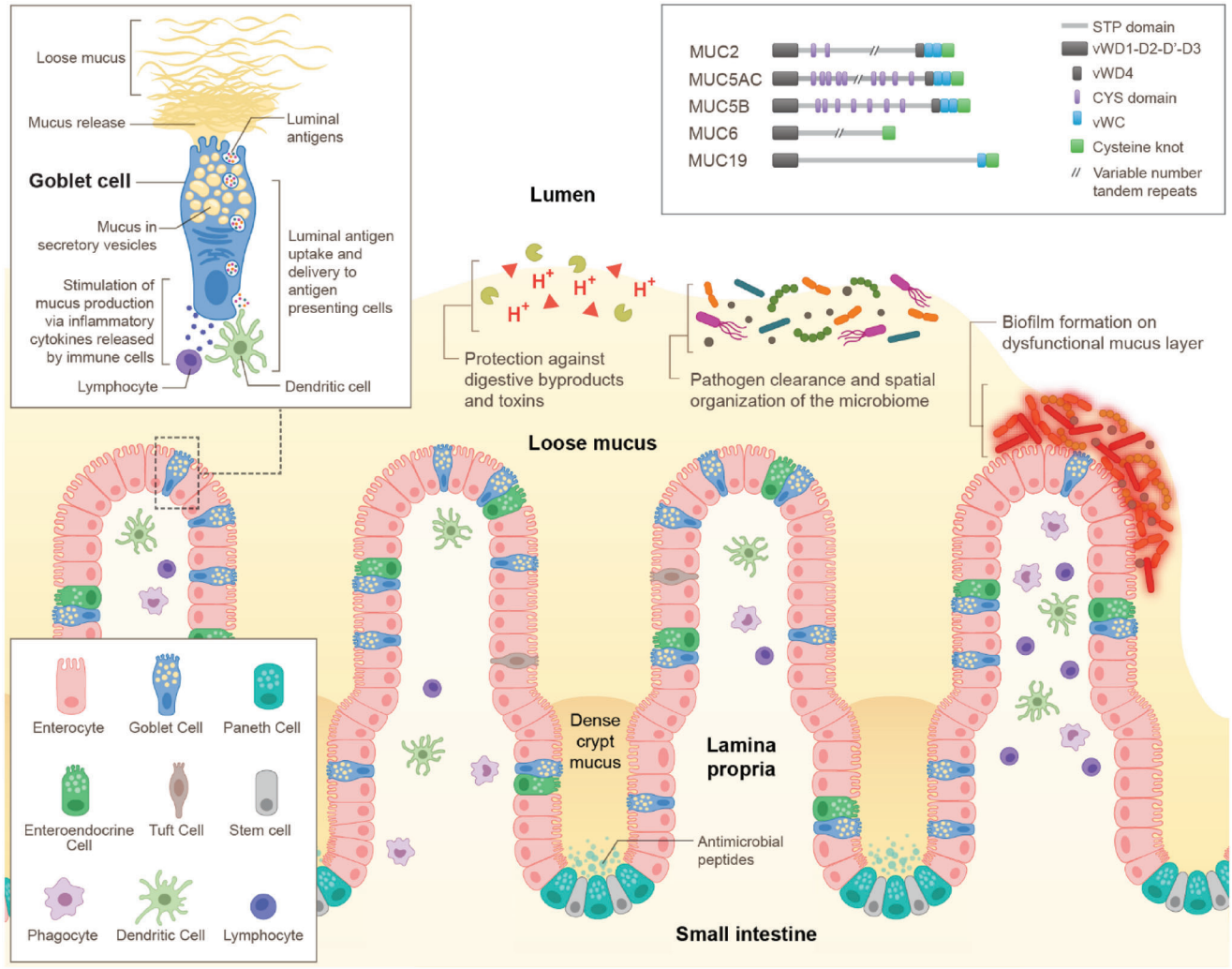

Figure 1. The biological role and structure of the intestinal mucus layer. The intestinal epithelium consists of diverse absorptive and secretory cell types including enterocytes, goblet cells, enteroendocrine cells (EECs), Paneth cells, M cells, Tufts cells, and stem cells. The small intestinal epithelium is covered by a thick layer of mucus which is secreted by goblet cells in part due to the stimulation of inflammatory cytokine release by immune cells. The mucus layer functions as a barrier to separate the commensal microbiota from the epithelium, keeping the epithelial cells bacteria-free and maintaining epithelial homeostasis. Goblet cells produce different subtypes of mucins, such as MUC2, MUC5AC, MUC5B, MUC6, MUC19), which contain multiple structural domains: STP (Serine, Threonine, Proline) domain, vWD (von Willebrand D) (D1, D2, D3, D4), CYS domain, vWC (von Willebrand C), Cysteine knot, and variable number tandem repeats. The top box with different mucin subtypes (MUC) is reproduced with CC-BY license from.[7] Copyright 2018, the Authors. Published by EDP Sciences.

the primary carbohydrates N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), sialic acid, galactose, and fucose, in addition to low concentrations of mannose and sulfate.[8] Mucin glycans are attached to a protein backbone that predominantly consists of tandem repeats of serine, threonine, and proline (STP) domains (**Figure 1**).[8] The resulting extensive O-glycosylation protects the mucins against degradation by proteases and is also responsible for the high diversity among the different mucins, as mucins of the same gene product may have significant variance in their glycosylation.[5b,9] Within the intestine, the composition of MUC2 O-glycosylation is regionally variable; mass spectrometry analysis revealed that human MUC2 is primarily composed of Core 3 O-glycans (GlcNAc(β1-3)GalNAc-ol) across all areas of the intestinal tract, while Core 4 O-glycans (GlcNAc(β1-3)-[GlcNAc(β1-6)]GalNAc-ol) are mainly located in the small intestine and Core 2 (Gal(β1-3)[GlcNAc(β1-6)]GalNAc-ol) in the colon.[10] Core 1 (Gal(β1-3)GalNAc-ol) and Core 5 (GalNAc(α1-3)GalNAc-ol) O-glycans are also present in MUC2 along the entirety of the intestinal tract, though to a lesser degree than Core 3 O-glycans.[10] Mucins are negatively charged due to the presence of sialic acid and carbohydrate-bound sulfates, and the resulting steric and charge repulsion causes their glycans to extend around the protein core in a distinctive bottle brush conformation.[1,11]

The reversible and covalent crosslinking of mucin glycoproteins facilitates network formation and thereby the hydrogelation of mucus. Most gel-forming mucins have cysteine-rich domains interspersed among the STP protein core that serve as reversible

crosslinking sites.[8] Additionally, the STP domains are bordered by von Willebrand factor (vWF) type C and D domains that are involved in reversible aggregation[12] and crosslinking with each other.[13] The carboxyl terminus contains a cysteine knot that can form disulfide bridges with other mucin monomers to form dimers and other higher-order structures.[13]

3. Material and Chemical Properties

The properties of mucus are foundational to its biological role and the viscoelastic properties of mucus mimics determine their function in relation to native mucus. Once crosslinked, mucins form a highly porous 3D gel matrix.[14] The spacing between mucin hydrogel fibers is fairly uniform in some mammalian samples,[15] while very heterogeneous in most others with variances depending on the location in the body.[16] The mesh size of human intestinal mucus is not well-characterized, however, porcine jejunal mucus has mesh spacing from 20 to 200 nm based on atomic force microscopy.[17] The influence of pH on the structure of mucus is of particular importance in vivo; mucus forms a more elastic gel at more acidic pH values and behaves like a viscoelastic liquid at neutral and basic pH.[18] Mucus also displays shear-thinning and self-healing behavior, and the gel viscosity is optimized for continuous shedding and flow of mucus while still trapping pathogens and debris.[15,19] Mucus porosity and aggregation are sensitive to calcium ion binding and concentration, although few mucus mimics have been evaluated for this property.[20]

Mucus is able to fulfill its biological role as a protective barrier due to its selective permeability. Mucus lines the different mucosal surfaces in the body, including respiratory, vaginal, and gastrointestinal tracts, forming a selective barrier against foreign particles, toxins, and pathogens.[1,11,21] In addition to steric filtration via pore size, mucus blocks penetration by microorganisms and particles through selective interactions and electrostatic interactions.[16] Although further information is needed on how mucins interact with other macromolecules, mechanisms may include binding via hydrogen bonding, electrostatic interactions, hydrophobic interactions, and chain entanglement.[22]

4. The Biological Role of Mucus

As a frontline barrier, the principal roles of gastrointestinal mucus are to protect epithelial surfaces against stress or damage and to provide lubrication and hydration to facilitate the passage of food and waste through the gastrointestinal tract (Figure 1). Moreover, mucus serves as a spatial barrier for the transport of nutrients into the epithelium while maintaining protection from pathogens, toxins, and harmful byproducts of digestion, such as hydrochloric acid and pepsin.[15]

4.1. Interactions with the Microbiome

Intestinal mucus houses the majority of commensal microorganisms and serves as a key mediator between the human microbiome and epithelial cells lining the track (Figure 1).[23] The composition of the microbiome varies along both the length of the gastrointestinal tract and the radial axis from the epithelium to the lumen.[24] The 3D structure of mucus permits spatial organization of different populations of microbes and may help to optimize the positions of the microbiome to serve as a barrier between pathogenic bacteria and the epithelial surface.[23] In the colon, the microbiome is located in the upper, loosely adherent mucus layer, while bacteria are normally absent in the inner dense mucus layer due to the decreased mesh size, thus creating a size exclusion barrier between the epithelium and microbes.[6]

Mucin glycans play a critical role in interactions between bacteria and mucus and are consumed by both commensal and pathogenic microbes as a carbon and nutrient source.[25] Bacteria initiate mucin binding and mucolysis by employing flagella,[26] pili,[27] and Lactobacilli mucus-binding proteins (MUBs),[28] and various other adhesins and cell-surface proteins that are genus or species-specific.[29] A key function of mucus is to prevent the penetration of pathogens to the epithelium as mentioned earlier, and mucosal glycans selectively bind or otherwise inhibit antagonistic bacterial signaling molecules to stop proliferation and the formation of biofilms.[5a,30] For example, intestinal mucus can disrupt *Pseudomonas aeruginosa* biofilms by inducing an increase in inhibiting virulence by downregulating genes implicated in key processes such as quorum sensing and type III secretion, while depletion of MUC2 inhibits *P. aeruginosa* biofilm dispersal.[31] Additionally, mucin glycans can act via the sensor kinase RetS in *P. aeruginosa* to downregulate type VI secretion among other transcriptional changes, resulting in reduced virulence.[32] Due to the diversity of glycan structures and microbial species present in the microbiome, further work is needed to continue to identify the mechanisms involved in mucus and mucin interactions with bacteria that impact tissue functions.

Interactions between bacteria and mucins can also result in alterations to barrier permeability and the physical properties of intestinal mucus. In mice, variance in microbiome species composition was associated with differences in mucus phenotype affecting permeability.[33] Along the human gastrointestinal tract, this phenomena is exemplified by *Helicobacter pylori*, which increases the pH of gastric mucus via secretions, to decrease its viscoelasticity to permit increased motility and mucus penetration.[34] Inversely, commensals, particularly Lactobacilli species, stimulate MUC2 secretion in the colon to maintain epithelial barrier integrity.[35]

4.2. Immunological Functions

Beyond associations with the microbiome, gastrointestinal mucus has been implicated in immunological signaling and surveillance processes. Increased mucus production is associated with the secretion of interleukin-22 (IL-22) by type 3 innate lymphoid cells in response to pathogenic bacteria and may also be stimulated via cytokine secretion by T helper type 2 cells (TH_2).[2,36] Moreover, mucus-secreting goblet cells within the small intestine can sample the contents of the mucosal layer and subsequently present the antigens to dendritic cells of the lamina propria.[37]

5. Relevance to Human Disease

Mucus is essential for the protection and maintenance of barrier surfaces, and subsequently mucus dysregulation can result

in disease. Insufficient thickness of the mucus barrier causes epithelial surfaces to be more vulnerable to pathogens and can result in biofouling, the accumulation of microorganisms, and infection due to improper clearance of antagonistic bacteria and parasites.[9,38]

Dysfunction within the mucus layer of the gastrointestinal tract has been associated with ulcerative colitis, Crohn's disease, and colorectal cancer. Increased bacterial penetration of the mucosal layer occurs within both forms of inflammatory bowel disease (colitis, Crohn's) despite significant variation in the physical properties of the mucus layer.[39] Ulcerative colitis is associated with a thinner colonic mucus layer with decreased microbial diversity, altered mucin 2 (MUC2) glycosylation, and differences in mucus phospholipid concentrations and types.[39a,40] A thicker colonic mucus layer and polymorphisms linked to improper reactive oxygen species-mediated mucin secretion are observed in Crohn's disease.[39a] While it is not yet clear if there are any alterations in the material properties or quantity of gastrointestinal mucus with colorectal cancer, significant differences in mucin and glycan expression have been observed; Mucin 1 (MUC1), mucin 17 (MUC17), and mucin 5AC (MUC5AC) are overexpressed in adenocarcinomas, while mucin 4 (MUC4) and MUC2 are considerably downregulated.[39b] The glycans tumor associated glycoprotein 72 (TAG72) and MUC1-associated GalNAc-Ser/Thr (Tn) and Sialyl-Tn have showed significantly higher expression in adenocarcinomas as well.[39b]

The gut microbiome plays a significant role in human health and various diseases.[41] In fact, gut and microbiome dysfunction and dysbiosis are also commonly observed in patients with neurological disorders such as Parkinson's disease,[42] Multiple Sclerosis,[43] Autism Spectrum Disorder,[44] and Alzheimer's disease[45] and often develop prior to the onset of neurological symptoms, although the relationships between microbiome and the neurological disease have yet to be determined.[46] It is not known what variations in mucus phenotype may lead to the observed changes in microbe composition, although hypotheses for mechanisms include misfolding of mucin proteins, alterations in the signaling pathways responsible for gastrointestinal stem cell maturation and goblet cell development, and changes in expression of vesicle-associated proteins involved in both mucus release and neurotransmitter systems.[46]

6. Current Methods of Obtaining Mammalian Free-Flowing Mucus and Biological Mimics

Native human mucus is generally collected as sputum from patients with cystic fibrosis or from the female genital tract (**Table 1**). However, neither source is without fault as there are often issues of uniformity, invasiveness of collection to the human participants, significant limitations to the quantity of mucus that can be isolated at given time, and issues of relevance to the properties of gastrointestinal mucus. While collection is less intrusive, sputum is not ideal for most applications as the pathology of cystic fibrosis includes an altered phenotype of airway mucus that differs significantly from gastrointestinal mucus.[47] Mucus from patients with cystic fibrosis is generally much more viscoelastic and contains a higher concentration of DNA, therefore the material requires further processing to accurately reflect the properties of native human mucus.[11] Bronchoscopy and endotracheal tube sampling can additionally be used for the direct collection of airway mucus, although both methods are invasive, must be performed by a medical professional, and similarly differ with regard to their rheology from gastrointestinal mucus.[48] A procedure for the collection of human intestinal mucus through colonoscopy was recently developed, although this approach has similar disadvantages in terms of the amount of sample that can be obtained, variability, and intrusiveness.[49] Moreover, the colonoscopy approach requires precise conditions for the mucus obtained to be acceptable for further use (i.e., there must be zero inflammation), and most study participants were ultimately disqualified.[49]

Porcine gastric mucus is commercially available and is most commonly used to mimic human gastrointestinal mucus.[50] Commercial mucus is advantageous in that it is readily available at much higher quantities, however this is at the expense of its rheological properties. Porcine gastric mucus differs in mucin composition from that of the small intestine, does not gel properly, likely due to damage (likely due to breaking of molecules or affecting key glycoprotein chemical blocks due to harsh condition) during the commercial purification process, and is also

Table 1. Benefits and limitations of methods used for the collection of mammalian mucus.

Native mucus source	Mode of collection	Advantages	Limitations	Refs.
Human	Sputum	Direct human source, less invasive, no specialized clinical procedure	Altered material properties, possibility for bacterial contamination, higher DNA content, different mucin content than gastrointestinal mucus	[11, 47]
	Direct removal from intestinal epithelial surfaces	Accurately replicates material properties and mucin composition of the target tissue	Invasive, low yield, labor intensive, lack of uniformity, highly specific conditions for collection	[48–49]
Porcine	Commercial gastric product	Uniformity, convenience, high yield	Mucin composition may not match desired application, improper gelation, material properties not preserved	[20b, 50, 51]
	Direct removal from epithelial surfaces	Can control for mucin composition to match desired application, accurate material properties	Greater potential for contamination, labor intensive, variation between individuals, low yield	[20b, 52]

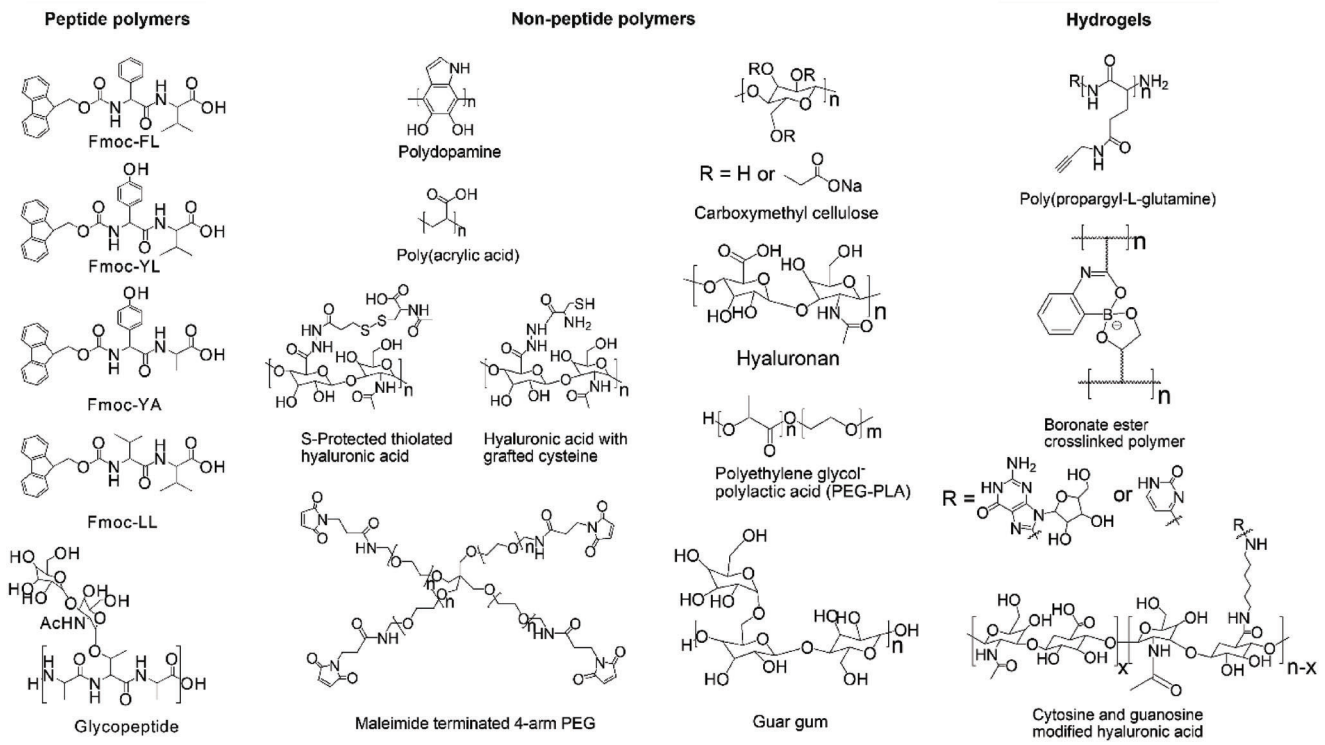

Figure 2. Chemical structures of some mucus mimics described in Table 2.

lacking in lubricity.[20b,51] Gastrointestinal mucus from cows and horses is also available but has similar functional shortcomings due to the nature of the commercial purification process.[15]

Mucus can also be directly scraped from the intestines of animals when a commercial product is not available or suitable, but this method presents the possibility of contamination with cellular debris that can result in an immune response or cytotoxicity when used for in vitro applications.[20b,52] Moreover, rheological properties such as viscosity can vary significantly between individual animals.[52] To more accurately mimic native human mucus, mucins have been purified from porcine intestinal mucus and reconstituted into a gel. While this method has been shown to more faithfully preserve the material properties of intestinal mucins, low yield, cost and time are issues.[20b] Further, to prevent bacterial proliferation and mucin degradation of the retrieved mucus, sodium azide was added to the isolated mucus. Enzymatic degradation can also be prevented by adding protease inhibitors to the sodium chloride buffer used to solubilize the retrieved mucus.[53]

Due to the importance of the mucosal layer to gut homeostasis and overall human health, robust models of the epithelial surface are necessary to study relevant pathologies and therapeutics. Existing in vitro models have generally emphasized cell layers, scaffolding, and 3D structure, while the mucus layer has typically received less attention despite its importance.[54] Recently, an in vitro mucus model for dynamic bacterial culture was reported.[55] Further, the role of the mucus layer was elucidated by mimicking host-pathogen interactions in an in vitro model.[56] Generally, there is a trade-off between efficiently synthesizing mucus with more accurate material properties and generating the correct mucin composition for the tissue being targeted. This balance has not been fully addressed by existing methods of mucus collection. The thickness and amount of mucus collection depend on the ratio of cocultured cells. In a cocultured in vitro model (HT29-MTX and Caco2 cells), the thickness of the derived mucus depended on the ratio of the HT29-MTX and Caco2 cells.[57] For example, when cultured alone for 21 days, HT29-MTX cells produced a mucus layer with a thickness of 175±37 μm. However, when cocultured with Caco2 cells, the height of the derived mucus layer decreased. Different ratios (9:1 and 8:2) of Caco2 and HT29-MTX, produced layers with 48±13 and 94±10 μm thickness, respectively.[57] While native mammalian and commercial sources of mucus are available as summarized earlier, they are often insufficient in their replications of in vivo rheology or difficult to obtain, therefore synthetic mucus mimics are needed to recapitulate the typical in vivo mucosal environment and achieve greater efficiency in production. Beyond use in epithelial models, mucus analogues could be additionally utilized as therapeutics to address an insufficient mucosal layer and mimicking the antimicrobial properties of native mucus may lead to treatments against biofilms or alternatives to standard antibiotics.

7. Synthetic Mimics of Mucus and the Mucosal Layer

There are several previous reports of mucosa-mimetic materials (**Table 2**) (**Figure 2**)[5a,15,58] and dynamic hydrogels.[59] Generally, mucus mimics can be categorized as cell cultures, in vitro models, nonpeptide polymers, and peptide-backbone polymers. Most prior work on intestinal mucus analogues has concentrated

Table 2. Overview of mimics discussed in previous reviews (Werlang et al. (2019),[5a] Lock et al. (2018),[58] Boegh and Nielson (2015)[15]).

Classification	Chemistry and/or materials	Advantages	Limitations
Mucus-secreting cell culture	Caco-2 HT29(-MTX) co-culture[65]	Reflects the properties of human mucus, suited for drug permeability applications, homogenous product	Low product yield, differences in in vitro versus in vivo mucin expression, length of time to culture, glycan expression is altered in cancer cell lines[66]
	Caco-2 HT29 Raji B triple co-culture[67]		
	Heterogeneous primary epithelial culture[68]		
	Human intestinal spheroids[69]		
In vitro models	Simulated mucus applied to cell culture on permeable filter inserts[52,70]	Easier to collect reference data, evaluation of mucus barrier	Not all simulated mucus biocompatible with cultured cells, differences in vitro versus in vivo mucin expression
Nonpeptide polymers	Carboxymethyl cellulose[71]	Forms similar barrier to native mucus, lubricates well.	Lack of glycosylation results in variable biological effects differing from native mucus, must have grafted-glycans to replicate normal in vitro interactions with microorganisms
	Hyaluronan[72]		
	Guar gum[73]		
	Polyethylene glycol-polylactic acid (PEG-PLA)[74]		
	Polymethyl methacrylate (PMMA) derivatives[75]		
	Poly(N-acryloyl-D-glucosamine)[76]		
Polypeptide backbone polymers	Ala-Thr-Ala tripeptides with sugars attached to Thr residues[77]	Polymer structure replicates the STP domains of native mucins, biocompatibility, accurate replication of mucus-microorganism interactions	Low molecular mass, high polydispersity among the group of potential polymers that can be synthesized
	Poly(α-GalNAc-Ser) via α-amino acid *N*-carboxyanhydride (NCA) polymerization[78]	Molecular mass similar to native mucins, low polydispersity, good yield, biocompatibility, replication of mucus-microorganism interactions	No gelation
Hydrogels	Poly(γ-propargyl-*L*-glutamine) polymer[79]	Easily prepared, properties fit many applications, stable for long-term use	Potential for racemization
	Polypeptides synthesized via NCA with added Cys residues[80]	Replicates cysteine regions in native mucins, provides opportunity for crosslinking and therefore proper gelation	
	Diol-boronic acid crosslinked hydrogels[81]	Shear-thinning and self-healing behavior, tunable mechanical properties, biocompatibility	Sensitive to oxidation, which may alter material properties
	Cytosine and guanosine modified hyaluronic acid prepared via nucleobase-mediated hydrogen bond crosslinking[82]	Rheological properties, biodegradability	Less suitable for long-term applications

on drug delivery and mucoadhesion, with a particular focus on replicating the tunable crosslinking, self-healing and shear-thinning behavior, and biocompatibility of native mucus. Toward this direction, significant advances in the design and synthesis of mucin mimetic materials and their biomedical utility were recently highlighted.[60] In addition to mucin analogues, the adhesive performance of different materials to the mucosal membranes were evaluated.[61] For the study, different hydrogels were prepared from different synthetic polymers, such as polyethylene glycol diacrylate (PEGDA),[62] and polyvinyl alcohol (PVA)[63] and were used as tissue substitutes and their mucoadhesive performance assessed. Multiple layers of hydrogels coatings, prepared on 2D glass surfaces by layer-by-layer (LBL) deposition mimicked mucosal tissues.[64]

7.1. Mucin-Containing Hydrogels

One strategy to overcome the shortfalls of nonhuman mucins involves combining partially purified porcine mucins with the native components of airway mucus: including albumin as a protein source, water, ions, and dipalmitoylphosatidylcholine (DPPC).[83] Glutaraldehyde is used as a bifunctional crosslinking agent and its concentration, and the duration of the time allotted for crosslinking modulate the viscoelastic properties of the mucus. As glutaraldehyde induces irreversible covalent crosslinking, the mucus product is highly stable, however this is a notable deviation from the disulfide bond networks seen in native mucus.

The lack of gelation in commercial mucin products has also been addressed by the addition of four-arm polyethylene glycol (PEG) thiols.[84] Though in vivo studies have yet to be performed, 4-arm PEG-thiol induces the formation of a stable viscoelastic gel that mimics the native chemistry of human mucus, has a similar gel pore size and rheological properties, and has tunable kinetics.

The gelation of porcine gastrointestinal mucins can be improved via the incorporation of chitosan.[85] Porcine gastric mucins (MUC5AC and MUC5B) were scraped directly from stomach epithelium samples, purified, and complexed with chitosan of molecular weights ranging from 1.3 to 16.1 kDa. The addition of chitosan induced gelation, while commercially

purified mucins only formed weak hydrogels. The lowest molecular weight chitosan-reinforced mucins (degree of polymerization 8) were not cytotoxic toward HT29-MTX cultures and slowed the distribution of cholera toxin B and dextran uptake in the monolayers in comparison to cultures containing HT29-MTX-secreted mucus alone, indicating that the chitosan-complexed mucus had improved barrier properties. However, heterogeneity of chitosan distribution remains a challenge, and the mucin composition of porcine gastric mucus differs from human intestinal mucus.

Purified or isolated mucins do not reproduce the native mucin properties, as they lack many components of native mucins as the complex extraction process leads to cleavage of disulfide bridges, resulting in a decrease of their viscoelastic properties.[63,86] In such cases, mucins can be supplemented with different synthetic polymers, additives or modified with different functional motifs to facilitate hydrogelation for the synthesis of artificial mucus like materials with properties closer to native mucus. For example, polyvinyl alcohol (PVA) was used to promote the hydrogelation of purified mucins from porcine stomach.[63] PVA gels alone do not form porous structures, however, when supplemented with mucins, they form hydrogels with ≈5 µm pore sizes. Similarly, hydrogels formed from PVA/mucin demonstrated higher adhesion to chitosan surface than PVA hydrogels alone due to charge complementarity.[63] Like PVA, polyacrylic acid (PAA), when supplemented with porcine MUC2 or MUC3, bovine serum albumin (BSA) and lipids (cholesterol and phosphatidylcholine) can form artificial porcine colonic mucus with comparable gelation, viscoelasticity and shear-thinning behavior to native intestinal mucus isolated from porcine intestine.[87] Biosimilar mucus models can also be generated by reconstituting porcine mucin with additives such as lipids and protein components of native mucus and applying the artificial mucus to a transwell membrane.[88] Such biosimilar mucus exhibits improve gelation in comparison to mucin solution in buffer, indicating that biosimilar mucus may be a more accurate model of the innermost, denser intestinal mucus layer. The mucus layer that was produced was 1.3 mm in thickness, much thicker than the native human intestinal mucus layer.[88] Similarly mucus-like hydrogels can also be formed from bovine submaxillary mucin (BSM) when modified to its corresponding aldehyde or hydrazide derivatives by hydrazone crosslinking.[89] The resulting hydrogel displays self-healing behavior and had a mesh size (38.8 ± 6.67 nm) which is within the range of native intestinal mucus. In addition, these gels were effective at inhibiting viral infection in vitro (HIV-1 and HSV-2).[89]

Recently hydrogel properties of native mucus and the reconstituted mucin gels were compared.[53a] Different relevant hydrogel features such as structural, mechanical and biochemical properties were assessed by preparing hydrogels from natively purified, commercial and synthetic mucin and compared with native mucus.[53a] Broadly, reconstituted MUC2 hydrogels exhibited similar material properties (rheology, biochemical properties, transport) compared to native intestinal mucus.

Mucus-mimetic surfaces have also been generated by incubating pig gastric mucin on an asymmetrical GM1 ganglioside-containing model membrane bilayer, resulting in a 30 Å thick layer of deposited mucus.[22] The adhered mucin layer was stable and was maintained even after solvent exchange and polymer deposition due to favorable interactions between the mucin glycans and the ganglioside sugar groups. Porcine mucins may also be applied to membrane surfaces derived from egg-phospholipid liposomes to achieve similar improvements to stability.[90] Such models are well-suited for mucus permeation and drug screening studies but are not ideal for applications requiring the isolation of artificial mucin.

7.2. Peptide Polymers

Due to the innate high cysteine content, keratin hydrogels can be generated by intramolecular and intermolecular disulfide bond shuffling to form crosslinked polymer networks.[91] The cysteine content can be modified to tune the mechanical properties without any additional crosslinking agents. Further, keratin hydrogels undergo rapid gelation, have a tunable in vivo degradation rate in a mouse model, and are not immunogenic. However, their cytotoxicity has only been assessed against rat and mouse osteoblast and fibroblast cell lines, and the pore sizes of the hydrogels were between 10 and 30 µm depending on keratin concentration and cysteine content, which is notably higher than native mucus.[16–17]

Fluorenylmethyloxycarbonyl (Fmoc) conjugated dipeptide self-assembled hydrogels were originally investigated as a submucosal dissection filler but may be modified to serve as mucosa-mimetic materials due to their tunable thixotropic and self-healing properties.[92] These dipeptide hydrogels have tunable mechanical properties that can be altered by modifying the strength of their hydrophobic interactions and the level of hydrogen bonding through control of peptide sequence. Moreover, these peptides demonstrate biocompatibility, biodegradability, and bioactivity and are suitable for injection. The following dipeptides have been assessed, with each found to have a unique mechanical profile: Fmoc conjugated phenylalanine-leucine (FL), tyrosine-leucine (YL), leucine-leucine (LL), and tyrosine-alanine (YA). Fmoc-YL demonstrated the highest postinjection mechanical rigidity and stability in vivo, while Fmoc-FL was associated with the highest ex vivo mechanical rigidity, which is slightly correlated with hydrophobicity. All of these Fmoc-conjugated dipeptides demonstrated shear-thinning behavior, and Fmoc-YL and Fmoc-YA showed the most rapid and robust self-healing behavior.

7.3. Nonpeptide Polymers

Polydopamine-based mimics that are polymerized by endogenous catalase can be generated in situ in mammalian small intestines due to the increased catalase concentration in the small intestine relative to other areas of the gastrointestinal tract.[93] Application to ex vivo porcine and human intestinal tissues selectively generated a polydopamine coating on the epithelial side. Polydopamine was biocompatible against human cell lines and within an in vivo porcine model, resistant to mechanical stress, and stable within simulated gastrointestinal fluids. Following in vivo polydopamine treatment in a porcine model, dopamine was not absorbed into the bloodstream, indicative of its safety. Furthermore, no in vitro cytotoxicity was observed within 48 h against human cell lines and no oral toxicity occurred over a 28

day treatment period with rats. Moreover, polydopamine was useful for a variety of applications due to the ease of integration of different functional agents and has been tested for enzyme delivery to improve lactose tolerance, altered nutrient absorption via modified crosslinking properties, and prolonged drug release. However, since lower dopamine polymerization occurred in the colon due to its naturally lower catalase concentration, in vivo uses of polydopamine as a mucus mimic are only applied to the small intestine. Moreover, to assess the in vivo tolerance and properties within the porcine model, polydopamine was applied directly to the gastrointestinal tract with a catheter, and such an invasive delivery method may pose a barrier to future use in human patients. Following further refinement, dopamine monomers could be delivered orally as a therapeutic, although additional work is needed to determine the dopamine dosage and drug formulation.

A thiolated linear polyglycerol (LPG) backbone crosslinked with ethoxylated trimethylolpropane tri(3-mercaptopropionate) formed a hydrogel with disulfide bonds analogous to native mucins.[94] LPG-based mimics have rheological properties of a typical gel, although the rheology properties can be modulated by adjusting the LPG backbone length and the ratio of LPG to the crosslinker. Additionally, the LPG backbone contains hydroxyl sites that can be readily functionalized with sugars or other functional groups characteristic of mucins. Although LPG hydrogels had a mesh size of 15–80 nm, comparable to the 20–200 nm mesh sizing of intestinal mucus, the biocompatibility and biological properties have yet to be investigated.

Cis-poly(norbornene) glycopolymers form an extended linear structure that mimics the native bottlebrush configuration of mucins and can be functionalized with galactose at a tunable density to replicate native in vivo glycan-microorganism interactions.[95] Cis-poly(norbornene) hydrogels demonstrated a similar or greater cholera toxin binding ability to purified porcine MUC2, MUC5AC or MUC5B, depending on the degree of galactose functionalization. Although glycopolymers accurately mimic the biological function of mucus to disrupt bacterial virulence, their biocompatibility remains undetermined.

7.4. Developments in Cell Culture Models

Human-derived colonic epithelial stem cells (organoids) at the air–liquid interface in culture produce a uniformly thick mucus layer (≈300 µm), when treated with vasoactive intestinal peptide once they have differentiated for 10-days in culture.[96] The generated mucus layer was harvested and appeared to be loosely adherent, like the in vivo upper mucus layer. The thickness of the mucus layer was modified via the duration of culture time, and the mucus product had similar in vivo biological interactions and properties as native gastrointestinal mucus, such as the strong expression of MUC2 and mild expression of MUC5AC. The mucus product protected the integrity of the epithelial layers from *E. coli* and *Clostridium difficile* Toxin A for 4 h and was anti-inflammatory in the presence of *E. coli* for 24 h. Although the glycan composition of mucus secreted by organoids better reflects that of native gastrointestinal mucins than mucins secreted by cancer cell lines, the overall yield of mucus remains low, and the model may not be as suitable for applications requiring the isolation of significant quantities of artificial mucus.

Commercial porcine mucin II can be combined with alginate and crosslinked via calcium chloride to form a hydrogel that has similar viscoelastic moduli to native mucus, including rapid gelation.[97] When applied to an epithelial cell monolayer within a polyethylene glycol and dextran aqueous two-phase system (ATPS) coculture platform, the alginate-mucin hydrogel supported Caco-2 cell biocompatibility in the presence of the bacterial species *P. aeruginosa* and *S. flexneri*, suggesting it may mimic the barrier properties of native mucus. However, the alginate-mucin composite hydrogel has yet to be tested within other cell culture platforms.

8. Perspectives and Conclusions

Due to the importance of mucus in gut homeostasis, intestinal disease pathology, and proper functioning of the epithelial innate immune system, accurate and efficient models of the intestinal mucosa are needed to examine these functions. As existing methods of collecting native mammalian mucus often have limitations related to heterogeneity, invasiveness, and low yield, synthetic substitutes may have more utility for such models. Although 2D or 3D mucus-secreting cell cultures, such as Caco-2/HT29-MTX cocultures or human intestinal organoids may be utilized in in vitro intestinal models to mimic the intestinal epithelial mucosa, their secreted mucus layer is insufficient and bacterial overgrowth occurs rapidly due to the lack of a mucosal biochemical and spatial barrier, limiting their utility to studies of the interactions between the gut microbiome and the intestinal epithelia and intestinal innate immune system.[98] Therefore, such models could be supplemented with synthetic mucus to support long-term study of the gut microenvironment and microbiome homeostasis.

To model the pathology of gastrointestinal diseases where the mucus layer is altered, mucin analogues could be restructured or altered in terms of glycan composition. Materials with tunable densities will be of particular importance to this aim to match the changes to mucus thickness, microbial penetrability, and MUC2 expression observed in various intestinal diseases. For example, MUC2 expression is decreased in ulcerative colitis, which could potentially be simulated by utilizing a lower concentration of the functional component in a mucus-like hydrogel when applicable.[99] Engineering sugar grafting could additionally be employed to match the glycan profile of each diseased mucus state to mediate microbial interactions more emblematic of each pathology.[100]

Further, synthetic mucus mimics could additionally be utilized as therapeutics to restore proper mucosal barrier functioning. Disruption or insufficiency of the mucosal layer is implicated in diseases such as inflammatory bowel disease and ulcerative colitis, and artificial mucus delivery could be used to prevent further bacterial propagation and reduce inflammation.[39a] Synthetic mucus could also be utilized in models of the gut-brain axis to provide insight into how the dynamics of the microbiome and mucus phenotype impact neurological diseases.

With regard to oral drug delivery, the intestinal absorption of therapeutics remains a challenge due to the steric and electrostatic barrier properties of the intestinal mucosa, particularly for high MW or nonpolar formulations.[16] Synthetic mucus mimics could be leveraged to improve the physiological relevance of

mucus-secreting intestinal models to assess the mucoadhesion and mucus permeation of therapeutics prior to in vivo clinical bioavailability testing.

The material properties of synthetic mucus-like hydrogels and cell culture products have been characterized and compared with the performance of the in vivo native product, but much remain to be learned about the biological functionality and microbial interactions. Many substitutes have yet to be tested in terms of bioactivity, and further assays and modifications will be needed to fully evaluate the biochemical properties of these substitutes to determine their functional potential. Additionally, as glycosylation is the foundation of mucus' interactions with the microbiome, nonglycosylated polymers may not be able to accurately replicate this function, and further investigation is needed into grafting mucus-like hydrogels with relevant glycan groups. Gastrointestinal mucus is fundamental to facilitating interactions between the epithelium, the immune system, and the microbiome, and further innovation of mucosa-mimetic materials will provide improved opportunities to more reliably investigate the intestinal microenvironment and gastrointestinal disease pathology and treatment.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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