Engineering mucus to study and influence the microbiome

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Abstract | Mucus is a 3D hydrogel that houses the majority of the human microbiome. The mucous environment plays an important role in the differentiation and behaviour of microbial phenotypes and enables the creation of spatial distributions. Dysregulation of mucus is further associated with various diseases. Therefore, mucus is the key ingredient to study the behaviour of commensal and pathogenic microbiota in vitro. Indeed, microorganisms cultured in mucus exhibit phenotypes substantially different from those exhibited in standard laboratory media. In this Review, we discuss the impact of mucus on the microbiome and examine the structure and glycosylation of mucins — the main building blocks of mucus. We investigate the impact of glycans on mucin function and highlight different approaches for the engineering of synthetic mucins, including synthesis of the backbone, the design of mucin-mimetic hydrogels and the engineering of glycans. Finally, mucin mimetics for 3D in vitro cell culture and for modulating microbial community structure and function are discussed.

Humans have at least as many bacterial cells as human cells[1]. The human microbiome (BOX 1) has a profound impact on human health, and changes in the composition of the microbial community are associated with various diseases, ranging from diabetes to depression[2,3]. The development of rapid and efficient DNA sequencing techniques has revolutionized the study of the human microbiome[4], enabling the identification, categorization and tracking of human-associated microorganisms[5]. Many microbial species are commensal and even beneficial; however, the microbiome also contains potential pathogens, and the mechanisms of virulence suppression in healthy individuals remain elusive thus far[6].

High-throughput DNA sequencing of clinical samples enables the identification of groups of microorganisms associated with health and disease[7]; however, to understand their function and behaviour, microorganisms need to be cultured in vitro. Cultures of clinical samples are often dominated by certain genera, but incorporating the natural mucous environment can help to preserve species diversity[8,9]. Interaction with the surrounding environment and other community members of different species is often crucial for survival and required to provide access to trace nutrients, metabolites and biochemical and biophysical signals[10,11]. Even microorganisms that are able to survive in traditional in vitro culture methods can exhibit behaviours different from those exhibited in their natural environment[12,13]. Therefore, culturing microorganisms in a relevant 3D context is essential for understanding their behaviour[14].

For decades, human tissue culture models were limited to 2D methods that did not facilitate cell differentiation and spatial separation. Polymers, such as Matrigel, can provide the 3D spatial scaffolding necessary for cell differentiation and enable environmental regulation of cell fate[15]. In such 3D systems, the definition and division of space allow the creation of signal and nutrient gradients, which can drive progenitor cells to differentiate into a variety of cell types[16]. Synthetic or natural 3D matrices can also present different biophysical and biochemical cues, such as growth factors that influence cellular development and cell fate[17]. Recognizing the importance of the extracellular matrix for in vitro cell culture substantially advanced the study of healthy and diseased tissues and enabled the engineering of physiologically relevant models of human tissues[18,19], such as organoid systems.

On the basis of lessons learned from human tissue culture methods, the 3D context of microorganisms — mucus — must be considered in the development of microbiota culture models. The mucous barrier (mucous gel) provides the specialized nutrients and spatial differentiation necessary to maintain microbial species diversity and coexistence[20]. The incorporation of mucus into in vitro microbial culture systems has the potential to facilitate viable multispecies culture systems and increase the number of culturable species.

The use of mucus and soluble mucins for in vitro culture systems has been limited by the difficulty of acquiring fresh mucosal samples and of generating a sufficient quantity through the culture of mucus-secreting cells.

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Box 1 | **The microbiome**

The human microbiota constitutes the vast majority of the 10–100 trillion microbial organisms inhabiting the human body. This microbial community is composed of a variety of different organisms, including bacteria, archaea, fungi and viruses[3]. It has been estimated that 400–1,000 different bacterial species coexist in a delicate equilibrium. However, microorganism–microorganism and microorganism–host interactions are still poorly understood[5,174]. Many of the organisms in the microbiota are not cultivable in vitro, that is, they do not grow in standard culture media[175,176]. Thus far, the microbiota has been mainly characterized using DNA-based analysis of specific microbial markers, including 16 S ribosomal RNA (rRNA) genes, 18 S rRNA genes or other marker genes and genomic regions, amplified and sequenced from biological samples[177].

Microbiota and microbiome

In the current literature, the terms microbiota and microbiome are used interchangeably, even as synonyms[168,178–180]. However, microbiome has been previously defined as a distinct concept, for example, the pool of genomes of the microbiota members[5] or the pool of microbiota organisms and their genes[181]. Owing to the availability of powerful genomic technologies, most studies of microbiota are specifically based on their genes and genomes, and therefore, both terms — microbiota and microbiome — have become operationally similar concepts.

Microorganisms

Microorganism is a catch-all term that includes bacteria, viruses, fungi and other microscopic organisms.

Processed mucins isolated from pigs and cows are commercially available; however, industrial-scale protein isolation can damage the structure of mucins and reduce their gel-forming functionality[21,22]. Alternatively, synthetic mucin mimetics can be designed to replicate the characteristics of native human mucus.

Synthetic mucins can be used to recreate and investigate how mucus can suppress microbial virulence and promote species diversity. Well-defined mucin-inspired in vitro systems provide powerful tools for the systematic analyses of microorganism–mucus interactions, enabling studies of single-species behaviour, community interactions and human–microbial co-culture.

Mucus houses the microbiota

The human body produces more than a litre of mucus per day, lining all surfaces in the body that are not covered by skin — over 2,000 ft^2 (FIG. 1a). Mucus is a hydrogel composed of gel-forming mucins, salts, lipids, proteins and immunological factors with varying concentrations depending on function and localization[23]. The canonical role of mucus is to lubricate, hydrate and protect epithelial surfaces. The properties of mucus change in response to pH[24]; mucus becomes thicker in acidic conditions, for example, to protect the stomach epithelium from its own acid, and it becomes more lubricative in neutral conditions, for example, to aid in swallowing (FIG. 1a).

Mucus is also a major ecological niche for the microbiota, acting as the main interface between human cells and commensal microorganisms. Most of the trillions of microorganisms that live on or in the body reside inside the mucus of the digestive tract[25]. Many of these microorganisms are essential for human health, helping to digest food and build vitamins. Mucus forms a habitat in which beneficial microorganisms can thrive and benefit the body, and it protects the body against damaging microorganisms[26]. Among the beneficial organisms are also potentially pathogenic microorganisms, which are kept under control by mucus.

Therefore, a functioning mucous barrier is essential for health, and mucus dysregulation can lead to disease. For example, underproduction of mucus or a change in the properties of mucus can result in dry or thick mucus[27], which leads to unshielded and dehydrated epithelial surfaces that are prone to infection and wounding (FIG. 1b). Dysregulation of mucus can further result in dry eye syndrome[28,29], xerostomia (dry mouth)[30] and respiratory diseases, such as cystic fibrosis[31,32], asthma[33,34] and chronic obstructive pulmonary disease[35,36]. Moreover, mucin dysregulation is implicated in diseases of the digestive tract, including Crohn's disease[37], ulcerative colitis[38] and gastric[39] and colorectal[40,41] cancer. In general, loss of functionality of the mucin barrier makes the body more accessible for environmental toxins and pathogens, which can lead to chronic inflammation or infection.

In vitro studies with mucous gels, reconstituted from animal sources[22], have enabled the discovery that mucus is able to directly influence bacterial behaviour, making a fundamental contribution to our understanding of host–microorganism interactions in health and disease. Indeed, mucus can regulate cross-kingdom virulence through its ability to influence how microorganisms swim, settle and communicate with each other. Therefore, mucus can be considered a sophisticated bioactive material with powerful abilities to manipulate microbial behaviour and phenotypes.

Mucins

Mucins provide mucus with its functional properties. Mucins are glycoproteins that, when hydrated, form the mucous hydrogel. The elongated protein backbone assumes a brush polymer architecture, as it is densely grafted with sugar side chains, called glycans[42] (FIG. 2a).

Broadly, there are two classes of mucins: mucins that remain tethered to cell membranes and mucins that are secreted, usually by goblet cells[43]. Tethered, or transmembrane, mucins are found on many cell surfaces and play an important role in immune recognition[44]. Secreted mucins form the mucosal hydrogel and are found throughout the body (FIG. 1a); for example, in tears[45] (MUC5B), saliva[46] (MUC5B), lungs[47] (MUC5AC and MUC5B), the intestines[40] (MUC2 and MUC5B), the stomach[48] (MUC5AC and MUC6), the ear canal[49] (MUC2, MUC5AC and MUC5B) and the female genital tract[50] (MUC5B) (FIG. 1a). Transmembrane and secreted mucins have structural similarities, such as domains rich in Ser and Thr residues that are heavily *O*-glycosylated. However, tethered mucins have a cytoplasmic tail that keeps them anchored to the cell surface, and the larger secreted mucins form a gel. Gel-forming secreted mucins endow mucus with viscoelasticity and non-Newtonian flow properties[24,51].

Mucin structure and glycosylation

Several gel-forming mucins with structural variations exist; however, they share key structural features: a linear protein backbone with crosslinking domains and dense, complex sugar side chains[43] (FIG. 2). Gel-forming mucins contain over 5,000 amino acids and thus are

extraordinarily large and intricate compared with a typical human protein with 300–500 amino acids. Thus far, some features have not been well characterized. However, the structural commonalities of gel-forming mucins can be used to inform a generalized mucin model and to design a broadly applicable mucin mimetic.

Mucins contain several crosslinking domains (FIG. 2a). The best-characterized domains are disulfide bonds supplied by Cys residues, which crosslink mucin monomers to form dimers and larger-order structures. These long chains can entangle, forming the basis of the mucin gel network[22]. Additionally, hydrophobic Cys-rich sequences that engage in reversible crosslinking are dispersed across the polymer[23]. Finally, mucin polymers also contain several von Willebrand factor (vWF) type C and D domains, which are *N*-glycosylated domains formed by disulfide bonds of Cys groups within the same region. These domains, which are common in blood clotting factors, are thought to engage in reversible aggregation akin to crosslinking[52,53]. This collection of covalent and reversible crosslinking domains throughout the protein backbone establishes the dynamic mucin gel.

Approximately 80% of the weight of mucins comes from glycosylation. The protein backbone is mainly composed of tandem Ser–Thr–Pro-rich (STP, sometimes PTS) domains, of which half of the residues are the hydroxy amino acids Thr or Ser (FIG. 2a). These provide initiation sites for *O*-glycosylation, often called mucin-type glycosylation, because of its strong association with the characteristic structure of the protein[54]. First, *N*-acetylgalactosamine (GalNAc) is α-linked to a hydroxy amino acid. Unlike in human *N*-glycans, there are no known amino acid sequences that mark the initiation of these *O*-glycan structures — a reaction that is catalysed by at least 21 enzymes[55]. Once the base sugar Thr/Ser-GalNAc is established, glycosyltransferases build the conserved core motifs and elongate them to form complex branched structures with distinctive and diverse terminal modifications[56] (FIG. 2b). These glycan cores and their extensions are composed of two hexose sugar monomers, fucose and galactose, and the amino sugar monomers GalNAc, *N*-acetylglucosamine (GlcNAc) and *N*-acetyl neuraminic acid (sialic acid, NeuNAc) (FIG. 2a). α-Glycosidic and β-glycosidic bonds on different sites of these monomers quickly build up combinatorial complexity, and bond orientation is an important component of the final functionality of the glycan[57] (FIG. 2c). There are also some mannose-rich *N*-linked and *C*-linked glycans in other regions of the protein, but they constitute only a small fraction of the total sugar mass[54].

Over 200 different *O*-glycan motifs can be found in mucins, each with a potentially unique regulatory capability[58]. The regulation of mucin glycosylation is not yet fully understood; however, certain motifs have been attributed to specific mucins and disease states[59]. Mucin glycosylation patterns further vary among individuals, and blood-type-specific antigens have been identified in mucin glycan motifs[46] (FIG. 2b). The chemical structures of the glycans of gastric mucin have been resolved[58], but owing to the difficulty of isolating these glycans in sufficient quantities, their individual bioactivities cannot yet be profiled. However, comparisons can be made with human milk oligosaccharides[60], which are a pool of complex sugars. In contrast to mucin glycans that are tethered to a protein, milk oligosaccharides are soluble and terminated with lactose at the reducing end. Milk oligosaccharides perform various biological functions, such as inhibiting the growth of some bacterial species[61], and they share structural homology with mucin glycan[62]. The homology of mucin glycans to human milk oligosaccharides and blood antigens suggests that they may also be involved in various biological activities.

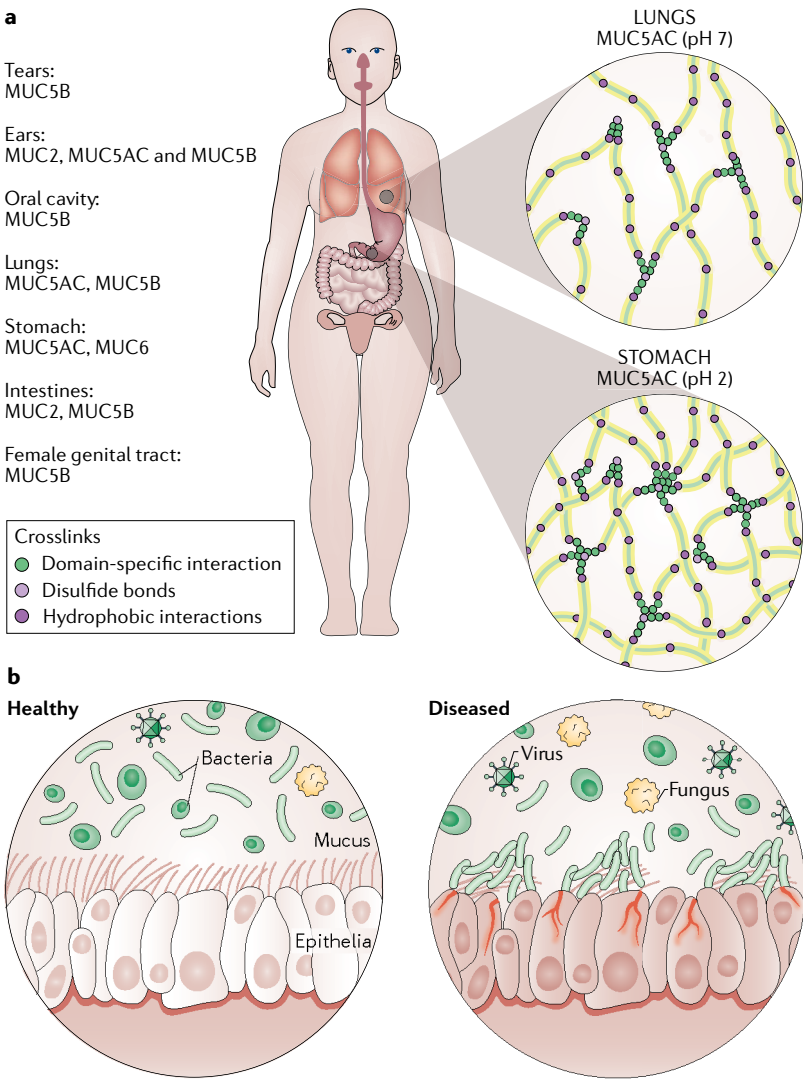

Fig. 1 | **Mucus houses the microbiota. a** | Mucus, which is made of gel-forming mucins (MUC), covers all non-keratinized epithelial surfaces of the body. Mucins form gels through covalent and reversible crosslinking, adapting to the different regions of the body. In low-pH environments, for example, in the stomach, electrostatic repulsion of the sugar side chains of mucins is reduced, causing the gel to partially collapse and to become thicker. **b** | Mucus promotes healthy interactions between microorganisms and epithelial cells. Mucus dysregulation is associated with increased susceptibility to viral infections, respiratory allergies, cystic fibrosis, irritable bowel disease, gastric ulcers, cavities and certain forms of infertility. Panel **b** is adapted with permission from REF.[22], Annual Reviews.

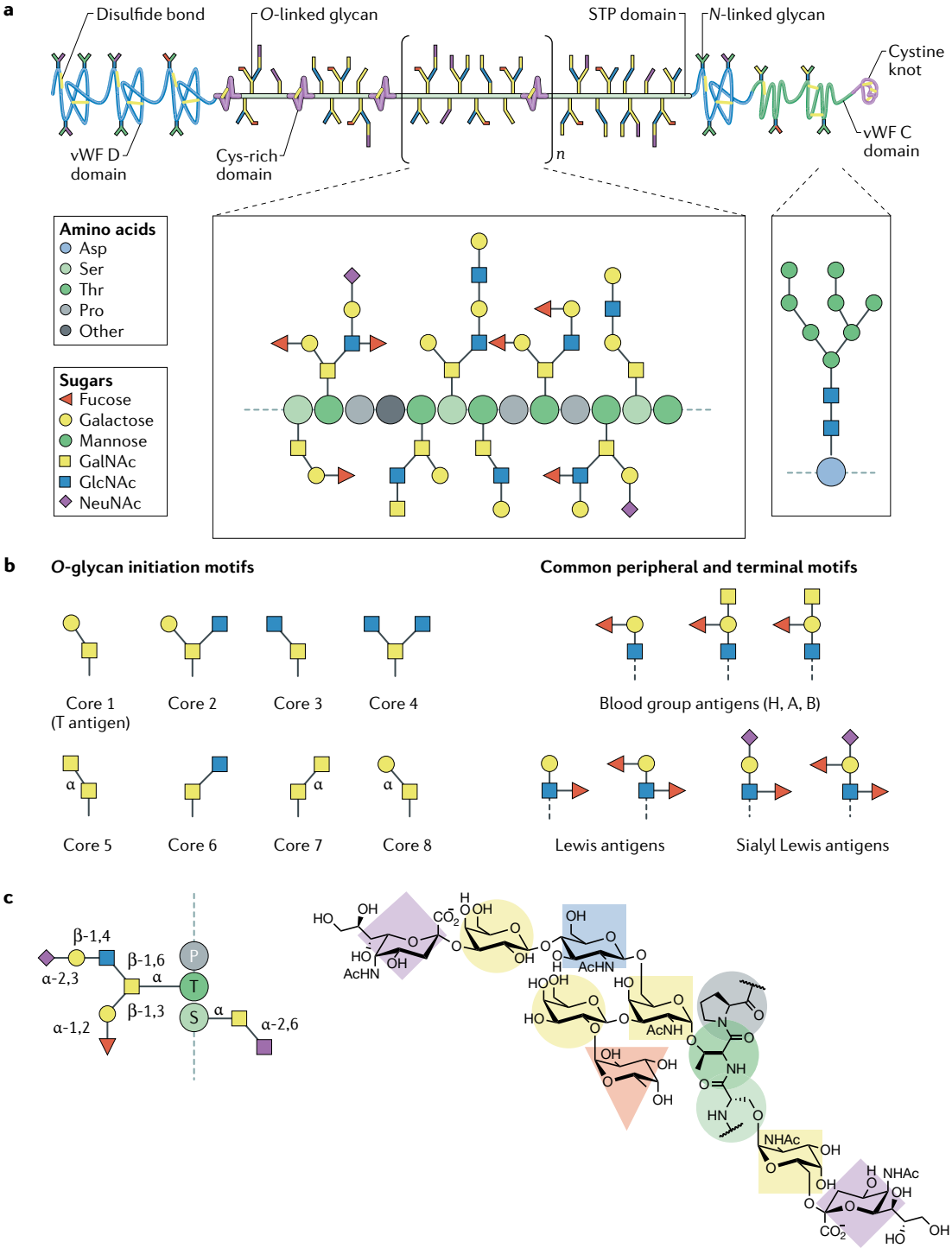

Fig. 2 | **Mucin glycoproteins contain a library of complex glycans. a** | The general structure of secreted mucins is a brush polymer made of Ser–Thr–Pro-rich regions, called STP domains. Complex *O*-glycans are attached to Ser and Thr in these domains. In this schematic, representative structures of isolated gastric mucins are shown[58], because the exact sites of glycosylation on the proteins are not yet determined. The STP domains are interspersed with hydrophobic Cys-rich regions that provide reversible crosslinking sites. The carboxyl terminus of each mucin contains a cystine knot that forms disulfide bridges with other mucin molecules. The STP domains are further flanked by von Willebrand factor (vWF) type D and C domains that can also engage in reversible interactions between different polymers akin to crosslinking. Some Asp residues in the vWF domains are glycosylated with *N*-linked sugars. **b** | Mucin contains eight core glycan structures that initiate all *O*-glycans. Mucin further displays common sugar-based motifs, such as blood group antigens and Lewis antigens. **c** | The *O*-linked glycans on mucins are predominantly made of five sugar monomers, starting with an α-linkage of the amino sugar *N*-acetylgalactosamine (GalNAc) to Ser or Thr. Complex glycans are then built off the initial sugar, with varying monomer identity and linkage patterns, affecting the bioactivity of mucin. GlcNAc, *N*-acetylglucosamine; NeuNAc, *N*-acetyl neuraminic acid. Panel **a** is adapted with permission from REF.[23], Elsevier.

Mucin–microorganism interactions

Mucus not only shields the body from potential pathogens but also plays an active role in shaping the behaviour of commensal microorganisms. Mucus has evolved the ability to mitigate damage caused by pathogenic microorganisms without killing them. The mechanisms of how mucins influence microorganisms and immune functions are currently being investigated, and several mechanistic hypotheses have been proposed, which need to be considered in the design of functional mucin mimetics (FIG. 3).

Barrier

Mucins regulate the transport of foreign bodies to epithelia by acting as a size exclusion barrier and by selectively binding targets[63] (FIG. 3a). Mucin glycans display many antigens that act as decoys for epithelial surfaces, trapping microorganisms in the gel. Bacterial adhesion depends on the identity of mucin antigens, which vary among individuals and mucin types[64,65]. Mucosal turnover then prevents infection by clearing old mucus and trapped microorganisms[66].

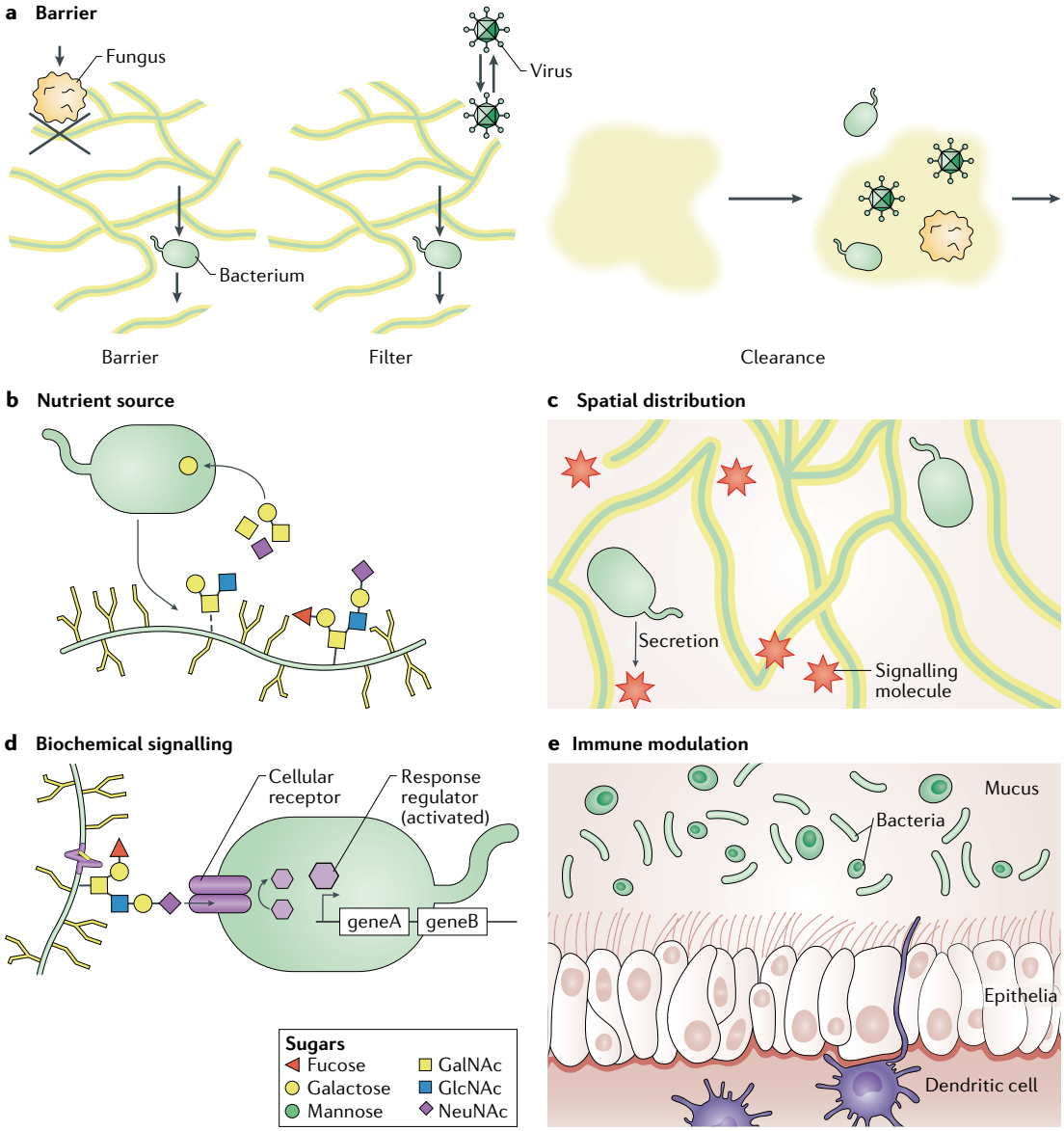

Fig. 3 | **Mucin gels influence microbial phenotypes. a** | Mucus is a selective barrier that protects the epithelium and regulates the exchange of nutrients, toxins and living cells between the body and the environment. The small gel pore size can prevent the penetration of microorganisms and large foreign bodies. Mucin is negatively charged and displays hydrophobic domains; therefore, mucins can selectively bind certain viruses, peptides and other moieties, acting as a filter. Mucus can further trap foreign bodies, allowing them to be cleared and flushed out of the body, while goblet cells produce new mucins. **b** | Some bacteria can consume mucin glycans, using them as a nutrient source. **c** | Mucin gels separate microorganisms. It is hypothesized that mucin gels can affect the diffusion of signalling molecules, affecting microbial communication by changing local molecule concentrations. **d** | Mucins display a large number of glycans that could directly signal to microbial receptors, triggering transcriptional responses to attenuate virulence. **e** | Without crossing the epithelia, dendritic cells sense and are stimulated by mucin antigens. GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NeuNAc, *N*-acetyl neuraminic acid.

Nutrient source

Mucus is thought to serve as a source of nutrients for commensal microbiota[60,67,68] (FIG. 3b). Several bacterial species produce glycosidases, which are enzymes that can cleave sugars off glycoproteins, allowing microorganisms to consume mucin glycans[69–72]. This exchange of mucin glycans between the mucus and microorganisms may promote the longevity of commensal species by giving them a competitive advantage[73–75]. However, many species are not able to produce such enzymes, and, although they can consume released sugars[68], mucus may impact them in other ways.

Spatial distribution

The mucin gel creates a complex 3D matrix that can shape the spatial distribution of cells and solutes, thereby influencing the physiology of and interactions between microbial cells. Mucin-rich gels, in the digestive tract, for example, have a pore size of approximately 200–500 nm, which is smaller than the average length of a bacterium (1 μm)[76,77]. Therefore, microorganisms are separated in the mucous gel, which promotes niche creation and differentiation in microbial communities[78].

Moreover, chemical gradients are established, similar to in the extracellular matrix of mammalian cells. These gradients can affect bacterial physiology by altering local concentrations of quorum-sensing molecules and other environmental signals (FIG. 3c). Quorum sensing describes the process by which bacteria secrete, sense and respond to soluble signalling molecules[79]. The formation of biofilms, production of toxins and sharing of genetic material — behaviours associated with increased pathogenicity — are all modulated by the concentration of signalling molecules[80–83]. For example, it has been shown that the spatial separation of *Pseudomonas aeruginosa*, mediated by mucus, can prevent the opportunistic pathogen from sensing signalling molecules, which may potentially reduce its virulence in healthy lungs[84].

Quorum sensing varies among species and can range from sensing small molecules to sensing peptides[85], and can be species-specific or enable cross-species interactions[86]. It is possible that mucus discriminately binds signalling molecules to favour particular species or that microbial separation interferes with interspecies sensing to prevent competition. More research is needed to fully understand the impact of the mucin matrix on bacteria; however, mucus may adopt an active role similar to that of the mammalian extracellular matrix in affecting cellular behaviour by defining spatial gradients of nutrients and signalling factors.

Biochemical signalling

In addition to providing structural definition, mucins provide biochemical signals through their glycans that can impact microbial behaviour, for example, by mitigating bacterial virulence and promoting species coexistence (FIG. 3d). Glycans mediate many intraspecies and interspecies interactions[87] and, when grafted, can enable receptor clustering, which can induce changes in cellular phenotypes[88]. Without *O*-glycans, the mucin backbone is not able to mitigate bacterial virulence[89], indicating that *O*-glycans play a key role in affecting cellular behaviour. Indeed, variations in mucin glycan identities in patients has been shown to alter the effect of mucus on bacterial virulence[90].

Mucins strongly influence the phenotypes of commensal microorganisms in vitro. Mucins in their native structure can be isolated from animal samples and reconstituted into gels for use in biological assay[91]. For example, mucin gels can prevent several species, including *P. aeruginosa*, from producing biofilms[92–94]. Mucin gels can also reduce virulence traits in other species, such as *Candida albicans*[95], and promote the survival of competing species[96]. Interestingly, although mucin gels suppress the virulence of these microorganisms, they do not limit bacterial growth, as seen with human milk oligosaccharides[61]. This fact suggests that mucins do not function as broad-spectrum antimicrobial agents but rather influence microbial physiology to mitigate virulence to the host.

Immune modulation

Finally, mucus participates in immune modulation, regulating the interaction between the human immune system and hosted microbiota[44,47,97]. Glycan recognition is an essential component of the immune response, enabling immune cells to distinguish self from non-self[98,99]. For example, bacterial cell-surface glycans contain foreign sugar monomers that can be recognized by immune cells[100]. Similarly, incompatible human blood types arise from different glycan motifs on the surface of red blood cells[101]. The *O*-glycosylated domains of mucins contain a variety of antigen motifs[46] (FIG. 2b) that endow mucus with the ability to affect immune responses[102,103]. For example, MUC2, a gel-forming mucin in the intestines, directly conditions immune cells, promoting homeostasis in the gut[104] (FIG. 3e). Additionally, MUC2 can regulate the production and activity of β-defensin 2, which is a peptide with antimicrobial and immune-modulatory functions[105]. Given the diversity of glycan structures and their importance in immunity, gel-forming mucins are expected to play an integral role in regulating the immune system and its effect on the microbiome.

Engineering synthetic mucins

Research on mucus and mucin mimetics has traditionally focused on their role as scaffolding polymers and their biophysical properties for lubricity and hydration and for mediating selective filtration. However, gel-forming mucins also have bioinstructive abilities. Therefore, application-driven design enables the creation of functional synthetic polymers.

Soluble gel-forming mucins have two main components that contribute to their function: the elongated backbone and grafted glycans[106]. Variations of these components determine the stiffness, charge and weight of the polymer, resulting in different pore sizes, viscoelastic properties, lubricity and pH-responsiveness of the mucin hydrogel. Glycan identities and densities affect mucin–microorganism interactions in terms of binding, nutrient supply and interactions with immune cells. The net charge and pore size strongly influence the penetration properties of the mucous barrier. Therefore, the microscopic and macroscopic properties of mucin hydrogels can be tuned by varying each of these design factors.

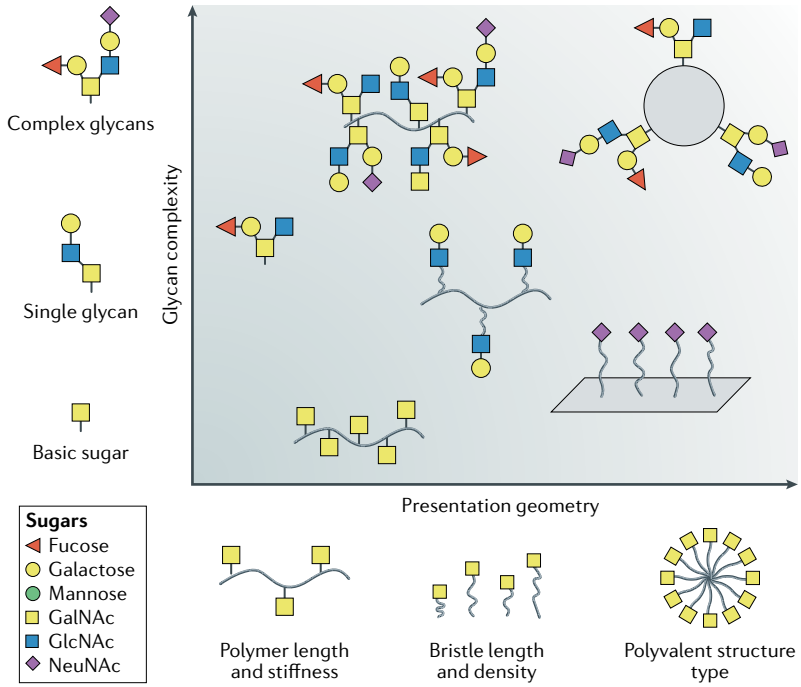

Fig. 4 | **Synthetic mucins.** Mucin-inspired agents can be synthesized with different architectures, backbones, crosslinkers and sugar moieties to tailor their properties and functions. Depending on the desired application, mucin mimetics can be engineered with different levels of complexity. GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NeuNAc, *N*-acetyl neuraminic acid.

The composition and architecture of mucin mimetics determine their function (FIG. 4). For example, to target a certain bacterial species, particular glycans need to be engineered and grafted on a hydrogel. In this case, the macroscopic rheological properties of the gel may be less important. By contrast, if the goal is to produce a lubricative material, the specific identities of sugar side chains are less important. Glycans can still be included in the polymer, because they increase hydration and can reduce friction[107], but alternative hydration strategies, such as the inclusion of hyaluronic acid[108], may also be sufficient as long as the hydrogel exhibits the desired rheological behaviour[109].

Direct synthesis would theoretically enable the replication of the whole mucin structure and its biological properties, but the structural complexity of mucin, particularly the diversity of glycans, makes direct synthesis currently impossible. However, the complex molecule can be broken down into structural units to understand the contribution of the different mucin components to its function and activity. This approach allows the rational and systematic design of mucin mimetics.

Engineering the backbone

Non-peptide mucin substitutes. The development of mucin substitutes initially focused on polymers that match the mechanical and physical properties of mucin and that exhibit a similar barrier-forming and lubricative behaviour[110]. Various polymers, such as carboxymethyl cellulose[111,112], hyaluronan[108], guar gum[113], the block copolymer polyethylene glycol–polylactic acid (PEG–PLA)[114], methacrylamide-based polymers (for example, poly(methyl methacrylate) (PMMA))[115,116] and poly (*N*-acryloyl D-glucosamine)[117,118], have been explored as mucin substitutes for mucoadhesion[119] and lubrication[109]. The diversity of these structures illustrates that there are many approaches towards building a lubricative, adhesive and hydrating polymer-based hydrogel.

However, these polymer mimetics have generally not been tested in microbiological assays. The functionality of mucin is dependent on its glycosylation[89], and non-glycosylated hydrogels are not able to recreate the ability of mucin to subdue microbial virulence[93]. Biological entities, such as viruses and bacteria as well as mammalian cells, primarily interact with the sugar side chains of mucins and not directly with the backbone. Therefore, easily synthesizable synthetic polymers have the potential to become relevant in microbiological contexts when grafted with biologically active glycan motifs.

Polypeptide backbones. Peptide-based mucin backbones can be chemically recreated with the idea that structural similarity reproduces functionality. Using a polypeptide backbone overcomes some concerns of biocompatibility for potential therapeutics, and native sugar linkages ensure that the chemical basis of microbiological interactions, such as nutrient use, are preserved. The mucin protein sequence contains many different peptides; however, for the design of a mucin-like glycopeptide, the most important components are the hydroxy amino acids Thr and Ser, which form the native bonds to α-GalNAc, initiating *O*-glycosylation. For example, a polymer can be synthesized by linking sugars to Thr residues in Ala–Thr–Ala tripeptides, followed by polymerization of the peptides[120]. Using this method, a glycopeptide can be engineered with a structure similar to that of STP domains; however, the synthesized polymer pool has high polydispersity, and the polymers have low molecular mass.

Alternatively, α-amino acid *N*-carboxyanhydride (NCA) polymerization can be applied to create long polypeptides with a molecular mass similar to that of mucin, low polydispersity and high yield[121]. In this method, the native linkage of α-GalNAc to Ser NCAs is first created before undergoing polymerization, which results in the production of poly(α-GalNAc-Ser). Non-glycosylated Ala NCAs can be incorporated into the polymer, such that monosaccharide functionalization directly correlates with the persistence length of the polymer. Thus, the elongated, highly functionalized structure of mucins can be recreated. These polypeptides are an important step in creating synthetic, glycosylated mucin backbones, but they do not form gels.

Creating gels. Mucin hydrogels have self-healing properties, enabled by the reversible crosslinking interactions in the gel network[122]; however, the exact mechanism of self-healing remains elusive thus far. Numerous methods have been developed to create functionalizable hydrogels[123–125] with a variety of mechanical properties relevant to mucin mimetics[126]. Copolymers can be used to integrate gel-forming functional groups into a mucin-mimetic backbone. For example, Cys residues can be incorporated into polypeptides built from NCAs to

provide crosslinking domains[126], similar to the Cys-rich regions in natural mucins. Alternatively, other dynamic crosslinking functionalities, such as diol-boronic acid complexes[127,128] or nucleobase-mediated hydrogen bonds[129], could be used to create self-healing gels.

Engineering glycans

Mucins contain hundreds of complex glycans, each with potentially specific biochemical functionalities[130,131]. The exact biological activity of each mucin glycan has not yet been profiled, making it difficult to design mimetics based on specific oligosaccharide functions. However, the biological activities of many homologous glycans, such as human milk oligosaccharides and blood type antigens, are known[132,133]. Such homologous structures can inform the design of functional glycans.

Basic sugar design. A single sugar can be sufficient to achieve a desired function if the target is well defined[134] (FIG. 4). For example, sialic acid is a mucin sugar monomer with well-profiled functions[135]. Mucins can bind viruses as a decoy for cell surfaces, enabled through the display of sialic acid at the end of their branched glycans, mimicking the chemistry of cell-surface glycans[136]. Inspired by this protective activity, brush polymers can be designed with sialyllactose exposed at the end of PEG side chains; this approach makes the sialic acid accessible for viral binding, reducing the infection of human cells in vitro[137]. In this approach, the polymer design is motivated by the biological function, and thus the key design variables, that is, the side chain length and grafting density of sialic acid, can be explored to maximize viral adhesion[138,139]. Alternatively to the brush polymer structure, liposomes displaying sialic acid can also prevent viral binding to red blood cells in vitro[140].

Complex sugar design. More complex glycans are often needed to create a specific biological function (FIG. 4). The creation of collections of glycans using purely synthetic approaches is difficult owing to the combinatorial number of potential structures and stereochemistries. Solid-phase synthesis has the potential to allow the creation of certain target structures, although this method requires access to advanced facilities[141].

Alternatively, glycosyltransferases can readily perform site-specific and stereoselective reactions without the need for extensive protections and deprotections. For example, recombinant sialyltransferase can be used to add sialic acid onto a galactose-displaying polymer, recreating a common terminal motif in mucin glycans[120]. Using this approach, an α-GalNAc-initiated backbone can be exposed to a series of glycosyltransferases that can then build the core motifs and larger glycans. On a larger scale, glycosyltransferases can be applied to build libraries of complex glycans[142], which could be grafted onto a desired synthetic backbone.

Synthetic biology

An attractive approach to generating mucins is the expression of the entire mucin gene together with relevant glycosyltransferases. Although systems capable of expressing long proteins such as mucin have not yet been engineered, recombinant cellular expression systems can be used to create short mucin-like glycoproteins with complex glycan structures, taking advantage of natural glycosylation processes. For example, human cell lines can be used to express structural fragments of mucin to engineer proteins that can bind pathogens; however, the glycan profiles of these proteins are undefined, as glycosylation relies on endogenous enzymes[143,144].

Short fragments of the mucin backbone can also be expressed in non-human cell lines in parallel with the expression of recombinant human glycosyltransferases. This approach allows the fabrication of glycoproteins that contain several of the most predominant glycan structures of mucin[145,146]. These glycoproteins can bind to microorganisms and their toxins as decoys[147,148], but the current variability in the glycan structures makes structure–function analyses difficult. Additionally, these proteins contain only portions of the mucin backbone, mainly the amino and carboxyl termini, and only small sequences of the glycosylated domains. However, they have advanced our understanding of mucins, elucidating the contribution of specific protein domains in gel formation[149] and secretion[150].

Progress in synthetic biology will enable tighter control of expression systems for the production of precisely defined mucin-mimetic glycoproteins. For example, mammalian cell strains can be engineered that can produce glycoproteins with the *O*-glycans restricted to the *α*-GalNAc initiator, which creates a clean starting point[151]. Series of recombinant enzymes that are known to encode the core motifs of mucin can then be transfected into the same cell lines to build up desired structures[152]. Such systems are often limited by a small yield, but advances in systems engineering will likely lead to an increase in production and consistency. Recombinantly produced grafted complex glycans can inhibit the binding of pathogens to their targets, and further development of these approaches will enable the recreation of other biochemical functionalities of mucin, such as its influence on microbial phenotypes.

Decoding mucin complexity

The creation and characterization of mucin-mimetic materials also enable the investigation of the functions of mucin polymers. The structure–function relationship of mucins is still a matter of ambiguity but may be clarified through the functional profiling of mucin-mimetic polymers.

To decode the complexity of the *O*-glycans of mucins, tools are needed that can systematically dissect glycans to allow top-down analysis. Moreover, the chemical synthesis of polymers consisting of glycan components enables the assessment of the role of each core complex and common modifications, such as sialylation and fucosylation. In addition to profiling the biological function of each glycan motif, the relevance of the different substructures needs to be understood. Progress in the synthesis of mucin glycans will enable systematic elucidation of the structural and chemical code underlying mucin glycan functions.

The presentation geometry of glycans is also important for mucin function (FIG. 4). In the mucus polymer

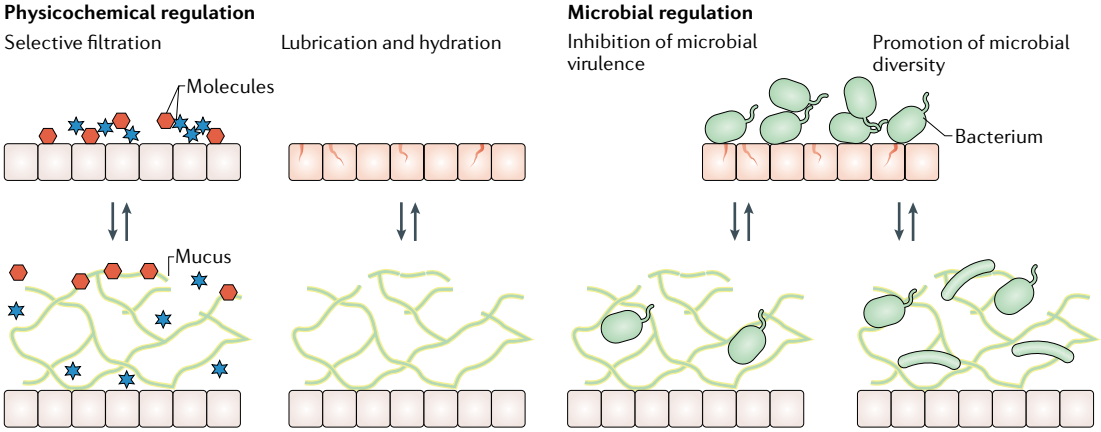

Fig. 5 | **Potential applications of mucin mimetics.** Mucin gels have several different functions in the body: they provide a barrier to prevent foreign bodies from reaching the epithelium, they hydrate and lubricate the epithelium, they attenuate microbial virulence and they can promote microbial diversity. Mucin-inspired agents could recreate and adapt these functions to study pharmacokinetics in tissue culture models in vitro, to create in vitro models of microbiota, for the treatment of topical infections and for the design of consumer products for dry eye and dry mouth conditions.

hydrogel, a spectrum of different glycans can be presented in minimal space to address a broad range of targets with individual specificity. Such a polymer configuration allows the creation of high local concentrations of sugars to increase affinity through polyvalency[153]. The advantage of this presentation can be assessed by exploring other configurations, for example, dendrimers, sheets or spheres, for glycan presentation[154–156] (FIG. 4).

Glycopolymers have been used to model the mechanisms of tethered sugar binding — interactions at the heart of mucin activity. For example, glycans found on mucins can be displayed on microarrays[157] to evaluate their functionality and interaction with lectins, which are proteins that specifically bind particular sugar domains[158,159]. Understanding the timing and mechanisms of lectin–glycodomain interactions gives insight into how glycan structures affect protein functionality[160–162], because lectin-type proteins are thought to be important in various glycan-based biological interactions[87], including mucin–microorganism interactions.

The development of gel-forming mucins can draw inspiration from cell-surface mucins by including functional analysis as an essential part of the design. MUC1 is a small, tethered mucin with a well-defined repeating *O*-glycosylation domain. MUC1 is widely studied, with a focus on glycosylation profiles, because of its association with cancer[140], which has prompted the production of recombinant MUC1 in cancer cell lines[163,164]. Alternatively, solid-phase synthesis can be applied to create an entirely synthetic, glycosylated MUC1 epitope[165], which can be used to mature a monoclonal antibody[166] and to study how glycosylation states impact antibody recognition of this important marker[167].

Outlook

Mucins have a profound effect on commensal microorganisms, and, therefore, synthetic mimics are required to support microbial cell culture in a more physiologically relevant context. Similar to the importance of the extracellular matrix for the structure and function of multicellular human tissues, the spatial organization and structure provided by mucus are needed to guide microorganism phenotypes and fate. The behaviour of microbial communities in vivo[168–170] substantially differs from their behaviour and growth in monoclonal laboratory cultures. The creation of synthetic mucin substitutes will assist biologists in studying human commensal bacteria in an environment that matches the in vivo matrix. Accurate in vitro models will improve our understanding of the mechanisms of microbial behaviour in a physiological — not laboratory — context.

Mucin also has to be considered in organoid models in vitro to decipher host–microbiome interactions[18,19,171]. Mucin mimetics can provide nutrients, regulate virulence and promote species coexistence to investigate how commensal and pathogenic bacteria influence human epithelia (FIG. 5). In addition, mucin mimetics can be used to establish spatial gradients, which are required to understand the pharmacokinetics of drug absorption through the gut and lungs.

On the basis of the regulatory role that mucus plays in the body, synthetic mucin-inspired agents could be used as therapeutics (FIG. 5). For example, dry eye syndrome and dry mouth syndrome, which are caused by an underproduction of mucus, could be treated with mucin-mimetic materials that are able to recreate the lubricative properties of mucus[112,172] and, in the case of dry mouth syndrome, the ability of saliva to protect against cavities[173]. Mucin can further prevent infectious bacteria[94] from forming biofilms; therefore, mucin mimetics could be applied as treatments for infections to hydrate and protect epithelia while reducing the pathogenicity of microorganisms.

Therapeutics based on natural or engineered mucin glycans could provide an alternative to traditional antibiotics. Mucins limit infections by domesticating existing virulent populations, rather than by trying to eliminate them. This domestication bypasses the selective pressure that drives drug resistance in microbial populations. Mucin-inspired treatments that emphasize the sustained

interactions between microorganisms and the host could complement antibiotics for the treatment and prevention of infectious diseases. Alternatively, they could be used as prebiotics to positively influence the environment and thereby augment the beneficial effects of the human microbiota.

Mucus plays an essential role in regulating the interactions between human tissues and the microbiome. The exact mechanisms of its influence remain elusive thus far; however, it is known that mucin dysregulation can cause disease. To study mucin–microbiome interactions in vitro and thus to understand the impact of mucin on microbiota, synthetic mucin mimetics are required to design relevant in vitro models.

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Competing interests
The authors declare no competing interests.

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