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Perspective

Mucus-Inspired Dynamic Hydrogels: Synthesis and Future Perspectives

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ABSTRACT: Mucus hydrogels at biointerfaces are crucial for protecting against foreign pathogens and for the biological functions of the underlying cells. Since mucus can bind to and host both viruses and bacteria, establishing a synthetic model system that can emulate the properties and functions of native mucus and can be synthesized at large scale would revolutionize the mucus-related research that is essential for understanding the pathways of many infectious diseases. The synthesis of such biofunctional hydrogels in the laboratory is highly challenging, owing to their complex chemical compositions and the specific chemical interactions that occur throughout the gel network. In this perspective, we discuss the basic chemical structures and diverse physicochemical interactions responsible for the unique properties and functions of mucus hydrogels. We scrutinize the different approaches for preparing mucus-inspired hydrogels, with specific examples. We also discuss recent research and what it reveals about the challenges that must be addressed and the opportunities to be considered to achieve desirable *de novo* synthetic mucus hydrogels.

1. INTRODUCTION

Efforts to synthetically mimic biological materials can help us gain in-depth understanding of biological processes and develop artificial tools for advancing bioengineering research.[1−3] The chemistry required to mimic biological components is of course an important foundation for constructing an artificial version of them.[4] Mucus, a biological dynamic hydrogel composed[5,6] of different glycoproteins called mucins, occurs at the biointerfaces that cover the underlying epithelial cells of various organs, such as the eyes, the airways, the gastrointestinal tract, and the reproductive tract. In humans, ocular mucus protects the eyes from bacterial infections and dryness; respiratory mucus helps to protect us from foreign particles entering through our airways; gastrointestinal mucus protects us from acidic gastric fluids in the stomach and from pathogens entering through the mouth; and cervical mucus protects women from sexually transmitted pathogens.[7−10] Depending on their location, these types of mucus function not only to protect organs from infections caused by external foreign particles but also to hydrate them, lubricate the epithelia, and help nutrients, gases, and proteins pass to and from the underlying epithelium to enable their functions.[11]

The efficacy of mucosal hydrogels in these biological functions depends on their dynamicity, and once defects appear in these gels, they fail in their critical roles in maintaining human health.[12] In-depth knowledge of how biophysical properties of different types of mucus change over time and with health conditions, and of how these changes alter their functions, is essential to understanding these functions and would enable their relatively straightforward restoration to help treat disease. For instance, mucus in the respiratory tract is always in motion caused by the movement of cilia, and by this mucociliary clearance (MCC) mucus protects the underlying cells against infection by foreign pathogens.[13] However, respiratory diseases like cystic fibrosis (CF), asthma, and chronic obstructive pulmonary disease (COPD) render lung mucus so viscous that the ciliary sweeping force cannot move it.[14] Under these compromised conditions, MCC ceases and the thickened mucus becomes more hospitable to foreign particles and the infections they can cause.[15] Patients exhibiting this problem are rapidly rising in number, and these diseases rank among the most life-threatening. Furthermore, to achieve successful infection, respiratory viruses including SARS-CoV-2 must pass through the mucus layer by overcoming the mucus defense mechanism.[16] While available vaccines[17] are working with great promise to mitigate the Covid-19 pandemic caused by SARS-CoV-2, a basic understanding of the virus's mode of entry would point us toward the pathways to prevent its infection. Gastric mucus hosts trillions of microbiota that not only help in digestion but also keep us healthy.[5,18] In-depth knowledge of how of mucus regulates microbiota and enables their coexistence with the underlying epithelium is essential for a better understanding of these bacteria's biological functions. Besides, for efficient drug delivery, the administered drugs need to cross this protective mucosal layer to reach the underlying epithelial cells, and their success rate depends on how easily the drugs pass through this layer.[19,20] Extending our present knowledge may offer new opportunities for drug development in the area of mucosal drug delivery systems, e.g. for local administration. In addition, ovulatory mucus is essential to

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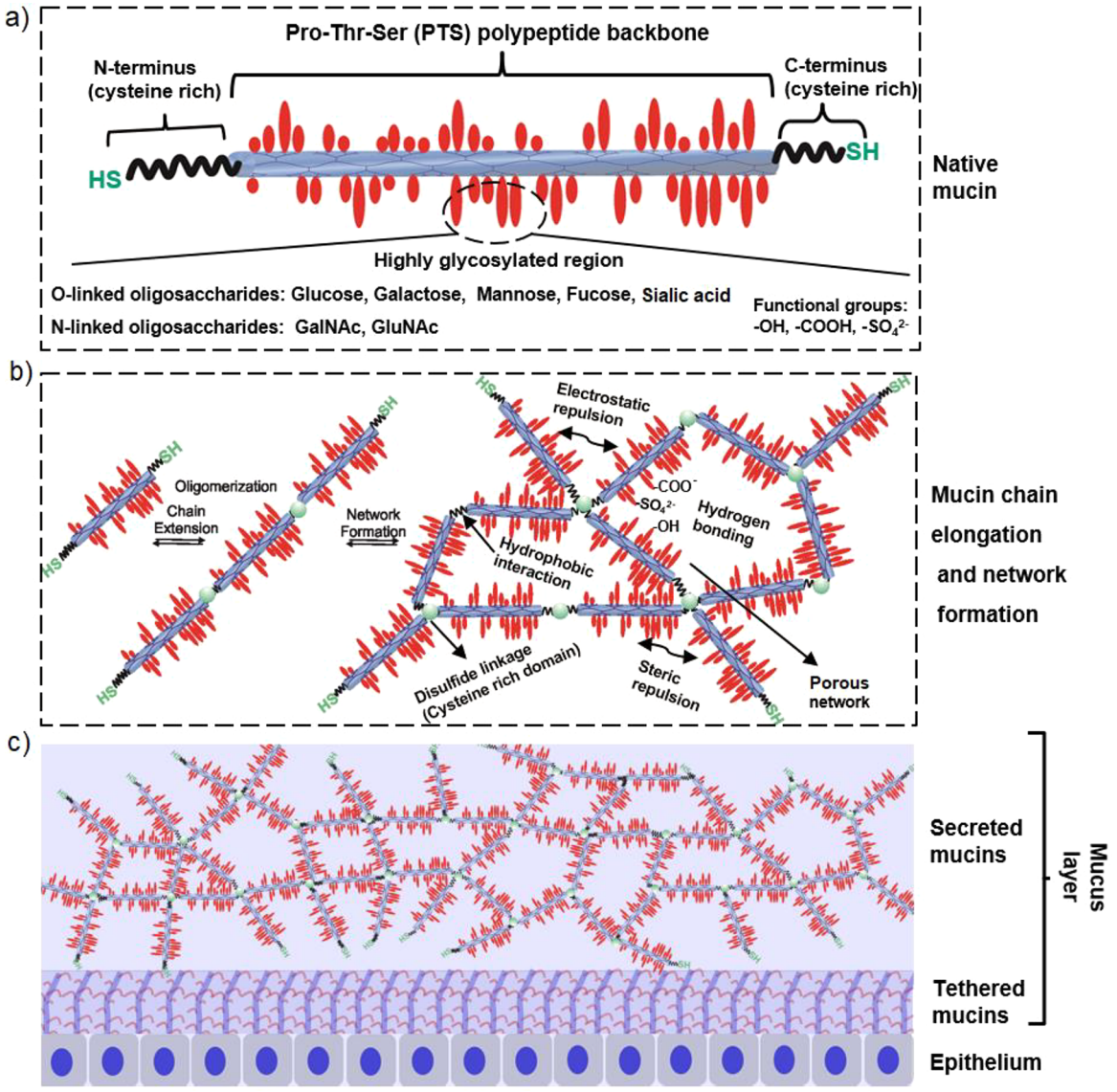

Figure 1. (a) Representative chemical structure of MUC5B, an important mucin present in respiratory mucus, together with its chemical functionalities. (b) Schematic illustration of the oligomerization of secreted mucin via formation of disulfide bonds and further cross-linking to form a three-dimensional cross-linked network, together with other noncovalent interactions responsible for achieving the network structure (Image credit: Dr. Wiebke Fischer). (c) Schematic representation of respiratory mucus, showing the positions of different mucins layered over epithelium cells. Tethered mucin is connected to the epithelial layer and packed densely, while secreted mucin forms a network structure.

successful fertilization, while nonovulatory mucus restricts semen from crossing the cervical mucus layer.[21]

With mucus playing such a wide array of critical roles throughout the human body, it is clear that the advancement of mucus research has much to offer, and this advancement will require a clear understanding of the properties of different types of mucus. In this context, different *in vitro*, *ex-vivo*, and *in vivo* mucus models have been developed to understand how drugs and foreign particles penetrate mucus.[22,23] The established models can help us understand the complex role of mucus hydrogels, but in general they suffer from one of two drawbacks: either suitable ingredients are not available in the quantities required for the bulk preparation of hydrogels, or the synthesized hydrogel cannot fully mimic the properties and functions of native mucus. Therefore, the large-scale study of mucus-mimetic hydrogels cannot proceed without the development of synthetic mucus that can be produced in sufficient quantities. In this perspective, we first highlight the basic chemical composition and structures of mucus hydrogel, along with the specific interactions that make it so complex and are responsible for its unique functions. Inspired by the physicochemical properties of mucus, we describe the different approaches to mimicking these hydrogels. We go on to discuss how researchers envision realistically mimicking mucus hydrogels in terms of their physicochemical properties as well as their functions. We conclude with a discussion of how synthetic mucus hydrogels could be used as artificial tools to advance research in this field.

2. MUCUS HYDROGELS: STRUCTURE AND FUNCTIONS

Mucus hydrogels are inherently complex in terms of their network structure, selective permeability, and specific biological functions. Therefore, a detailed basic understanding of these hydrogels is a prerequisite for any synthetic approach to

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Table 1. Overview of Different Human Mucus Types and Their Associated Mucins (G' Represents the Hydrogels' Storage Modulus, and G'' Represents Their Loss Modulus)[43−50a]

Mucus Types	Organ	Gel-forming mucins (Secreted mucins)	Bulk rheology (Pa)		Mesh size (nm)[b]	pH[b]
			G'	G''		
Respiratory	Lung, nose	MUC5B, MUC5AC, MUC19, MUC2	14.9 ± 9.2 (ref 49)	4.3 ± 2.7	100 nm to several μm	6.5−7.9
Gastrointestinal	Stomach	MUC5AC, MUC6	NA	NA	500 nm	1.0−4.0
	Intestine	MUC 2, MUC 6	26.7 ± 6.3 (ref 49)	8.1 ± 3.1	NA	5.5−7.5
Cervical	Vaginal tract	MUC5B, MUC5AC, MUC2, MUC6	20 ± 10 (ref 50)	5 ± 3	20−200 nm	5.4−8.2
Ocular	Eye	MUC2, MUC5AC, MUC5B, MUC6	NA	NA	550 ± 50 nm	7.3−7.6
Buccal	Mouth	MUC5B, MUC19	NA	NA	NA	6.5−7.5

[a]The presented rheological data are according to the corresponding literature. [b]Data are reproduced as shown by Ghosh and co-workers in ref 38. NA stands for "not available".

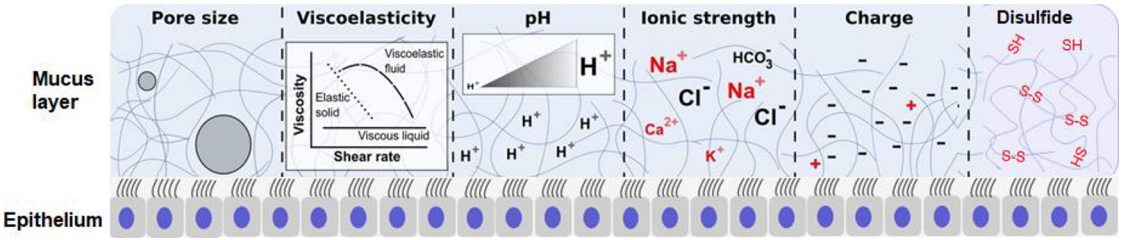

Figure 2. Physicochemical properties of mucus depend on its pore size, viscoelasticity, pH, ionic strength, charge, and thiol and disulfide concentration. These properties therefore significantly influence the barrier properties and other functions of mucus. Adapted with permission from ref 38. Copyright 2017 Elsevier B.V.

mimicking them. In this section we will discuss the precise composition and unique properties of mucus through the lens of a (bio)chemist.

2.1. Composition of Mucus Hydrogels. Irrespective of their origin, mucus hydrogels consist of ∼93−97% (w/v) water and ∼3−7% (w/v) solid residues that include glycoproteins, lipids, and mineral salts.[24,25] Mucin glycoproteins are the primary component for gel formation and the major contributor to the structural and functional characteristics of the mucus gel. The chemical characteristics of mucin glycoproteins are well-known and have been discussed elsewhere.[5,6,26−29] Generally, mucins consist of a high-molecular-weight (200−500 kDa) polypeptide backbone composed mainly of proline, threonine, and/or serine (PTS) amino acids, with extensive glycosylation in their side chains producing an average chain length of 100 to 500 nm. The glycosidic hydrophilic domains of mucin contain O-linked *N*-acetyl galactosamine along with N-linked sulfate-bearing glycans that are connected to the peptide backbone by their hydroxyl groups, whereas its nonglycosidic hydrophobic domain contains the cysteine residues (Figure 1a). Around 50−80% of mucins' dry weight is contributed by oligosaccharide, indicating the intense glycosylation present in mucus. The functional groups present on the oligosaccharide residues are sialic acid, L-fucose, galactose, low amounts of mannose, and sulfates, contributing to the overall charge of the mucins. In the human lung, the mucin layer, with an average thickness of ∼20−200 μm, is essentially distributed in two different sublayers (Figure 1c): the transmembrane mucins that connect to the epithelium by anchoring its hydrophobic domain, and the gel-forming mucins that are secreted as highly reversibly cross-linked structures.

The C- and N-terminals of these secreted mucins have cysteine-rich von Willebrand factor (vWF)-like domains, which allow for dimerization or multimerization through disulfide linkages to attain a very high-molecular-weight extended structure (Figure 1b).[30−32] The mucin chain elongation is facilitated by the electrostatic charge−charge repulsion together with additional steric interaction of bulky sugar residues with the protein's core. It appears that the extended polymer structure is in the range of 2−50 MDa in mass, with a length of ∼0.5−10 μm. With the context of this perspective in mind, we focus here only on the hydrogel-forming secreted mucins, excluding from our discussion the tethered mucins[33] that play important roles in cellular adhesion and signal transduction.

MUC5B, MUC5AC, MUC2, MUC6, and MUC19 are the secreted mucins present in human mucus samples. They differ from each other in their extent of glycosylation, cysteine-rich domains, protein sequences, and size (Table 1).[34] The properties and biological functions of different types of mucus differ based upon the different types of mucin present in them as well as these mucins' relative contributions in the case of mucus composed of multiple mucins.[35]

2.2. Rheological Properties of Mucus Hydrogels. Semiflexible secreted mucins form dynamic disulfide bonds[36,37] among themselves, and through this cross-linking process they become entangled to form a three-dimensional network structure. This network structure is further stabilized by diverse molecular interactions (Figure 1b) such as electrostatic forces, hydrophobic interactions, and hydrogen bonding, leading to a viscoelastic hydrogel.[38−41] Rheological properties are one of the key properties for hydrogel characterization. Bulk rheology of mucus is generally characterized by using cone-and-plate geometry; properties such as viscoelasticity, mesh size, and surface tension are measured. The bulk rheology largely focuses on two properties: (i) elastic or storage modulus (G'), which quantifies the gel's propensity to regain its original network structure after stress-induced deformation, and (ii) viscous or loss modulus (G''), which expresses the gel's ability to retard flow. These typically depend on the frequency of deformation. Based on the impact of frequency of deformation on hydrogels, they are divided into two categories: shear-thinning and shear-thickening hydrogels. The viscosity of shear-thinning hydrogels

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decreases upon increasing frequency of deformation, while higher deformation frequency increases the viscosity of shear-thickening hydrogels. At macroscale, the mucus hydrogel appears as a viscoelastic shear-thinning material that shows elastic behaviors under low shear strain, whereas under high shear strain it shows a viscous liquid nature.[42] At microscale, mucus hydrogel typically functions as a physical barrier, trapping pathogens and foreign particles and preventing them from reaching the epithelial cells beneath. Bulk rheological properties of mucus are essential to its proper macroscale functions, such as mucus clearance and lubrication. The rheological properties of mucus strongly depend on the composition and glycosylation of mucins as well as the presence of specific pathogens and antigens.[43−50] In the mucus network structure, the entangled mucin's polymers create various empty spaces, imparting a porous network structure to the mucus. In bulk rheology the average mesh sizes are calculated[51,52] assuming a homogeneous network structure throughout a sample of hydrogel. But in a practical sense the mesh size of mucus is highly heterogeneous (Figure 2), with an average size in the range of 20−1800 nm across different organs. Based on our literature survey, we summarize bulk rheological properties of different mucus in Table 1.[43−50] Microrheological studies therefore play an important role in characterizing biological fluids' local mechanical properties at a spatial resolution not achievable with bulk rheological techniques. In this context, particle tracking microrheology[53−55] (PTM) has been used extensively to gain insight into such properties as actual mesh size and the local environment inside hydrogels, traits that influence the diffusion scale of small drug molecules and pathogens across the mucosal barrier.

2.3. Selective Barrier Permeability. The permeability of different substrates through mucus networks is highly specific, depending on not only particle size but also several other parameters such as electrostatic interactions, hydrophobic interactions, hydrogen bonding, and other binding interactions (Figure 3).[56−58] Mucus allows nutrients to pass through it and allows gas exchange with underlying cells, while preventing the entry of foreign particles. The various functional groups of mucus interact with the reactive functional groups of particles, restricting their penetration through mucus irrespective of particle size.

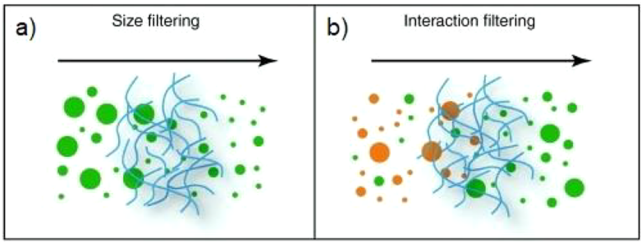

Figure 3. (a) Size filtering regulates mucus permeability: particles that are smaller than the mesh size of mucus are able to cross, whereas larger particles are rejected. (b) Interaction filtering regulates mucus permeability: interactions such as hydrophobic, electrostatic, hydrogen bonding and mucoadhesive interactions play the key roles for mucus penetration. A subgroup of particles (orange) show strong interactions with the mucus hydrogel and are trapped, whereas other particles (green) interact weakly and thus are allowed to cross. Reprinted with permission from ref 56. Copyright 2011 Elsevier Ltd.

For instance, in interactions based on what are termed mucoadhesive properties, the thiol groups of cysteine residue can interact with other materials, such as drugs or drug carriers, that contain thiol-reactive functional groups, helping these materials to adhere to mucosal surfaces. Understanding these properties is essential for mucosal drug delivery research,[59] whose success depends on how effectively drug carriers adhere to mucosal surfaces for sustainable drug delivery. Such insights will also assist in designing engineered nanoparticles that can cross mucosal barriers for successful mucosal drug delivery. This field has developed significantly, and the relevant research has been nicely documented.[60−62] The overall charge of mucus depends on the pH of the environment (Figure 2), which influences the charge-selective permeability (Figure 3). For instance, under normal conditions mucus is negatively charged owing to its negatively charged carboxylate and sulfate groups, so it shows electrostatic attraction to positively charged particles, thereby restricting their mobility. It is also reported that mucus penetration depends not only on nanoparticles' net charge but also on the spatial distribution of charges on the nanoparticles.[63]

2.4. Dynamics in Mucus Hydrogels. A dynamic hydrogel is a hydrogel whose presented covalent and noncovalent bonds are continuously changing among themselves to generate a dynamic situation in the hydrogels. Dynamic hydrogels with different types of dynamic bonds have been used widely in various emerging biomedical fields and have been reported elsewhere.[64−72] We will focus here on the covalent and noncovalent interactions that make a mucus hydrogel dynamic in nature. In a dynamic mucus hydrogel, the dynamicity strongly depends on the individual components, types of mucins present and their relative abundance, the number of thiol and disulfide groups, salt concentration, and the pH and charge of the environment. The highly complex properties and functions of mucus are regulated by the dynamicity of its hydrogels. Slight changes within the constituents of mucus can significantly alter its physicochemical properties and can have further consequences on its function as a selective barrier, a lubricant, and the body's first line of defense against infection. In the following discussion we will highlight how the biophysical properties of different types of mucus depend on health status, and how changes from healthy to diseased states can alter their functions.

For instance, in the respiratory tract, the viscoelastic properties of mucus facilitate the entrapment of foreign materials in the hydrogel network and are responsible for maintaining the mucociliary defense mechanism (mucociliary clearance, or MCC) (Figure 4b).[73] Studies have suggested that an optimal rheological profile for mucociliary clearance comprises an elastic modulus (G') in the range of 1 Pa and a viscosity of 12−15 Pa·s,[74−76] whereas, under disease conditions, G' is increased manyfold.[77] This is due to the formation of additional cross-links among the thiol groups of cysteine residues to form disulfide bonds (Figure 4a). Therefore, the MCC mechanism becomes restricted under disease conditions. Thornton and co-workers established[22] that, for respiratory mucin networks, their oligomeric mucin composition (MUC5AC, MUC5B) shows variation between healthy conditions and diseases such as COPD and cystic fibrosis. For instance, MUC5B predominates over MUC5AC in healthy mucus, whereas under disease conditions MUC5AC outstrips MUC5B in quantity.

The properties of cervical mucus are regulated by the secreted mucins such as MUC5B, MUC5AC, and MUC6. Human

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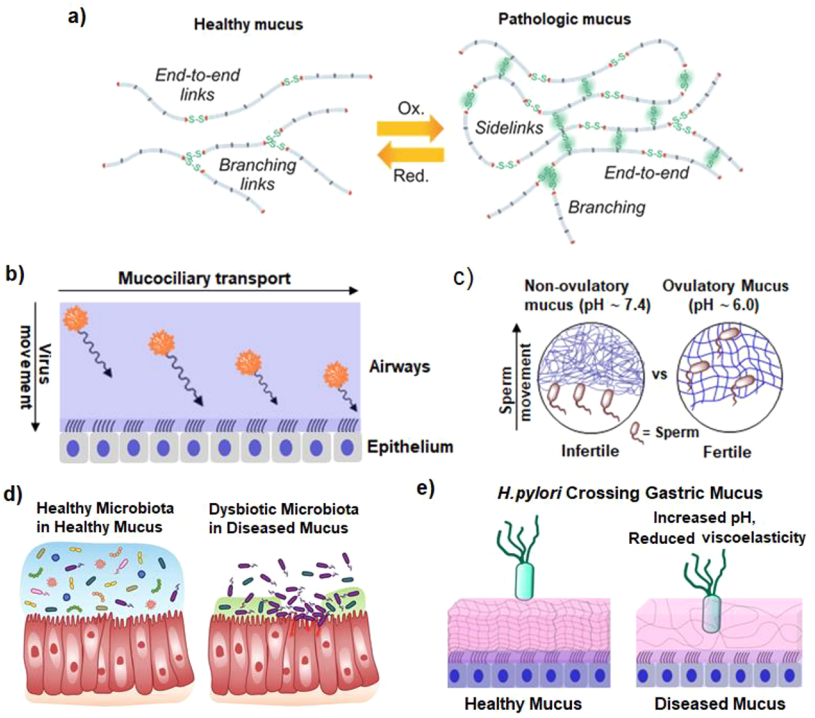

Figure 4. (a) Schematic representation showing oxidation-promoted transformation of healthy mucus to pathological mucus by the cross-linking of mucin's internal cysteine, via the formation of both end-to-end and side-to-side disulfide bonds. From [*Sci. Transl. Med.* **2015**, 7, 276ra27]. Reprinted with permission from AAAS. (b) Schematic representation showing the pathway of viral infection in the airways and the mucociliary defense mechanism. (c) Schematic showing ovulatory and nonovulatory mucus and their selective fertilization properties. (d) Schematic representation of the microbiota communities in healthy and diseased mucus. In healthy conditions microbiota move freely, whereas under disease conditions they attach to the epithelium cells. Reprinted with permission from ref 18. Copyright 2020 Federation of European Biochemical Societies. (e) This cartoon illustrates a possible mechanism by which *H. pylori* crosses the mucus gel to infect the underlying cells.

cervical mucus exhibits change in its network structural organization based on the menstrual cycle, selectively permitting entry of various pathogens and spermatozoa (Figure 4c).[78,79] Ovulation is accompanied by changes to the structure and glycosylation of mucus, or, more precisely, a decrease in the number of sugar residues containing sulfate groups and in the number of sialic acid residues, resulting in an increase in pH. The rheological properties of cervical mucus (Table 1) also strongly depend on the pH of the environment, which is connected with the menstrual cycle. Ovulatory mucus carries a pH of ∼6.0 and appears as a viscous liquid, whereas nonovulatory cervical mucus has a higher pH of ∼7.4 and appears as a semisolid.

The primary secreted mucin present in gastric mucus is MUC5AC. The mucus undergoes pH-dependent sol-to-gel transition, and a decrease in pH can increase the mucus's viscoelasticity owing to a reduction in the negative charges of the carboxyl groups on sialic acids. The thicker double layer of mucus in the stomach and colon functions as a protective barrier, defending the organ-lining epithelium from both pathogens and the acidic stomach environment. Apart from pH, other parameters such as electrostatic charge interactions and the presence of foreign particles influence the network structure of gastric mucus.[80−82] Mucus houses trillions of microbiota, and it is interesting that mucins' glycosylated structures not only supply bacteria with food but also allow them to swim freely in mucus hydrogel, preventing their aggregation to form bacterial colonies while also preventing them from being washed away by mucus defense mechanisms (Figure 4d).[5,18,83] Under healthy conditions, mucus controls these bacteria with specific glycan signals that not only prevent interaction among bacteria but also tune their movement so that none of them can cross the mucosal barrier to infect the underlying cells.[84,85] Ribbeck and co-workers extensively studied[5,18,83−85] mucus macrobacteria to understand the specific role of mucins' glycosylation patterns and spatial organization for such selectivity. Under compromised conditions this defensive control breaks down as mucus loses its ability to control the bacteria, enabling them to easily infect the underlying epithelium.[15] For instance, *Helicobacter pylori* is a well-known bacterium present in the gastrointestinal tract, where it is responsible for major inflammation.[86] To achieve cell entry, *H. pylori* increases the pH of its surroundings, eventually reducing the viscoelasticity of mucus hydrogels to facilitate entry (Figure 4e). Upon successful entry, it destroys the functionality of gastric mucus.

Secreted mucins are also present in ocular and buccal mucus (Table 1), which protect the eyes and mouth from bacterial infection and maintain the surface lubricity essential for their functions. Deficiencies of mucins in these types of mucus leads to dryness in the eyes and mouth. Research on this topic is progressing quite promisingly and has been well-documented.[87−90] However, our limited scope does not permit us to discuss these two mucus types in further detail.

The discussion above highlights the dynamic behavior and origin of mucus from the structural point of view. Greater overall insight into these properties will be essential to designing mucus-mimetic hydrogels.

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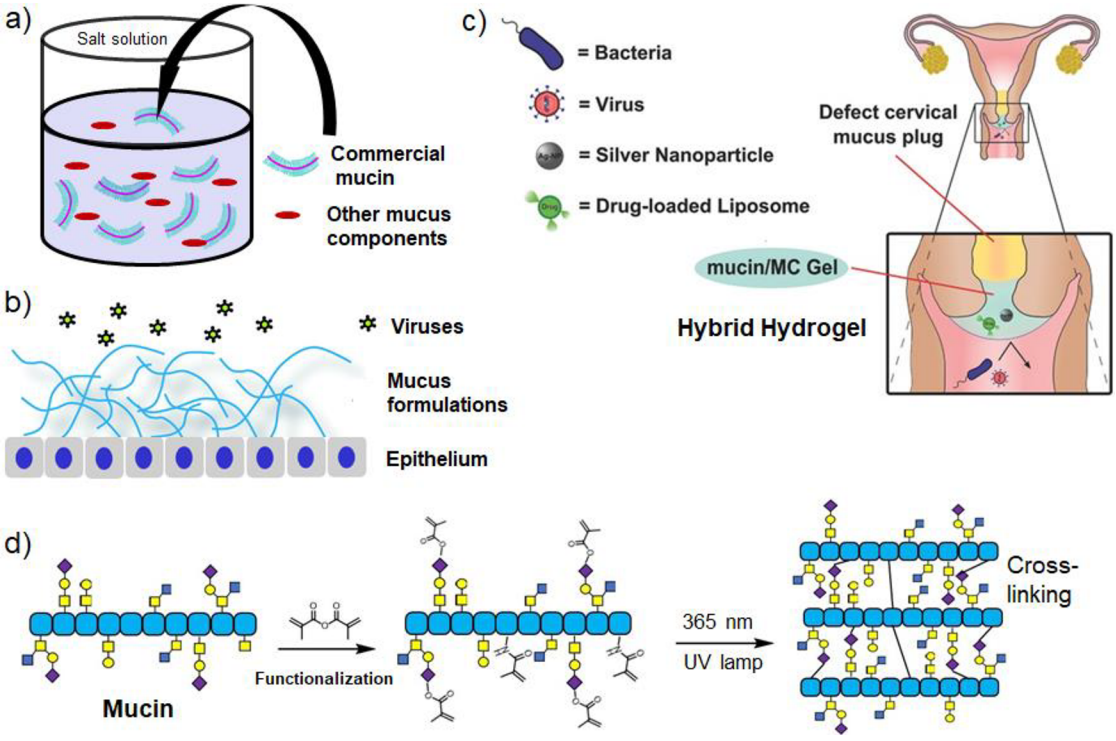

Figure 5. (a) Schematic showing preparation of mucus formulation by mixing commercially available mucins, other mucus components, and salt. (b) Schematic representation of in vitro infection assay using mucin formulation. Adapted with permission from ref 95. Copyright 2012 American Chemical Society. (c) Schematic visualization of hybrid mucin-methylcellulose (mucin-MC) gel applied to female reproductive system to protect from sexually transmitted infections. Reprinted with permission from ref 96. Copyright 2016 Wiley-VCH GmbH. (d) Chemically modified mucin (representative structure) for phototriggered network formation. Adapted with permission from ref 98. Copyright 2020 Wiley-VCH GmbH.

3. MUCUS-INSPIRED DYNAMIC HYDROGELS

Inspired by the physiochemical properties of mucus hydrogels, a series of mimetic versions have been reported in the literature. In this section we discuss these hydrogels, which can mimic the physiochemical and/or functional properties of native mucus. It is well understood that all of the covalent and noncovalent interactions present in mucus are reversible and that they change rapidly depending on health and redox status, making mucus hydrogel dynamic in nature. With this dynamic chemistry of mucus in mind, this perspective covers the reported hydrogels, which fall into two categories: hydrogels based on natural mucin, and mucus-mimetic synthetic hydrogels where dynamic disulfides serve as the cross-linking chemical bonds responsible for network formation. We divide the reported mucus-mimetic hydrogels into two categories: synthetic hydrogels using native mucin as a component (section 3.1) and synthetic hydrogels using nonmucin components (section 3.2).

3.1. Synthetic Hydrogel Using Native Mucin as a Component. *3.1.1. Mucus Formulations.* It is well-known that the properties of native mucus depend substantially on the types and proportions of mucins present in it. This relationship has inspired the idea of using native mucins to prepare mucus-inspired synthetic hydrogels in two types of formulation: one which is essentially a physical mixture of the exact components of native mucus, and another resulting from rehydration of purified native mucins. Generally, mucins are purified from their parent native mucus following a standardized extraction procedure.[91] Commercially available native mucins such as porcine gastric mucin (PGM) and bovine submaxillary mucin (BSM) have been widely used to prepare mucus-mimetic synthetic hydrogels (Figure 5a). Studies describe[92−94] that the rehydration of commercial mucins produces weak hydrogels of acidic pH and low ionic strength, but mucus-like rheological properties cannot be achieved even at high mucin concentrations. The mucin isolation procedure must therefore have changed its physicochemical properties significantly so that the initial structure could not be recovered after rehydration.

Lieleg and co-workers reported[95] one such formulation using native mucin (PGM) as one of the components, and studied its protective barrier properties against pseudoviruses. For this study, they placed the formulated solution on top of cells, followed by the addition of a drop of virus solution, and then measured the number of infected underlying cells; native mucus was used as a control. It was observed that the presence of ∼1 wt % of mucin in the formulation was enough to prevent infection of the underlying cells (Figure 5b). Even though such formulations mimic mucus's physical properties and its defensive properties against pathogens, they fall far short of native mucus because of their very low mechanical strength. This shortcoming restricts them from use in broad applications, especially those requiring materials with high mechanical strength for optimal performance. Such issues have been partly addressed either by adding a mechanical adjuvant to the formulated mixtures or by using a chemical cross-linker capable of forming a covalent network structure. Lieleg and co-workers introduced[96] a macromolecular hybrid system comprising mucin glycoproteins, methylcellulose (MC) as a mechanical adjuvant, glycerol, and NaCl. In this system, the MC exhibited thermoresponsive hydrogel formation at physiological temperature and the mucin retained its properties, making this hybrid gel system mucus-mimetic (Figure 5c). Owing to the inclusion of native mucins, this hybrid hydrogel demonstrated selective

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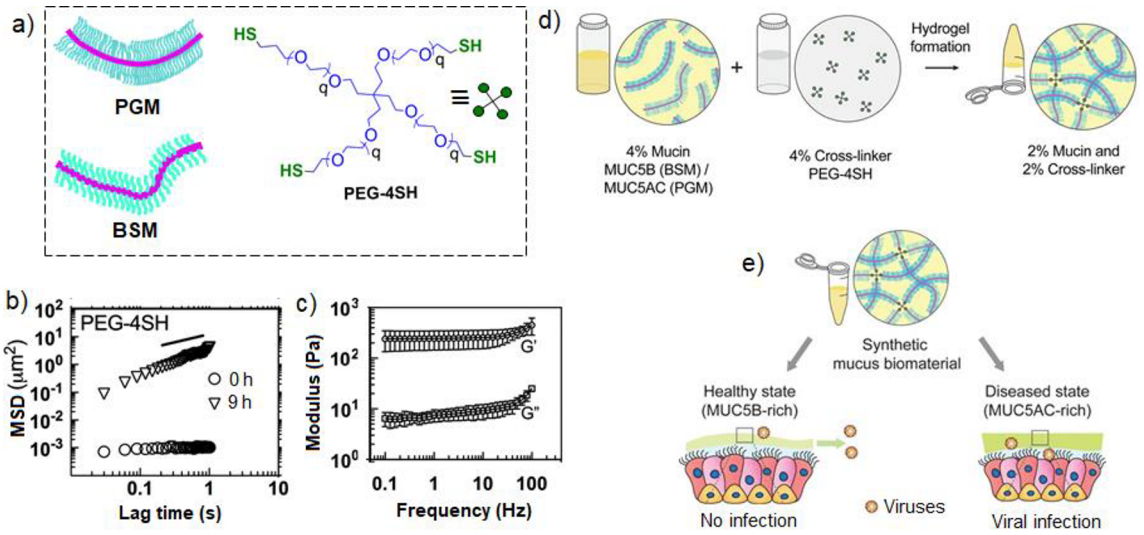

Figure 6. (a) Schematic representation of native mucins porcine gastric mucin (PGM) and bovine submaxillary mucin (BSM) and the chemical structure of 4-arm PEG thiol (PEG-4SH) used to study hydrogelation. (b) Particle tracking microrheology (PTM) has been investigated to understand time-dependent network formation using a muco-inert nanoparticle. Difference in time-averaged mean square displacement (MSD) value as a function of lag time, τ (s), confirms the network formation. (c) Bulk rheology measurements of hydrogel, produced by using PEG-4SH as cross-linker. G' represents hydrogel elasticity, and G'' represents hydrogel viscosity. Reprinted with permission from ref 99. Copyright 2019 The Royal Society of Chemistry. (d) Schematic illustration of the synthetic route for bioinspired mucus biomaterials. 4% (w/v) mucin solution is mixed with a 4% (w/v) cross-linker, resulting in a mucin-based hydrogel with 2% (w/v) mucin and 2% (w/v) cross-linker. (e) Changing the ratio of mucin composition produced healthy mucus rich in MUC5B as well as disease mucus rich in MUC5AC. Reprinted with permission from ref 100. Copyright 2021 American Chemical Society.

permeability for pathogens and allowed diffusion of drug molecules in a manner similar to native mucus. However, the hydrogel's other properties were dominated by the nonmucin components and were not as mucin-dependent, making the hybrid system significantly different from native mucus.

Fiegel and co-workers reported[92] another similar formulation to mimic tracheal mucus under healthy conditions, using the exact same composition as native tracheal mucus. They observed that the formulation itself formed a very weak hydrogel and that its rheological properties remained unchanged over time. To address these issues, they introduced a bivalent cross-linker that could cross-link with the hydroxyl groups of mucin proteins via acetal linkage and mimic the native tracheal mucus both rheologically and in terms of surface tension. It is therefore clear that the network structure in mucus is responsible for such properties. Fiegel and co-workers showed that they could control the rheological properties of hydrogels by tuning the amount of cross-linker as well as the cross-linking time. In another approach, Crouzier and co-workers appended vinylic functional groups onto bovine submaxillary mucin (BSM) by chemical modification, forming elastic gel via photoinduced cross-linking using UV light of 365 nm wavelength (Figure 5d).[97,98] Though the synthesized cross-linked hydrogels could partially mimic native mucus and could be used in various biological applications requiring hydrogels with significant mechanical strength, the cross-linking approach used by Crouzier and co-workers departed from that of native mucus, restricting the resulting hydrogels from broader application. For instance, due to lack of dynamic disulfide bonds in the cross-linking site, these hydrogels could not be used in applications where hydrogel dynamicity plays a major role.

3.1.2. Native Mucin-Based Cross-linked Hydrogels. To mimic the chemical environment of native mucus, native mucins were chemically cross-linked with thiol-containing cross-linkers to obtain mucus-mimetic synthetic gel. One such type of hydrogel has been reported[99] by Duncan and co-workers using commercially available partially purified animal mucin (PGM or BSM) along with PEG-based or Dextran-based cross-linkers of molecular weight 10 kDa with various geometries (Figure 6a). In their system, they mixed a mucin-cross-linker solution, of both the mucin and cross-linkers with ~2 wt % of each component, to mimic the concentration of mucin in native mucus, and then they monitored the time-dependent hydrogel formation at room temperature by particle tracking microrheology (PTM) using PEG-coated poly(styrene) nanoparticles (PEG-NPs), as well as by bulk rheology measurements. They measured diffusion using the particles' mean square displacement (MSD) just after mixing (hour 0), then again after 9 h. After the hydrogel had formed, the MSD of PEG-NP fell as a result of steric interactions with the meshlike mucin polymer network. Using 4-arm PEG thiol (PEG-4SH), Duncan and co-workers noted that incubation for 9 h with PGM was followed by a notable drop in the magnitude and slope of MSD (Figure 6b), indicating network formation, whereas other cross-linkers showed no significant change of MSD over time, indicating their inability to form networks. Similar results were observed in bulk rheology measurements of different sample combinations. In the case of PEG-4SH as cross-linker, the bulk rheology was in the range of the viscoelastic region of mucus (Figure 6c). This can be explained by considering the branched architecture of PEG-4SH, which enabled association between multiple mucin chains in a dynamic that was quite unlikely for the other PEG cross-linkers. In spite of the excess number of thiol groups in Dextran-SH as compared to PEG-4SH, the former could not form a network structure. One could therefore conclude that a more flexible backbone such as PEG is essential for interacting with multiple mucins at the same time and facilitating hydrogel formation.

Using native mucin and a commercially available cross-linker, Duncan and co-workers established a methodology that could partly emulate native hydrogels, showing rheological properties,

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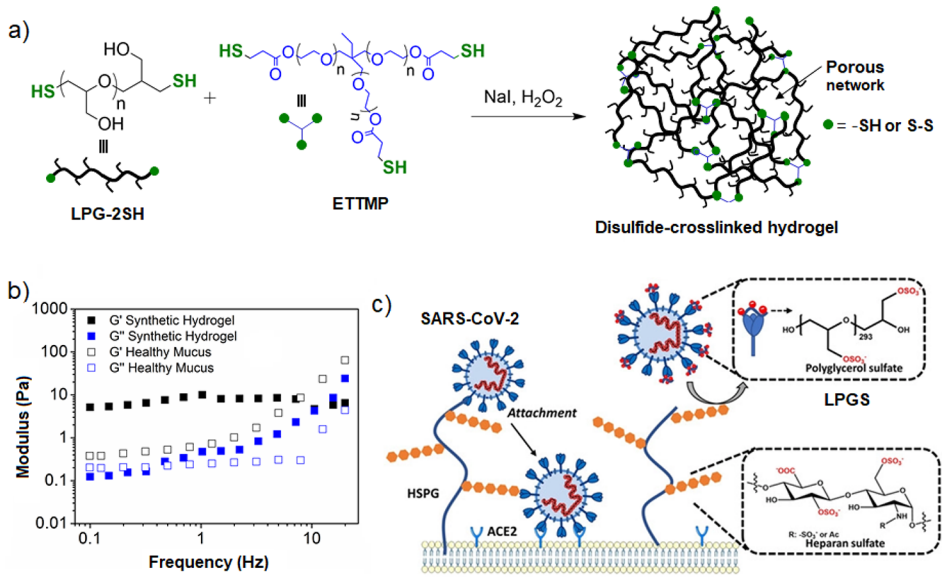

Figure 7. (a) Representative chemical structures of linear polyglycerol dithiol (LPG-2SH) and ethoxylated trimethylolpropane tri (3-mercaptopropionate) (ETTMP); an illustration of their resulting hydrogel network, showing cross-linking via formation of disulfide bonds. (b) Bulk rheological properties of synthetic mucus hydrogels and healthy mucus (sputum sample) at 25 °C. Adapted with permission from ref 104. Copyright 2021 Wiley-VCH GmbH. (c) Chemical structure of linear polyglycerol sulfates (LPGS), heparan sulfate and their inhibition mechanism for blocking the entry of SARS-CoV-2. Reprinted with permission from ref 107. Copyright 2021 Wiley-VCH GmbH.

network structure, and observed pore size that were all in the range of mucus. They also showed that the observed properties could be tuned by individually adjusting the amounts of mucin and PEG-4SH. In a follow-up work, they investigated[100] the impact of the relative proportions of the mucins MUC5B and MUC5AC in healthy and disease-state human respiratory mucus. Using the same cross-linking strategy with 4-arm PEG thiol, they synthesized different hydrogels by varying the ratio of MUC5B to MUC5AC in order to mimic both healthy (MUC5B-rich) and disease-state (MUC5AC-rich) respiratory mucus (Figure 6d). The rheological and transport properties of the synthesized hydrogels were compared with those of human respiratory mucus collected from human airway cell cultures. The synthesized healthy mucus hydrogel, rich in MUC5B, showed mucus clearance rates similar to those seen in mucus collected from cell culture; these rates were also in the range of physiological values reported in the literature. Using PTM analysis, Duncan and co-workers found that a relative increase in MUC5AC content caused a drop in particle diffusion, indicating a tightening mesh network and increased hydrogel viscoelasticity changes that have been observed under disease conditions. Furthermore, despite its very tight network of MUC5AC-rich hydrogel, the synthetic hydrogel was quite inefficient in restricting influenza A virus (IAV) infection of underlying cells in comparison to MUC5B-rich hydrogels, which were essentially low-viscosity elastic hydrogels (Figure 6e). This is because MUC5B, enriched with sialic acid,[101] constricted the free movement of IAV. Therefore, IAV diffusion through mucus hydrogels was not dependent on the compactness of the hydrogels; rather, the interactions with the glycan play the key role in enabling diffusion.[102,103]

The established synthetic models may enable new therapeutic interventions for lung diseases by facilitating a greater understanding of these diseases' mechanisms and the harm they cause to airway function. However, synthetic hydrogels' use of nonhuman mucins is a major issue, as these mucins differ from human mucin in the extent of their glycosylation. In addition, the mucins in the synthetic mucus models are not fully purified and so contain nonmucin adulterants like albumin and immunoglobulin, whose quantities may vary from batch to batch. Furthermore, existing models have not determined the concentration of thiol groups in different hydrogels; this is a key gap in understanding, as these groups may serve as a marker for the extent of lung disease. Moreover, the concentration of cross-linker used was quite high relative to the mucin concentration, so the effect of the additional thiol groups on hydrogel dynamics was not examined in these studies. Overall, this synthetic model system is perfect for preliminary research, but it cannot address all of the effects of native mucus, such as virus inhibition.

3.2. Mucus-Mimetic Hydrogels from Completely Synthetic Materials. Other synthetic approaches to mucus-mimetic hydrogels have been developed without the use of native mucins. The advantage of this route is the availability of materials, which can be functionalized to create tunable hydrogels based on specific requirements. Our group has recently reported one such example (Figure 7a).[104] Linear poly(glycerol) (LPG) is known as an alternative to poly(ethylene)glycol (PEG); it offers additional advantages for functionalization owing to its pendant hydroxyl groups and has been used to develop various functional biomaterials.[105,106] In a recent report from our group, dithiol-containing LPG (LPG-2SH) was oxidized together with a PEG-based triarm cross-linker, ethoxylated trimethylolpropane tri(3-mercapto-propionate) (ETTMP 1300), in the presence of hydrogen peroxide as an oxidizing agent. Upon varying the ratio of LPG-2SH and ETTMP, the authors successfully synthesized a hydrogel that could mimic the viscoelastic properties (Table 1) and mesh size of native human respiratory mucus, according to bulk rheological data (Figure 7b). Using PEG dithiol as a control, this research also highlighted the impact of LPG in such hydrogel formation. The advantage of this route is that one could synthesize the hydrogel in bulk, as its components are easily accessible. Though the synthesized hydrogels mimic the bulk rheological properties of native mucus, a few issues need to

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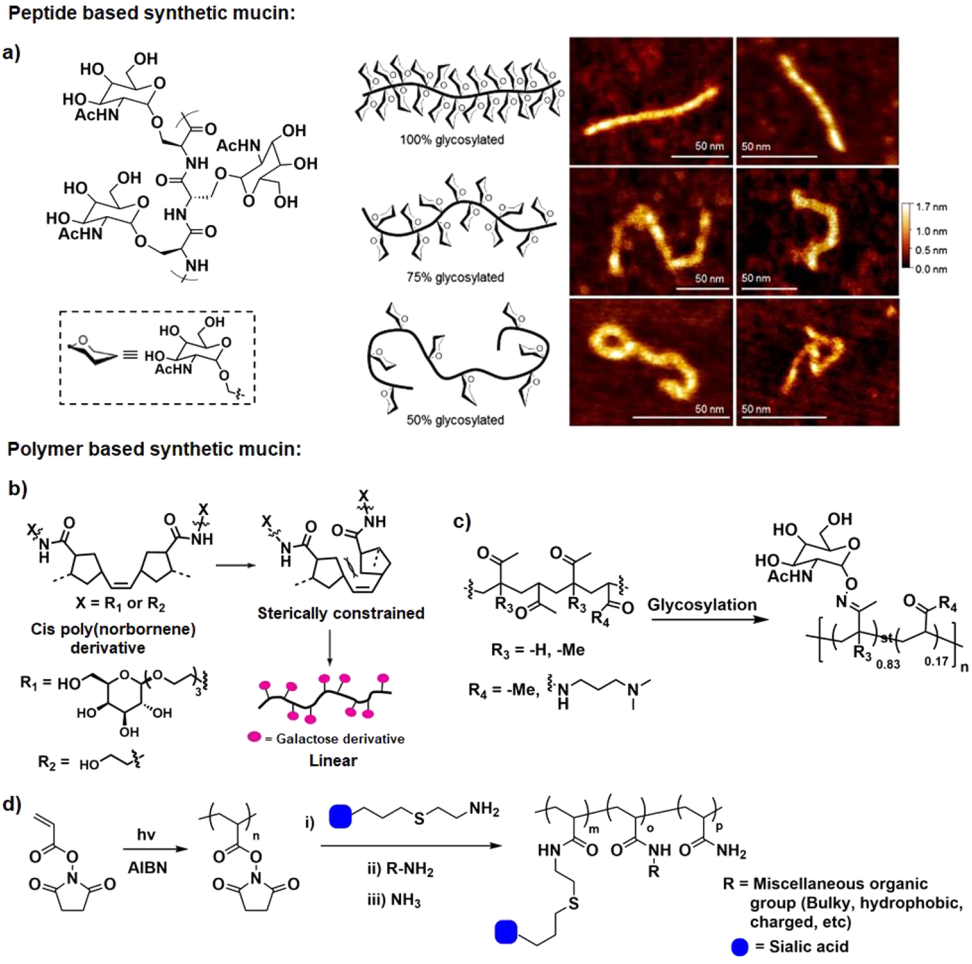

Figure 8. (a) Schematic representation and chemical structures of peptide-based synthetic mucins. AFM images of synthetic mucins with different degrees of glycosylation to show that the steric crowding induced by the sugar units is responsible for straight-chain fibers. Reprinted with permission from ref 133. Copyright 2015 National Academy of Sciences. (b) Chemical structure of cis-poly(norbornene)based mucin mimetic glycopolymers. Allylic strain helps them to attain linear conformation. Adapted with permission from ref 136 (https://pubs.acs.org/doi/10.1021/acscentsci.0c01569). Copyright 2021 American Chemical Society. The ACS is solely responsible for the copyright permission to reuse this material. (c) Structures of copolymer backbones derived from methyl vinyl ketone (MVK) and isopropenyl methyl ketone (IMK), functionalized with glycans. Steric repulsion between adjacent glycan chains endows mucin glycoproteins with linear bottlebrush structures. Adapted with permission from ref 134. Copyright 2021 American Chemical Society. (d) Schematic showing synthesis of poly[N-(acryoyloxy)succinimide] and postpolymerization glycosylation on them produced mucin-mimetic polymers. Adapted with permission from ref 98. Copyright 2020 Wiley-VCH GmbH.

be addressed to improve the hydrogel and make it more applicable as a synthetic model system.

For instance, macrorheological properties, important for understanding actual mesh size and for quantifying thiol groups to understand the hydrogels' dynamicity, are not discussed. The major drawback of the reported hydrogels is the wt % of the solid components used, which was quite high (>30 wt %) as compared to the mucin wt % in native mucus (2−3 wt %). This could be because the reported hydrogels used small polymer chains for hydrogelation, while native mucins' megadalton-range size effectively helps them become entangled and create a network structure, even at very low wt %. Owing to the lack of functional groups on LPG-dithiol that are present in native mucin, this study does not discuss the protective properties of the synthesized hydrogels. The further functionalization of LPG-dithiol with different mucin-mimetic functional groups (such as sialic acid, sulfate groups and so on) will help address this gap in understanding. Recently, our group has reported the sulfated version of linear polyglycerol (LPGS) as an excellent inhibitor against respiratory viruses such as HSV-1 and SARS-CoV-2 at remarkably low IC_{50} values (Figure 7c).[107,108] This unprecedented report could further motivate the installation of sulfate groups,[109,110] as the pendant functional groups of LPG-dithiol and their use in preparing hydrogels that could match native ones in virus entrapment. Furthermore, the hydrogels that our group reported are formed by the oxidation of thiol-containing polymers and cross-linkers, so there is always a chance for uncontrolled oxidation to yield more cross-linked hydrogels. This problem could be solved by avoiding the oxidizing agents for disulfide bond formation. To achieve this, one could easily think of introducing more reactive thiol groups[111] or an activated disulfide such as 1,2-dithiolane[112−118] and/or 2-pyridyl disulfide[119−124] among the hydrogel components; this approach could produce hydrogels under mild conditions via either thiol-triggered or phototriggered disulfide formation. We discuss such possibilities in detail later in this article (Figure 9).

In addition to mucus-mimetic synthetic hydrogels that are based on dynamic covalent linkage, the current literature includes mucus-mimetic synthetic hydrogels that are based on

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the other noncovalent interactions present in native mucus. In this section we have highlighted examples of these latter synthetic hydrogels, but due to the limited scope of this perspective we have not discussed them in detail. For example, synthetic hydrogels that can mimic vaginal mucus are reported. Patrick and co-workers developed[125] a pH-responsive, *in situ* forming polymeric gel as a synthetic cervicovaginal mucus-mimetic. The cross-linked hydrogel was based on phenylboronate-salicyl hydroxamate affinity pairing and sensitive toward pH change, showing a facile reversible sol−gel rheological transition upon changing the pH from vaginal (pH 4.0) to seminal (pH 7.4). This hydrogel could successfully hinder virion transport at seminal pH owing to the formation of a more cross-linked network. Apart from showing protective properties against virions, this hydrogel also could be used as a localized drug delivery matrix.

The further development of various mucoadhesive materials[126] and various innovative methods[127] developed to date in mucoadhesion will certainly strengthen mucus research, but because of our limited scope we have not explored these efforts in this perspective. Other approaches that may mimic some aspects of native mucus, such as porous network formation;[128,129] worm gel,[130] which could function as a suitable bioinert 3D matrix for pluripotent stem cells (PSCs); and PEG-based functional synthetic dynamic hydrogels for 3D cell culture applications,[131] are also excluded from our discussion.

4. CHALLENGES

We opened this perspective with an overview of native mucus hydrogels, including their chemical compositions and the complex network structures that allow selective permeability toward foreign particles. We then attempted to highlight the different approaches to mimicking native mucus in terms of their respective properties. These synthetic hydrogels have been used as model systems to understand some functional properties of mucus, but each has its own limitations. The strategy using native mucin produced promising mucus-mimetic hydrogels and could be used as a model system in mucus-related research. But large-scale availability of native mucin is quite limited, restricting these hydrogels' broad application. Besides, the purification of mucin from mucus is a complicated process, resulting in variation in the chemical composition of the purified mucins as compared to native mucins. For instance, the nonglycosylated domain of mucins is susceptible to proteolytic degradation,[132] altering its hydrogelation as well as lubrication properties. Furthermore, in most cases the native mucins used are nonhuman and differ in protein sequence from human mucin, and therefore the hydrogels based on them cannot mimic human samples with complete accuracy. Native human secretions' physical and chemical properties make them ideal in this context. But the large-scale collection of human mucus is not possible, and the subsequent processing that would be required to obtain native mucins usable for hydrogel formation nearly impossible. Human sputum could be one possible source of mucus for research, especially from chronic disease patients who produce large quantities of sputum, but its high patient-to-patient heterogeneity and intermixing with saliva can only lead to the collection of heterogeneous samples. The collection of small quantities of airway mucus by endotracheal tube could provide limited characterization of its physical properties. However, tracheobronchial mucus is extremely difficult to collect from healthy human lung airways.

As an alternative, considering their possible availability in bulk scale, mucus-inspired hydrogels from synthetic materials offer promise as a model system for mucus-mimetic research prior to *in vivo* studies. Our earlier discussion on mucus-mimetic hydrogels makes clear that, despite various attempts to achieve the selective properties of native mucus hydrogels, the optimal hydrogels that can realize this sort of biomimicry have not yet been developed. This is because the synthetic materials used to date have departed greatly from the structural and chemical compositions of native mucins, which are likely the key contributors to mucus hydrogels' functional properties. Synthesis of mucin-mimetic materials is quite challenging due to their very high (MDa-level) molecular weight, extensive glycosylation and corresponding high charge density, specific hydrophilic−hydrophobic balance, number and sequence of cysteine residues, and extensive heterogeneity in size.

In this regard, synthetic polypeptides have certain advantages, as they could exactly mirror native polypeptide sequences, and further glycosylation would lead to synthetic mucin. Bertozzi and co-workers reported[133] one such synthetic polypeptide that binds to cell receptors (Figure 8a). They also showed that extensive glycosylation and structural rigidity are the key parameters for attaining mucin-like long-chain fiber structure. The peptide-based mucin mimetic that they developed emulates native tethered mucins both chemically and structurally. However, the reported synthetic peptides are rather short, in great contrast to the complex gel-forming secreted mucins with their extended length. The current lack of a synthetic route for peptides of extended structure prevents their use as a mirror of secreted mucins. Mucin-mimetic polymeric systems that could mimic native mucins in terms of their glycosylation and morphology have been reported.[134−136] It is postulated that to attain a wormlike nanofiber structure, the polymer backbone must be rigid (Figure 8b), and that steric repulsion (Figure 8c) may also play a role here. However, due to the lack of suitable functional groups, neither mucin-mimetic synthetic peptides nor mucin-mimetic polymers have yet been used for the preparation of the desired hydrogels.

Besides, the formation of additional disulfide bonds is responsible for achieving the more viscous hydrogel observed in defective mucus. A series of mucus modulators have been developed[137−140] and showed promise for practical application. However, so far only *N*-acetyl cysteine (NAC) has been approved for commercial use. Mucus modulators can cleave the additional disulfide bonds to return the mucus to normal condition, but most of them suffer from poor selectivity in cleaving disulfide bonds. For instance, these modulators sometimes cleave not only the additional disulfide bonds present in diseased mucus but also the disulfide bonds in mucin proteins and other noncovalent bonds common to diseased and healthy mucus. As a result, the modulated mucus becomes less viscous than native mucus. Knowledge of the concentration of thiol and disulfide groups is important for understanding the dynamic nature of hydrogels and mucus health conditions. Ellman's reagent has been used extensively[141] to detect thiol groups and accurately quantify thiol concentration, but it is not suitable for these functions in mucus, as the mucus itself shows absorbance in the same region as Ellman's reagent.

5. OUTLOOK

Despite the challenges facing every route to the successful mimicry of mucus hydrogels, the field is rich with possibilities, due to the advanced modern research techniques that could help

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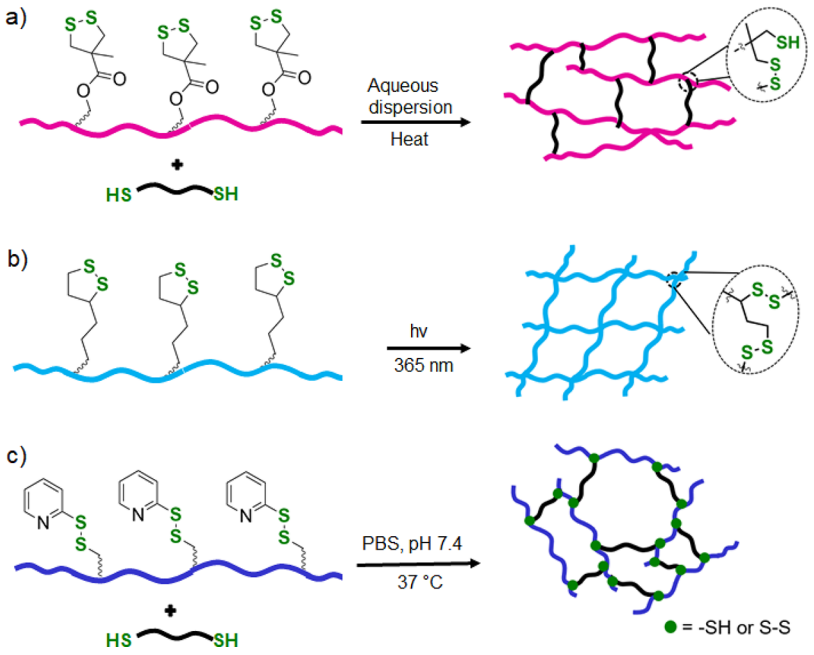

Figure 9. Schematic showing the incorporation of reactive functional groups such as 1,2-dithiolane derivatives and 2-pyridyl disulfide on various polymer backbones and their use in hydrogel formation under mild conditions. (a) Cross-linking of dithiolane-containing polymers by thiol-triggered disulfide-thiol exchange to form dynamic hydrogels. (b) 1,2-Dithiolane-containing polymers also undergo phototriggered disulfide formation followed by cross-linked network formation. (c) Thiol-pyridyl disulfide groups installed on the hyaluronic acid backbone and used for preparation of disulfide hydrogels. Adapted with permission from ref 161. Copyright 2011 American Chemical Society.

realize the laboratory synthesis of mucus-mimetic "dream" hydrogels at the bulk scale necessary to revolutionize mucus-related research. Here we discuss point-by-point the current research that could overcome the limitations in synthesizing mucus hydrogels and result in substantial improvements to the current state of the art:

Addressing the Problems Associated with Native Mucin-Based Hydrogels. Recently established protocols[142,143] for purifying mucin from native animals' mucus may help to overcome common issues associated with mucin purification. As a result, the hydrogels synthesized from them could be better defined and more reproducible. As for human samples, considering that nonovulatory mucus shares more or less similar properties with the other mucus types, and considering also that small-scale collection of such mucus is comparatively less complicated, nonovulatory mucus could therefore serve as an excellent model for biophysical studies of human mucus. In addition, human bronchial epithelial (HBE) cells cultured under an air−liquid interface can produce mucus whose composition is very similar to that of native secretions. For studies requiring only a small amount of mucus, these cell cultures can provide mucus capable of serving as a model system.[144,145] Generally, the cone-and-plate geometry method that is typically used for rheological measurement is difficult to apply to human samples, given the very small sizes of the available samples. Magnetic microrheology[146,147] that requires only tiny volumes of samples could solve this problem and offer the type of insights that are essential for characterizing mucus-mimetic model systems.

Overcoming the Limitations of Synthetic Mucus Hydrogels. Recently developed[148−157] controlled polymerization techniques for the synthesis of high-molecular-weight glycosylated polymers in bulk scale show some hope for synthesizing polymers that could mimic the extended lengths of mucins. Various controlled radical polymerizations have been developed for the synthesis of megadalton-range polymers with defined functional groups at chain ends, and various glycosylation techniques have been developed[158−160] that would help to achieve extensive glycosylation on these polymers (Figure 8d). It would be nice to imagine the use of such synthetic materials in the synthesis of mucus-mimetic hydrogels in the near future. Therefore, the optimal approach would be to use mucin-mimetic synthetic peptides or mucin-mimetic synthetic polymers (Figure 8) in hydrogelation, using different techniques to achieve the desired hydrogels, which could closely mimic native mucus. Research on the formation of various dynamic covalent networks under mild conditions, especially the disulfide bond formation, motivates the researcher to consider disulfide-based mucus-mimetic synthetic hydrogels under mild conditions, which could be used as a model system to study the dynamic environment of mucus. In this context, 1,2-dithiolane derivatives undergo thiol-triggered ring-opening of thiolane disulfide under room temperature to produce the cross-linked network.[113,114] Hence, 1,2-dithiolane-based polymeric systems have been designed, and corresponding hydrogels have been prepared (Figure 9a). The synthesized hydrogels also showed thiol-triggered de-cross-linking of the polymer, demonstrating dynamicity in the hydrogels. Moreover, 1,2-dithiolane derivatives also undergo phototriggered (365 nm wavelength) radical formation followed by disulfide bond formation to create a network structure when incorporated in a polymer backbone (Figure 9b).[112] One more popular disulfide formation strategy under mild conditions is the exchange reaction between free thiol and 2-pyridyl disulfide, which has been widely used for creating the disulfide network.[119−124] This strategy has also been used for hydrogel formation[161] by incorporating 2-pyridyl disulfide in the polymer backbone and using dithiol as a cross-linker (Figure 9c). Therefore, the

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incorporation of such functional moieties in a synthetic mucin-inspired polymer would lead to the dream hydrogel in terms of mucus-mimetic properties.

Addressing the Issues in Mucus Modulator Research. Recent studies have reported on thiol sensors[162] that could detect free thiols at even lower concentration levels, and quantify them more accurately, than Ellman's reagent. Considering their wide range of absorbance, these sensors could also measure the thiol concentration of mucus without any ambiguity. The ideal modulator would be one that could cleave only the exact number of excess disulfide bonds, an extremely challenging task in practice. One could therefore design a disulfide-based dynamic hydrogel with disulfide bonds of different reactivity[111] and investigate the recently developed sophisticated mucus modulators to understand their selective reduction properties. Advanced research on particle-tracking microrheology can certainly help in understanding the properties of dynamic hydrogels at nanoscopic levels and would advance mucus modulator research.

Explaining Mucus Permeability. Another unsolved question is how virus particles (~100 nm) larger than the mucus pore size (Table 1) pass through mucus and infect the underlying cells. The recent report[163,164] on the penetration of influenza A virus through the mucus layer offered some insight, indicating that, in addition to specific chemical interactions, the reorganization of viral surface proteins during mucus penetration plays an important role in viral entry. Various proven modern techniques, including super-resolution microscopy, would enable better understanding of the diffusion of various virus particles through the mucus barrier.

6. CONCLUSIONS

The importance and variability of mucus have inspired a range of synthetic methodologies for mucus-mimetic materials, each having strengths and drawbacks for the work of understanding and mimicking human mucus. Current techniques for native mucin purification and for synthesizing mucin mimetic peptides and polymers; established methodologies for preparation of dynamic hydrogels under mild conditions; and existing sophisticated thiol sensors will be the cornerstones of future research into synthetic mucus hydrogels. Future research into developing synthetic mucus-inspired hydrogels not only will help us to understand in detail the physiochemical properties and biological functions of native mucus but also may seed a broader initiative in mucus-related research. For instance, synthetic mucus models in combination with established modern techniques might reveal the entry pathways of various existing viruses including SARS-CoV-2; such insights would certainly open new avenues for protection against them. Additionally, synthetic mucus could be used as a functional coating to provide epithelial protection, could act as a lubricant for the treatment of disease associated with mucus membrane dryness, and could serve as a platform in mucus modulator research for the development of efficient modulators for treatment against respiratory diseases. We believe that all of these potential applications will inspire the next generation of scientific progress on synthetic mucus materials.

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Notes

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