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# Selective permeability of mucus barriers

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Mucus is a hydrogel that exhibits complex selective permeability, permitting the passage of some particles while restricting the passage of other particles including important therapeutics. In this review, we discuss biochemical mechanisms underlying mucus penetration and mucus binding, emphasizing the importance of steric, electrostatic, and hydrophobic interactions. We discuss emerging techniques for engineering nanoparticle surface chemistries for mucus penetration as well as recent advances in tuning mucus interactions with small molecule, peptide, or protein therapeutics. Finally, we highlight recent work suggesting that mucus permeability can serve as a biomarker for disease and physiological states such as pregnancy.

## Addresses

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## Introduction

Mucus is a selectively permeable gel that covers all non-keratinized surfaces in the human body, including the respiratory, gastrointestinal and urogenital tracts [1]. The mucus barrier has critical functions in protecting tissues from attacks by pathogens and toxins, while permitting transport of beneficial particles such as nutrients and sperm. Mucus's natural selectivity, while beneficial in normal contexts, also represents a core obstacle for engineers designing methods for drug delivery [1–4,5\*,6]. The selective permeability properties of mucus have important roles in health and disease, and unwanted changes in mucus permeability are associated with diseases such as cystic fibrosis (CF) [7], ulcerative colitis (UC) [8\*], and some forms of infertility [9].

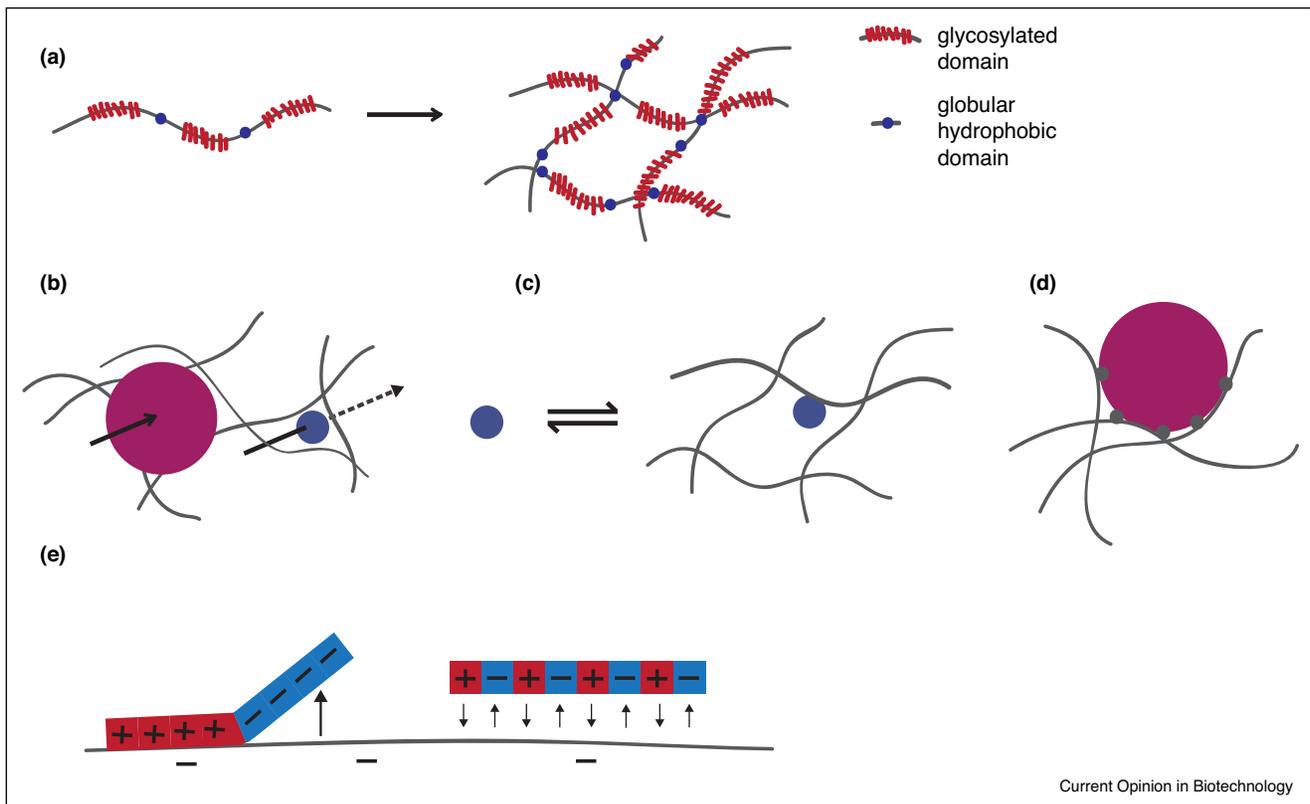
Little is known about the detailed molecular properties that distinguish particles that penetrate, or are rejected by, a mucus barrier. Mucus is a complex mixture of water, salts, lipids, nucleic acids, and a variety of proteins, including high molecular weight glycoproteins called mucins [1–4,6]. Mucins are the main gel-forming polymers of mucus and contain threadlike core protein domains which contain large numbers of O-linked oligosaccharide chains that confer negative charge to the mucins through carboxyl and sulfate groups [10]. Moreover, mucins also contain hydrophobic domains which appear to mediate self-assembly of mucin polymer networks [11]. Mucins, along with other components such as lipids and DNA, create a plethora of binding sites for many incoming and secreted particles. Our inability to predict mucus passage of natural biological substrates, such as viruses, nutrients, or toxins, or synthetic particles such as small molecule drugs or nanoparticles, is related to our poor mechanistic and quantitative understanding of how substrates with complex charge and hydrophobic surface properties interact with the mucus barrier [1].

This review will provide an overview of what we know about transport selectivity in mucus, focusing on insights into molecular mechanisms from within the past two years, and highlighting in particular how properties beyond simple net charge or hydrophobicity modulate permeability. Progress has also been made on the design of nanoparticles with compelling mucus penetration properties, which we discuss along with exciting applications of these surface chemistries. Finally, we discuss changes in selective permeability associated with diseases related to mucosal surfaces, and how these changes can be offset to improve drug delivery or measured for diagnostics.

## Mucus selectivity arises from its structure and biochemistry

Mucins are cross-linked both through reversible, hydrophobic interactions and disulfide bonds to form a polymer network (Figure 1a) with a mesh size ranging from 100 to 2000 nm, depending on the location in the body. The mesh size of mucus is typically highly heterogeneous, even within a given site [1]. For particles that are larger than the mesh size, mucus presents a geometrically constraining filter and hinders their passage regardless of surface chemistry (Figure 1b) [12]. Mesh size is not necessarily static, however. For example, increasing the mucus mesh size with the mucolytic N-acetylcysteine can enhance *in vitro* nanoparticle transport through mucus [13]. Similarly, synthetic nanoparticles coated with mucolytic proteases such as papain or bromelain can improve

Figure 1



Overview of mucus and mucus selectivity. **(a)** Mucins contain disordered, heavily glycosylated, polyanionic domains interspersed with globular hydrophobic domains. These polymers are chemically and physically cross-linked to form a polymer network. **(b)** Particles larger than the mesh size of mucus (magenta) have hindered passage, while particles smaller than the mesh size may pass through more freely. **(c)** Binding to polymeric mucin components slows diffusion. **(d)** Polyvalent binding strongly hinders diffusion, even if each individual binding interaction is weak. **(e)** Spatial arrangement of charge impacts the diffusion of particles in mucus [28].

mucus penetration by degrading the mucin polymers [14<sup>\*</sup>,15–17].

Steric filtering has little to no effect on the movement of molecules smaller than the mesh size of mucus. However, mucus impacts the diffusion of particles of any size via interaction filtering, in which binding interactions with components inside the mucus arrest diffusion and thus inhibit transport [12]. Put plainly, the more time a molecule spends bound, the less time it has to freely diffuse (Figure 1c) [18]. A related, equally important effect is that the same binding that slows diffusion may reduce the concentration of free molecule and inhibit activity, even after enough time has elapsed for local equilibration. The polyanionic mucin polymers are major contributors to interaction filtering, so net positively charged particles are generally considered to bind with mucus. Mucin-associated lipids and proteins can modulate the detailed interaction capacity of the mucins [19,20]. Other polyanions in mucus, such as DNA and bacterial polysaccharides, which are particularly prevalent in CF, and shed

epithelial cells, also contribute to interaction filtering [1,21].

The impact of interaction filtering on the diffusion of a particle depends on the specific biochemistry, but also, how many binding sites on the particle can interact with mucus. Specifically, mucus typically reduces the diffusivity of small molecules by no more than an order of magnitude [22]. However, for nanoparticles of ~100 nm in size or larger, mucus can reduce the diffusivity by several thousand fold, even if the particle is still below the mesh size [23<sup>\*\*</sup>]. This dramatic effect on diffusion arises when a particle presents multiple binding sites for mucus that can engage in mucus binding simultaneously. This phenomenon, termed polyvalent binding, implies that even if each individual interaction is weak, the net effect is near-irreversible binding that traps the particles (Figure 1d) [24]. The concept of polyvalent interactions applies to virtually any particle that presents multiple binding sites, and a useful rule of thumb is that most nanoparticles present multiple binding sites and

therefore, will be trapped in mucus unless they are designed, or have evolved, to escape retention by mucus.

### Biochemical features that regulate interactions with mucus

While size and polyvalency have a strong impact on the effect of interactions, the biochemical mechanisms behind mucus binding are shared between small molecules and nanoparticles. We therefore discuss these mechanisms using examples from both small molecules and nanoparticles. Particularly well studied for small molecules are electrostatic interactions between mucus and cationic antibiotics including certain antimicrobial peptides, polymyxins, and aminoglycosides. For these drugs, binding inhibits diffusive penetration and reduces efficacy [25]. However, while aminoglycosides and polymyxins may have as many as five protonatable amines and so are capable of strong charge-based binding [25], most cationic drugs are only singly or doubly charged, hence, the effect of electrostatic interactions on their transport is weaker and sometimes seemingly non-existent. Unsurprisingly, positively charged nanoparticles, with many more positively charged moieties than aminoglycosides, are typically trapped in mucus [1]. For example, adeno-associated virus serotype 2, a potential vector for inhaled gene therapy, is trapped in mucus, possibly due to electrostatic interactions [26].

While higher positive charge correlates with tighter binding in general, net charge is not necessarily a reliable predictor for mucus binding because different molecules with the same net charge may interact very differently with mucus. For example, Li *et al.* showed that a peptide with separated blocks of positive and negative charge interacted with mucin, while a peptide with the same sequence composition but alternating charges did not (Figure 1e). These experiments suggest that reducing large clusters of positive charge, such as by interspersing anionic groups, can ablate cation–mucin binding [27]. This is in line with results from antibody design and antifouling research, which show that nonspecific binding can be reduced by avoiding large surface patches of positive charge [28,29]. For mucosal applications, these results imply that balancing the charge of a molecule on the nanoscale may be an effective way to incorporate charge into a molecule while preventing undesired mucus binding. Antifouling appears to be particularly relevant in this context because just as reducing binding to chemically complex mucus components is one central goal of mucus research, hydrophilic antifouling coatings are designed to resist adsorption of proteins and other polymers with diverse binding chemistries [30\*].

Of note is that not all charged groups are necessarily created equal. While alternating glutamic acid–lysine (EK) peptide coatings are excellent for preventing non-specific binding for antifouling applications, alternating

glutamic acid–arginine (ER) peptide coatings are less effective, implying that identity of the charged moiety is important to consider. The difference between EK and ER may arise because protonated arginine provides more hydrogen bond donors, is more weakly hydrated than protonated lysine, and possibly interacts with hydrophobic aromatic groups [28]. We expect that such detailed biochemical features will be relevant for regulating interactions with mucus as well.

The example of the ER peptide begins to illustrate the importance of hydrophobic interactions, which occur in mucus primarily with hydrophobic domains of mucins or mucus-associated lipids [4]. As with net charge, correlations between mucus binding and quantitative estimates of hydrophobicity of small molecules such as the octanol–water partition coefficient are substantial but far from perfect [4,31\*] and we expect that spatial arrangements of proximal charge and other parameters such as double bonds or aromaticity for  $\pi$ – $\pi$  bonding come into play. For nanoparticles, any exposed hydrophobic surface typically means trapping: synthetic polystyrene [24] or metal oxide nanoparticles [32] and single-walled carbon nanotubes [32] are immobilized in mucus likely due to polyvalent hydrophobic interactions.

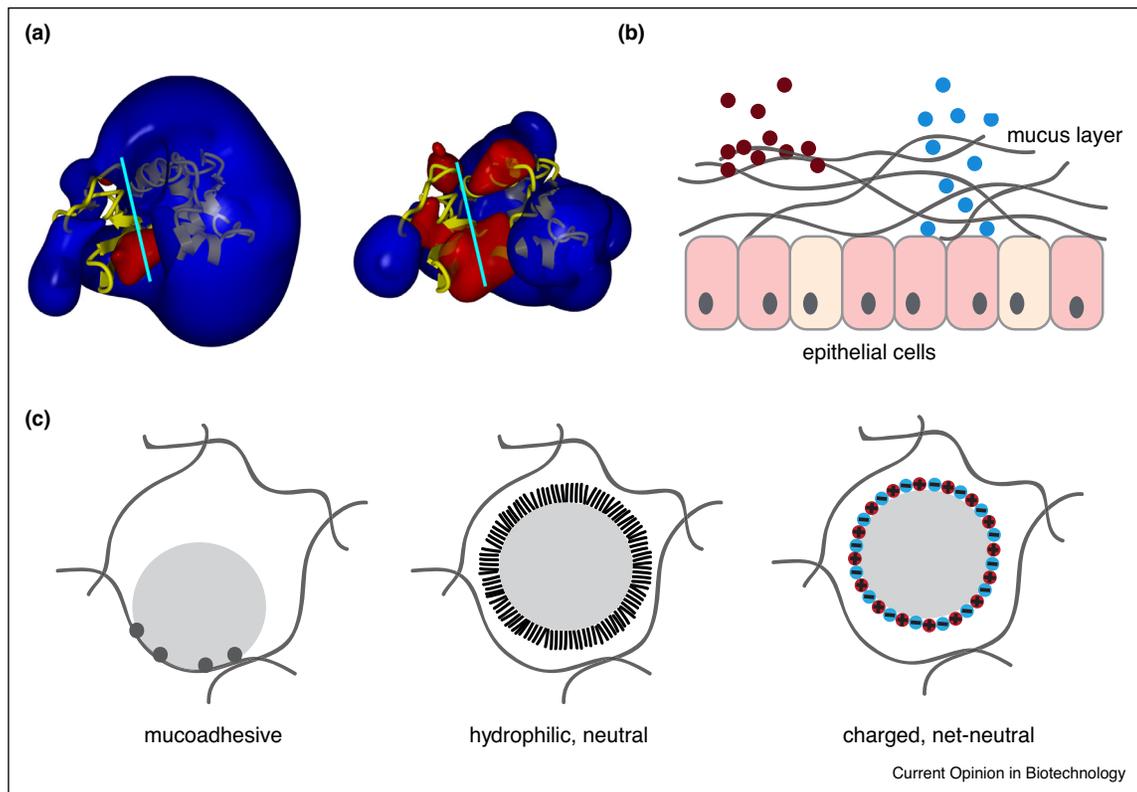
While hydrogen bonding is not clearly correlated with small molecule–mucus binding, it appears to be an important contributor for interactions between mucus and various polymers used as mucoadhesives for drug delivery, such as polyacrylic acid and alginate [33]. Hydrogen bond donors and acceptors on a diffusing particle may increase mucus binding via hydrogen bonding or decrease mucus binding by decreasing hydrophobicity. Antifouling coatings with hydrogen bond acceptors but not donors are better at reducing non-specific binding [34], and we anticipate this design principle may hold for mucopenetration.

### Engineering strategies for tuning mucus interactions

Many strategies have been developed to regulate mucus binding for drug delivery, which have been summarized in recent excellent reviews [1–4,6,35]. The main molecular targets for modification are charge and hydrophobicity, and a common theme that emerges among these strategies is the neutralization, or physical shielding from mucus, of cationic or hydrophobic groups. Alternatively, positive charge may be strategically increased to enhance mucus interactions.

For small molecules, peptides, and proteins, a few studies have been reported for strategic designs to reduce drug binding to mucus. The Smyth group PEGylated the aminoglycoside tobramycin, which slightly reduced its net charge and potentially shielded it from electrostatic interactions. Likely due to enhanced diffusion,

Figure 2



Strategies for tuning mucus interactions. **(a)** Electrostatic potential fields of wild type lysozyme (left) and charge-reduced lysozyme (right), with blue representing positive potential and red negative potential. Charge-reduced version of lysozyme showed reduced polyanion inhibition. Reprinted with permission from Gill *et al.* (2011) [39] Copyright (2011) PLOS. Published under CC BY license <https://creativecommons.org/licenses/by/4.0/legalcode>. **(b)** Strategies for tuning interactions between particles and mucus include, mucadhesion (left) in which a particle is designed to bind mucus, and mucopenetration (right), in which a particle is designed to be mucoinert. **(c)** Many nanoparticles have multiple binding sites with mucus and are therefore mucoadhesive (left). Mucopenetrating particles typically either have a hydrophilic and neutral polymer brush coating (middle), or highly charged, net-neutral surfaces (right).

PEGylated tobramycin had better *in vitro* anti-*Pseudomonas aeruginosa* biofilm activity than the unmodified variant [36] and this superior activity held up in a CF-like model combining mucus and a *P. aeruginosa* biofilm [37<sup>\*\*</sup>]. Another example is the cationic antimicrobial protein lysozyme, which is inhibited by polyanions in CF lung mucus [38,39]. An engineered charge-reduced lysozyme mutant reduces this inhibition while maintaining antimicrobial activity and has shown increased efficacy in mouse models of lung infection (Figure 2a) [40<sup>\*</sup>,41]. These two examples support the idea that the rational design of molecules may improve their mucosal function, and we argue that incorporating mucus binding into drug design will be an important future area, particularly for highly cationic drugs, but also potentially for hydrophobic drugs.

Certain drug delivery applications take advantage of mucus attachment, or mucoadhesion (Figure 2b, left), for extended-release drug formulations in sites of the body where mucus is regenerated and shed slowly and

when the encapsulated therapeutic can itself penetrate mucus [33]. These strategies involve the formulation of drug-loaded micro or nanoparticles composed of polymers that can engage in hydrogen bonding, hydrophobic, or electrostatic interactions with mucus, as well as physical entanglement or even the formation of disulfide bonds. However, mucoadhesion can become limiting in sites where mucus is shed rapidly, such as in the lungs and intestine [33].

For most applications, in particular for mucosal sites with rapid mucus turnover, strategies that prevent particle–mucus interactions and hence allow for free diffusion through mucus (mucopenetration) (Figure 2b, right), appear more effective [42<sup>\*</sup>,43,44]. Of note is that mucopenetrating particles may paradoxically be cleared from mucus more slowly than mucoadhesive particles because the inner layers of a mucus barrier, which these particles are designed to reach, are often cleared more slowly than outer layers [42<sup>\*</sup>,43–45].

The primary strategy for achieving mucopenetration is a hydrophilic but net-neutral surface that prevents both hydrophobic and electrostatic interactions. The most well-developed method for achieving such a surface is a dense brush coating of the neutral but hydrophilic polymer PEG (Figure 2c) [24,46,47]. Recent applications of this technology in mouse models include nanoparticle penetration through cervical mucus for anti-cancer drug delivery [48], inhaled gene therapy [7], and PEGylated nanoparticles for sustained anti-inflammatory drug release [42]. With the exception of current clinical trials for treatment of ocular diseases, however, PEGylated mucopenetrating particles have not yet reached the clinic.

Other recent experimental strategies to build hydrophilic but net-neutral surfaces include using zwitterionic coatings [49] and formulating nanoparticles from polycations complexed with polyanions (Figure 2c) [50,51]. The latter approach was further improved through combination with PEGylation [52]. Coating nanoparticles with neutral hydrophilic polymers other than PEG (Figure 2c) [53,54–56], has also been recently demonstrated to increase mucopenetration. For applications requiring nanoparticle uptake by cells, the Huang group has demonstrated that a balance of mucopenetration and properties such as hydrophobicity and positive charge that assist in cell uptake may be possible to achieve [57,58,59]. Other approaches balancing the competing interests of mucopenetration and cell uptake are to include negative charges removable by intestinal alkaline phosphatase, allowing for a zeta potential increase following mucus penetration [60,61,62], and making the mucoinert coating dissociable [56].

Looking forward, we anticipate that strategies used to engineer antifouling surfaces [30] may be a direct source of inspiration for future strategies to achieve mucopenetration. This prediction is supported by experiments with surface coatings of PEG, poly(hydroxypropyl methacrylate), and poly(2-oxazoline)s, which have each been used with some success for both antifouling [30] and mucopenetration [47,54,56]. In particular, polyzwitterions such as poly(carboxybetaine) and poly(sulfobetaine) [63] and EK peptides are excellent antifouling coatings [28] and we predict they may be beneficial for mucopenetration as well.

While mucopenetrating nanoparticles can successfully avoid non-specific binding to mucus components, particle penetration may still be blocked by antibody response. For example, when specific to otherwise mucopenetrating particles such as PEG [64], HSV and even influenza [65,66], antibodies including IgG and IgM can block diffusion of these particles by polyvalently crosslinking them to mucins via weak antibody–mucin binding. This response may impede repeated administrations of therapeutic nanoparticles, but conversely antibody trapping is

a promising new strategy to prevent viral penetration of mucus layers and subsequent infection [67].

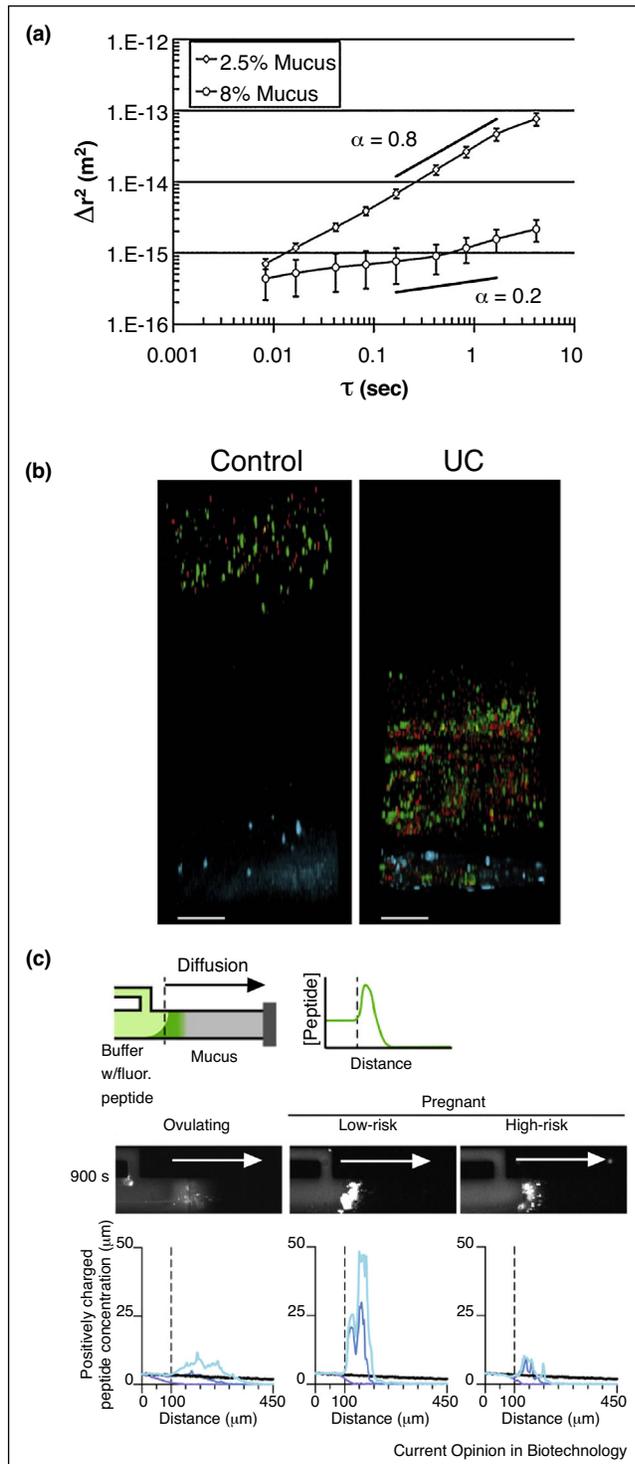
### Mucus permeability in health and disease

Understanding mucus permeability is important to predict or engineer transport through the mucus barrier, but it also presents translational potential for using mucus as a non-invasive diagnostic for mucosal health. The rationale is that the physicochemical properties of mucus barriers are intricately related to health and disease, and a number of pulmonary, gastrointestinal and urogenital conditions are associated with mucus barrier alteration. For example, cervical mucus changes naturally during pregnancy to form a thickened plug, suggesting a strengthening of the mucus barrier toward microbial ascension, thereby maintaining a relatively sterile environment in the intra-uterine cavity. Experimentally, cervical mucus from pregnant women is less permeable to both nanoparticles and small charged peptides than mucus from non-pregnant women [68]. Indicating an increase in the barrier function through a reduction in permeability. More broadly, mucin production is altered by a variety of microbial products and immunological factors [69]. While mucus permeability has not specifically been tracked as a function of these alterations to our knowledge, it is almost certainly changed by the altered mucus compositions.

While certain changes in mucus permeability are beneficial to health, mucus dysregulation is often associated with disease. In the lung diseases CF and chronic obstructive pulmonary disease, for example, lung mucus is thickened, resulting in decreased permeability [70–72]. Mucus is thickened due to the hyperconcentration of mucus components [73] (Figure 3a) including mucins, filamentous actin, bacterial DNA and polysaccharides [1], but also due to increased intermolecular disulfide cross-linking of mucins [74] and impaired secretion of bicarbonate, which sequesters calcium to prevent calcium-mediated mucin compaction [75]. Mucosal diseases can also be associated with increased mucus permeability. For example, an unstirred inner colonic mucus layer is thought to prevent contact between the colonic epithelium and bacteria. However, in UC, bacteria penetrate to the epithelium, which is likely mediated by a compromised mucin mesh (Figure 3b) [8]. Another example where increased permeability can be problematic is during pregnancy. Pregnant women at high risk for preterm birth have cervical mucus plugs that exhibit compromised barrier integrity compared to women undergoing healthy pregnancies (Figure 3c) [68]. The causes of increased permeability in these cases are not understood in molecular detail, but some possibilities include altered mucin glycosylation [76], mucin cleavage [77], pH changes [78], and microbiome composition [79].

Given that permeability is a sensitive indicator of health and disease, we anticipate that mucus permeability will

Figure 3



Mucus permeability is a marker for disease. **(a)** Mean squared displacement of 1  $\mu\text{m}$  carboxylated beads in normal (2.5% solids) and CF-like (8% solids) mucus. Hyperconcentrated mucus presents a greater barrier to diffusion. Reprinted from Matsui *et al.* (2006) [72] Copyright (2006) *National Academy of Sciences*. **(b)** Penetration of 2  $\mu\text{m}$  (green) and 0.5  $\mu\text{m}$  (red) nanoparticles through colonic mucus layer to epithelium (blue) in healthy (control) patient and patient with UC. Penetration of beads in UC patient indicates increased

serve as a valuable biomarker for mucosal diseases throughout the body. It is striking that the same molecular defect which drives lung morbidity in CF — a dysfunctional anion channel called CFTR — also causes intestinal mucus to be thick, adherent, and static, with a concomitant reduction in permeability [80]. Another example of global mucus dysregulation may be Crohn's disease and ulcerative colitis (UC), which are both also associated with periodontal disease [81] and thus potentially saliva dysregulation given the importance of saliva for maintenance of oral health [82]. The implications from this are twofold — first, that mucus dysregulation may be affected across the body and second, that one could potentially use easily accessible mucus samples, such as saliva, to gather information about less accessible surfaces, such as the lungs, or the intestine. We anticipate that valuable insight can be gained by measuring permeability across the body, and that this could help us find new ways of predicting and diagnosing disease [83].

Understanding the biochemical mechanisms that underlie mucus permeability dysfunction can also direct the design of intervention strategies [84]. Thickened lung mucus in CF, for example, presents an even more challenging barrier to therapeutic delivery than healthy mucus. Some current strategies therefore focus on increasing mucus permeability by altering the organization of the mucus gel. For example, dissociating disulfide bonds with N-acetylcysteine [13], counteracting calcium-mediated mucin compaction with bicarbonate or other calcium chelators [80,85,86], disrupting hydrophobic crosslinking with surfactants [11], and diluting mucus using osmolytes such as hypertonic saline and mannitol to increase mucus liquid content [87] are all strategies that thin mucus and in some cases have improved *in vitro* nanoparticle transport. Overall, countering the increased tenacity of mucus in disease shows great promise for improving nanoparticle delivery. However, the same treatments that increase mucus permeability also typically increase clearance rate, thus reducing the time available for particles to penetrate to the epithelium, so it will be important to find the optimal balance between

permeability of inner colonic mucus layer. Reprinted with permission from Johansson *et al.* (2013) [83] *BMJ Journals*. Published under CC BY license <https://creativecommons.org/licenses/by/4.0/legalcode>. **(c)** Diffusion of positively charged peptides into native cervical mucus of ovulating patients and pregnant patients at low and high risk for preterm birth. Top: schematic of microfluidic device for measuring diffusion. Fluorescently labeled cationic peptides diffuse from buffer into mucus-filled channel, and the diffusion is then quantified and plotted. Middle: representative 900 seconds diffusion timepoints for cervical mucus from women in each group. Bottom: averaged diffusion time courses over multiple patients from each group. Different colors on bottom schematic indicate different time points. Greater enrichment of peptides in low-risk mucus indicates greater adhesiveness to cationic peptides. Reprinted with permission from Smith-Dupont *et al.* (2017) [68] *NPG*. Published under CC BY license <https://creativecommons.org/licenses/by/4.0/legalcode>.

these two parameters. For the opposite situation, in which a thinned mucus barrier causes disease, it would be desirable to stimulate mucus secretion, but this area of research has received little attention thus far. Alternatively, therapeutically introducing mucus replacements may be a useful future direction of research.

## Conclusions

Mucus is a selectively permeable hydrogel that acts as a barrier to particle diffusion across multiple size scales. Mucus slows diffusion of small particles via interaction filtering, while potentially fully blocking penetration of larger particles via steric filtering and interaction filtering. While some basic principles, such as the role of net charge or hydrophobicity, are understood, the effects of spatial variation of charge and hydrophobicity on mucus binding are only beginning to be unraveled. Finely engineering appropriate mucus interactions may have applications in drug or nanoparticle design, where uniform mucoinert surfaces may be limiting for physiological effect. Finally, mucus properties change in distinct states of health and disease, which manifests as altered permeability to cells, viruses, and nanotherapeutics. Understanding and addressing the molecular mechanisms of altered permeability could therefore advance treatment of mucus-associated diseases, and permeability may also be a valuable biomarker for disease.

## Conflicts of interest statement

None declared.

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