The biology of mucus: Composition, synthesis and organization☆

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Abstract

In this review we discuss mucus, the viscoelastic secretion from goblet or mucous producing cells that lines the epithelial surfaces of all organs exposed to the external world. Mucus is a complex aqueous fluid that owes its viscoelastic, lubricating and hydration properties to the glycoprotein mucin combined with electrolytes, lipids and other smaller proteins. Electron microscopy of mucosal surfaces reveals a highly convoluted surface with a network of fibers and pores of varying sizes. The major structural and functional component, mucin is a complex glycoprotein coded by about 20 mucin genes which produce a protein backbone having multiple tandem repeats of Serine, Threonine (ST repeats) where oligosaccharides are covalently O-linked. The N- and C-terminals of this apoprotein contain other domains with little or no glycosylation but rich in cysteines leading to dimerization and further multimerization via S—S bonds. The synthesis of this complex protein starts in the endoplasmic reticulum with the formation of the apoprotein and is further modified via glycosylation in the cis and medial Golgi and packaged into mucin granules via Ca2+ bridging of the negative charges on the oligosaccharide brush in the trans Golgi. The mucin granules fuse with the plasma membrane of the secretory cells and following activation by signaling molecules release Ca2+ and undergo a dramatic change in volume due to hydration of the highly negatively charged polymer brush leading to exocytosis from the cells and forming the mucus layer. The rheological properties of mucus and its active component mucin and its mucoadhesivity are briefly discussed in light of their importance to mucosal drug delivery.

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Fig. 1. Mucus producing epithelial goblet cells. A. Histological section of colonic mucosa showing the epithelial cell layer containing mucus producing cells. Goblet cells appear as cells containing a large dilated rounded apical region containing mucus secretory granules. The goblet cells are interspersed among simple columnar colonocytes (Condon [36]: website for Basic Histology D502, Indiana University Purdue University Indianapolis). B. Transmission electron micrograph of a single typical intestinal goblet cell. Morphological features include a basal nucleus (N) and rough endoplasmic reticulum (RER), a central supranuclear Golgi apparatus (G), and the characteristic large dilated apical region containing translucent mucus droplets (MD) secretory granules (Moran and Rowley [114] Visual Histology Atlas website). C. A schematic representation of a typical goblet/mucous cell showing basal nucleus and mitochondria, a supranuclear Golgi body, the large dilated apical region containing the mucus granules and apical microvilli [113]. Fig. 1A is reproduced from Condon [36] http://www.iupui.edu/~anatd502/Labs.I04/epithelia/s20lab/s9940x1.jpg by permission from K. Condon. Fig. 1B is reproduced from Moran and Rowley [114] by permission from http://www.visualhistology.com/ Fig. 1C Reproduced from Molinoff and Atkinson [113] by permission of the author, P. Molinoff.
1. Introduction

Mucus is a complex viscoelastic adherent secretion that is synthesized and secreted by specialized goblet and mucous cells in the columnar epithelia that line the lumen of all of the organs and glands that are exposed to and communicate with the external environment [3,38] (Fig. 1). This includes the inner linings of the respiratory tract, the gastrointestinal tract, the reproductive tract and the ocular surface. Historically, mucus was certainly known since ancient times. For example Phlegm has origins in 3rd century Greek φληγμα (phlegma) and Hippocrates (460–370 BCE), extended the humoral theories first stated by Empedocles (504–433 BCE) postulating the existence of 4 humors, black bile, yellow bile, blood, and phlegm. A balance of these humors was associated with health and an excess or deficit of any one was associated with disease. The Chinese pharmacopoeia Book of Herbs (Pen Ts’ao) dating to 2500 BCE mentions the use of ‘ma huang’ which contains ephedrine and pseudoephedrine, found in current “Sudafed” cold remedies which are used to alleviate symptoms associated with mucus hypersecretion in upper respiratory infections [182].

Mucus has been described in organisms from all kingdoms [97]. Viscous, gel forming mucilages and structural glyco-substances are found in all forms of life, including viruses, bacteria, fungi, plants, insects, fishes, etc. but this review concentrates specifically on mammalian mucus and its mucin glycoproteins. Vertebrates contain mucous layers in their corresponding organ systems. Additionally, most aquatic organisms also possess an external mucus layer on their skin [128]. Mucus has also been studied from invertebrates (worms, snails, slugs, insects, anemones). For example the hogfish is able to explosively release liters of mucus from its skin which is used as a prey escape mechanism [186, 151]. Mucus layers have also been described in various pathogenic protozoa [161]. Zebra fish provides an excellent model system currently much in vogue to study the effects of mucus in vivo [24,78].

Mucus serves many protective functions for the underlying epithelia, such as lubrication for material transport and hydration over the epithelium particularly in the respiratory tracts, eyes and mouth that are exposed to and communicate with the external environment [3,38]. This arrangement is thought to facilitate mucus expulsion and clearance from the lung and airway passages by the action of cilia via mucociliary transport [25,146].

Mucus is secreted by goblet/mucous cells (for a recent review see [171]) which are interspersed with many other types of specialized epithelial cells (Fig. 1A), and have a similar typical highly polarized morphology with a broad apical region and narrower basal region, giving them their characteristic ‘wine glass goblet’ shapes (Fig. 1B and C). The apical region contains large, supranuclear, mucus containing storage and secretory granules, a central supranuclear endoplasmic reticulum and Golgi apparatus, a basal nucleus, and sub-perinuclear rough endoplasmic reticulum and mitochondria (Fig. 1B and C). These cells are surrounded by other columnar or squamocolumnar epithelial cells with specialized functions such as ciliated, absorptive, secretory or proliferative cells, and are attached to their neighboring cells by typical tight junction structures. The goblet mucous cell types are distinguished based on gross morphology. In general goblet cells are tapered, decreasing in circumference (e.g., get thinner) from apex to base, whereas mucous cells have near constant diameter from apex to base. Both types of cells have large stores of apical mucus secretary granules, occupying 75% of the cell volume [53]. Goblet cells regulate the production of mucins in response to external factors. For example, the response of goblet cells to enteric infections is discussed in [89]. Goblet cells, mucus and mucins also interact with the immune system as discussed in [121].

2.2. Mucus structure

Scanning electron microscopy (SEM) of gastric mucosal surfaces [58, 119] show the highly folded structure of stomach epithelium with gastric glands and fibrous mucus. Electron microscopic studies of mucus gels are strongly influenced by dehydration during sample preparation (see for e.g. [41,110]). Familiari et al. [55] developed a multi-step process to preserve the real microarchitecture of mucus covering the jejunum surface and the zona pellucida surrounding the mature oocyte and show the network of the dried mucosal fibers. Cryo-SEM [91] of pulmonary mucus preserves the structure of better showing a highly heterogeneous mesh with both large and small pores and a thick polymer scaffold. We examined the structure of hydrated mucus on the sub-micron length scale in vitro by atomic force microscopy (AFM) in a liquid cell under appropriate buffers. A tapping mode AFM measurement of a wet sample of human mucus taken from the discarded material obtained in the lavage following a stomach biopsy reveals a swollen network similar to that formed by the glycoprotein mucin [74] with aqueous pores of about 200–300 nm in diameter.

2.3. Mucus thickness

The thickness of the mucus layer varies considerably both within and between different organ systems. For example, in the respiratory tract mucus layer thickness is about 10 μm in the trachea [112] while in the gastrointestinal tract, the mucus layer increases in thickness from the upper to the lower GI tract varying from 200 μm to 800 μm respectively [5]. Table 1 gives thicknesses of the mucus layer in the GI tract. In the eye the mucus layer that covers the corneal and conjunctival surfaces has a thickness of 1–7 μm [64,73,82], and in the female reproductive tract the thickness and viscosity of the mucus varies with the ovulatory cycle and regulates sperm penetration [86] and uterine implantation of the fertilized egg [28]. These differences in mucus thickness may reflect the various protective functions and challenges of mucus within its specific organs.

Mucus in the respiratory, ocular and gastrointestinal tracts has been shown to exist in two layers with distinct rheological properties [155]. For example, in the gastrointestinal tract the first 100–150 μm thick layer which is more viscoelastic, is in intimate contact with the epithelial cell layer, and a second, less viscous layer of thickness 100–700 μm lies on top of it extending to the luminal surface. It has also been shown that bacteria have differing affinities for these two layers [80]. In contrast, in the respiratory tract, the lower viscosity periciliary mucus layer consisting of hydrated membrane mucins and mucopoly saccharides, is in contact with the airway epithelium and supporting the higher viscosity mucus on top of it, which is exposed to the air. This arrangement is thought to facilitate mucus expulsion and clearance from the lung and airway passages by the action of cilia via mucociliary transport [25,146].

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### Table 1

<table>
<thead>
<tr>
<th>Thickness (μm)</th>
<th>Location in gastric mucosa</th>
<th>Corpus</th>
<th>Antrum</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td></td>
<td>150</td>
<td>275</td>
<td>170</td>
<td>125</td>
<td>480</td>
<td>830</td>
</tr>
<tr>
<td>Firmly adherent</td>
<td></td>
<td>80</td>
<td>155</td>
<td>15</td>
<td>15</td>
<td>30</td>
<td>115</td>
</tr>
<tr>
<td>Loosely adherent</td>
<td></td>
<td>110</td>
<td>120</td>
<td>155</td>
<td>110</td>
<td>450</td>
<td>715</td>
</tr>
</tbody>
</table>

Average values reported by Atuma et al. [5]. Note: we have rounded the numbers to 5 μm, well within the range of values reported.
3. Mucus composition

Mucus is a complex dilute aqueous viscoelastic secretion consisting of many components: water, electrolytes, lipids and various proteins. Creeth [40] provides an excellent review describing the constituents of mucus and methods to separate them. Water comprises approximately 90–95% of mucus and serves as the solvent and diffusion medium for all the other mucus components.

3.1. Mucus electrolytes

Electrolytes are an important component of mucus and their composition can be different in mucus from various tissues, and are determined by the underlying secretory epithelium. These differences may be important to the specific environmental challenges found in each organ. Common to most mucus secretions are sodium and potassium and chloride and sodium bicarbonate, phosphate and magnesium and calcium. Common to most mucus secretions are sodium and potassium and chloride and sodium bicarbonate, phosphate and magnesium and calcium. Approximately 90% of mucus and methods to separate them. Water comprises approximately 90–95% of mucus and serves as the solvent and diffusion medium for all the other mucus components.

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3.2. Mucus lipids

Lipids constitute 1–2% of mucus. Many lipids have been described to be both covalently and non-covalently associated with mucus layers [71,102]. These include phospholipids, primarily phosphatidyl choline and phosphatidyl glycerol and small amounts of lysophosphatidyl choline generated by phospholipase A2 in some pathologic conditions, cholesterol, fatty acids both free and acylated to proteins. The lipids interact with mucus glycoproteins and affect the wettability (charged lipids) and hydrophobicity (neutral lipids) and barrier functions of the mucus layer [102]. Lipids may also contribute to the lubricating and surface tension properties of the mucus layer. The rheological properties of mucus are also strongly affected by lipids [65,172]. Lipids also serve to prevent evaporation of the aqueous phase, for example in the tear mucus layer covering the eye [39,82].

3.3. Mucus proteins

Mucin glycoproteins constitute the major functional component of mucus, however ancillary non-mucus proteins play an important role in the structural and protective functions of mucus. Recently many mucus proteins have been identified by high-throughput, proteomic mass spectrometric, chromatographic and 2D-PAGE methods. One hundred eighty six different proteins have been identified in airway, 147 or 685 in cervical, 111 in nasal and 145 in gastrointestinal tracts and are found in the mucus layers of all organs [29,143,147,154]. Many of the proteins present are serum proteins, presumed to arise from epithelial serum transudation. Most important proteins found can be broadly classified into functional groups including defensive proteins, growth factors, structural proteins and glycoproteins.

The major defensive proteins are involved in contributing to the mucus protective abilities against pathogens and particulate matter.

These include the alpha and beta defensins, which are antimicrobial peptides [14,105] and lysozyme (muramidase), an enzyme that digests muramic acid mucopolysaccharide bacterial cell wall components. The positive charge of lysozyme enables binding to the negatively charged mucus; its bactericidal properties were discovered by Alexander Fleming [57]. Lactoferrin, in all mucosae, is an intrinsic factor (in the stomach) that chelates iron which limits its availability for bacterial growth. Statherins, proline rich peptides and histatin defensive proteins have also been identified in mucus [76].

Immunoglobulins, secretory IgA and IgM are present in all mucus layers and are active against a variety of pathogens. Immunoglobulins have also been reported to increase the viscosity of mucus in airways [65] and IgA and IgA have been shown to bind differentially to cervical mucus [54]. Secretory IgA has been shown to be required to bind to the mucus layer for effective antibacterial defense [138,158].

The major growth factors present on almost all mucus layers are transforming growth factor beta, epidermal growth factor and hepatic nuclear factor [35]. One or more of these growth factors are present in mucus layers of all organs and may serve growth, maintenance, repair and regulatory functions to the underlying epithelium. Additionally, there are various cytokines present that are released from epithelial or immune cells in response to various stimuli, pathogens or disease states.

Structural proteins include protease inhibitors such as secretary leukocyte proteinase inhibitor and pancreatic secretory trypsin inhibitor. These proteins protect the mucus proteins from proteolysis, denaturation and loss of viscosity by enzymes of both host and pathogen origins. Also present are the trefoil (TFF) peptides. These are small cysteine rich peptides with a characteristic disulfide linked three loop clover leaf conformations [139,174]. They have been reported as agents associated both with mucus interactions, increasing its viscosity [157], protection from proteolysis and as epithelial repair growth factors.

3.4. Mucins

The major functional components of mucus are the mucin glycoproteins present at a concentration of 1–5%. They are responsible for the essential and predominant viscoelastic properties of the mucus layer. Details of mucin genes, glycoprotein structure and biophysical properties have been presented in considerable detail in previous reviews [6–8,38,45,72,123,134,137,158], Mucins are broadly classified as two types, secreted mucins and membrane bound mucins. Both membranes and secreted mucins share many common characteristic compositional and structural features based on the fact that their underlying protein sequence is governed by the same family of genes. Based on sequence and domain comparisons and similarities, and cellular origins, 22 mucin genes have been identified and can be grouped into two broad classes representing membrane-bound mucins, anchored by insertion through the plasma membrane and secreted mucins, that are packaged in and secreted extracellularly from secretory granules. The membrane mucins include MUC 1, 3A, 3B, 4, 11, 12, 13, 15, 16, 17, 20 and 21. The secretory mucins include MUC 2, 5AC, 5B, 6, 7, 8, 9, and 19 [3,134]. The secretory mucins can be further subclassified as 1) large gel forming mucins MUC 2, 5AC, 5B, 6, 19 and 2) small soluble mucins MUC 7, 8, 9. Immunohistochemistry has been used to identify expression of different mucins in many organs in healthy as well as diseased states with a significant focus on cancer (see for e.g. [10,16,34,81,99,100,104,129]).

3.5. Glycosylation

Both membrane and secreted mucins are highly glycosylated, consisting of approximately 50–80% (w/w) carbohydrates, primarily composed of GalNAc, GlcNAc, Fuc, Gal and sialic acids or NANA or NeuNAc (N-acetyl neuraminic acid) and relatively small amounts of mannose. The oligosaccharide chains possess approximately 2–20 monosaccharides in both linear and moderately branched structures (see for e.g. [21,104]). The oligosaccharides are attached to the mucin
protein core by alpha-1-O-glycosidic bonds from GalNAc to the hydroxyl side chains of serines (Ser) and threonines (Thr). In a recent paper, Jin et al. [79] present a structural map of human gastric glycosylation from healthy and diseased subjects showing a large structural diversity with over 200 O-glycan structures identified in the total sample of 10 individuals. To date, no single consensus O-glycosylation amino acid sequence motif has been identified, however O-glycosylation sites can be roughly predicted by statistical, and artificial intelligence analysis of databases of experimentally determined glycosylated proteins (NetOGlyc, OGlycBase) [26,27,70,84]. A large proportion of the Ser/Thr residues are predicted to be glycosylated. This results in a “bottle brush” configuration of the oligosaccharides arranged around the mucin protein core (see Fig. 2). Elongation and branching of the oligosaccharide side chains are governed and performed by a large family of glycosyltransferases [23,156,160]. It is believed that the vast diversity in mucin oligosaccharide structures is determined by the tissue specific expression patterns and combinations of the glycosyltransferases and their substrate specificities [13,63,137]. Additionally, the oligosaccharides may be modified by the addition of blood group (ABO, Lewis), secretor (H) epitopes [135], and terminal negatively charged sialic acids and sulfates. The large amounts, distribution and density of glycosylation, steric exclusion, and charge repulsion, impart a highly hydrated, extended, relatively stiff elongated linear “rod-like” conformation to the mucins [62,109]. These extended mucin structures have been directly observed by both electron and atomic force microscopy and exhibit molecular lengths of 50–1000 nm up to 8 μm [74,109,144] for a variety of secreted mucins from various sources. This extensive glycosylation also serves to protect the mucin protein core from proteolytic degradation. Keismer et al. [88] used electron and atomic force microscopy to show 190–1500 nm long tethered mucin polymers, and a finely textured glycoalyx matrix filling interciliary spaces of airway mucus. AFM methods for measuring binding forces of glycans to lectin and other groups deposited on AFM

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**Fig. 2.** Structure of mucin glycoproteins. The hierarchical structure of mucin depicted schematically. A. The various non-glycosylated and glycosylated domains of mucin as discussed in the text under mucin structure are indicated. B. The mucin monomer consists of a linear protein core with a serine/threonine rich tandem repeat region where oligosaccharides (red) are linked O-glycosidically. C. Mucin monomers dimerize via formation of C terminal S–S bonds of cysteine groups as indicated by the gray arrowhead. D. These dimers further polymerize forming multimers. Occasional branching can occur as indicated by an N terminal (blue circle) polymer branch. (Reproduced from Bansil et al. [8] under Creative Commons Attributable License).
cantilevers have also been applied to investigate the distribution of glycans from mucins such as sialic acid of ocular mucins [9] and gastrointestinal mucins [69].

Both membrane and secreted mucins also contain N-glycosylated multiantennary complex and hybrid oligosaccharide high mannoside structures, which are synthesized in the secretory pathway by the en bloc transfer of preformed mannosone oligosaccharides to the amide side chain of Asparagine (Asn) residues located in well-defined N-X-T/S sequence motifs at the ends of mucin proteins [34,150,183]. Secreted mucins also contain a novel type of glycosylation, C-mannosylation, modifications that involve a carbon-long carbon bond between the C1 of mannose and the indole ring of specific tryptophans (W) in W-X-X-W motifs [125].

The combined extensive glycosylation gives mucins a higher buoyant density in CsCl (1.45 g/cm³) than typical non-glycosylated proteins (1.2 g/cm³) or lipids (<1.0 g/cm³) yet less than nucleic acids (>1.6 g/cm³) [42,67]. This feature along with their large molecular sizes (>2 MDa) results in facile methods for purifying and analyzing mucins by isopycnic, density gradient ultracentrifugation [67] and size fractionation by size exclusion chromatography and agarose electrophoresis [67,130], respectively.

3.6. Mucin protein core, tandem repeat domains, non-repeat domains and cysteine rich domains

The apomucin protein core (100–500 kDa) for both membrane and secreted mucins, comprises approximately 20% (w/w) of the mucin mass. It is organized into two broadly distinct regions, 1) a centrally located tandem repeat region rich in Ser, Thr, and Pro (STP-tandem repeats) which contains O-glycosylation, and 2) the carboxy- and amino-terminal non-repeat regions, which may be rich in cysteine (Cys) residues with low amounts of Ser/Thr and relatively little O-glycosylation, but possessing N-glycosylation. The amino acid composition, sequences and structures of these terminal Cys-rich regions are more typical of secreted globular proteins [38,123].

The Ser/Thr repeat units can vary in length from 7 to 375 amino acids in MUC19 and MUC3A respectively. The number of repeats within a mucin molecule can also vary from 10 to 500 as inheritable Variable Number of Tandem Repeats (VNTR) polymorphisms [169]. The amino acid sequences of the repeat units are very similar (70–90% identical) within a particular mucin gene within an individual of a single species, but can vary significantly between homologous mucin genes in different species [137].

In secreted gel forming mucins, Cys-rich regions of the protein core are located at both the amino and carboxy ends of the central tandem repeat region. In some secreted mucins (MUC5AC and MUC5B) Cys-rich sequences (CysD) can also be found periodically interspersed within the Ser/Thr tandem repeats. The Cys-rich regions can contain up to 10% Cys and relatively little Ser or Thr residues that are arranged in highly conserved, specific patterns and can be used to classify mucins [47,97,123]. The cysteines can be involved with both intra- and intermolecular disulfide bonds or in contributing to protein flexibility between tandem repeat domains. These regions contain few Ser/Thr or O-glycosylation and have an overall amino acid composition and conformation characteristic of globular secreted proteins. Multiple Cys-rich von Willebrand Factor D (vWF-D) domains are found at the amino end of secreted mucin molecules [85,123]. These domains have been determined to be involved in the multimerization of both mucins and von Willebrand factor (vWF) [108,122,124].

At the carboxy terminal end of the central tandem repeat domains of secreted mucins, are Cys rich regions similar to vWF D domains, vWF C domains and carboxy terminal cysteine knot (CTCK) domains which are required for dimerization [12,181]. The mucin carboxy terminal vWF-D domains have similar sequence, structure and dimerization functions as those located at the amino terminal.

4. Mucin synthesis and secretion

In this section we consider in some detail mucin synthesis and secretion leading to the formation of a hydrated mucus layer. This subject has been recently reviewed [34,43]. We will consider how mucins are synthesized, co- and post translationally modified, transported packaged, stored and secreted. Synthesis of large amounts of high molecular weight, complex extensively glycosylated mucins represents a significant metabolic commitment by the cell. We consider mainly secreted gel forming mucins (MUC2, 5AC, 5B, 6, 19), but the general description also applies to membrane mucins. These pathways have been studied for a wide variety of secretory products, including mucin in different cell types, and organisms and have yielded basic mechanisms and components that are relevant and applicable to all mucins.

4.1. Synthesis pathways

Mucin synthesis and secretion proceeds by both constitutive pathways, characterized by continuous release of newly synthesized products and regulated pathways involving storage of mucin products that are released in response to various stimuli in a calcium dependent manner. In this description we consider primarily the regulated pathway, although the initial steps of synthesis and assembly through the trans Golgi vesicular budding, also apply to the constitutive path.

4.2. Mucin synthesis

The various steps in mucin synthesis are listed in Table 2. As with many other secreted glycoproteins, the synthesis of the protein core of mucin (apomucin) begins on membrane bound ribosomes of the rough endoplasmic reticulum (RER). This involves ribosomal docking to the translocon complex, a large (> 125 Å) [111] membrane associated assembly of over 25 proteins including Sec61p [118] through which the growing nascent apomucin is transported into the lumens of the endoplasmic reticulum (ER). A number of post translational modifications (PTM) of the apomucin protein take place co-translationally or very early in synthesis, namely N-glycosylation, C-mannosylation and dimerization.

4.3. N-glycosylation

N-linked glycosylation is accomplished co-translationally by transfer of preformed high mannoside oligosaccharides from dolichyl-pyrophosphate-Glc₃Man₉GlcNAc₂, to the side chain amide group of asparagines (N) in consensus motif N-X-T/S (where X is any amino acid except proline) sites located in the mucin Cys-rich regions catalyzed by the enzyme oligosaccharide transferase which is closely associated on the ER luminal side of the translocon complex [32,150,178]. Immediately after N-glycosylation, N-linked oligosaccharides are further modified in the ER by removal of two Glc residues by [177] α1,2- and α1,3
mannosylation may be required for export from the ER and decreased secretion, suggesting that C-sulfation can constitute 50% [4.6]. O-glycosylation and transported to the ERGIC-53p (ER to Golgi Intermediate Compartment Protein 53 kDa) by condensation by the formation of vesicles that bud off by means of cargo receptors VIP36 (Vesicular Integral Protein 36 kDa) and ERGIC-53p (ER to Golgi Intermediate Compartment Protein 53 kDa) and transported to the cis-Golgi on microtubules [120,136,177].

4.4. C-mannosylation

C-mannosylation, an unusual novel PTM also occurs cotranslationally. It involves transfer by the enzyme C-mannosyl transferase [49] of a single mannose residue from dolichol phosphate mannose to the indole C2 of the first tryptophan (W) in the consensus W-X-X-W motifs [83]. These motifs are also usually found within the Cys-Rich domains of mucins. Although the precise function of C-mannosylation is unknown, mutation or absence of the mannosylation site or expression of mucins in C-mannosylation deficient cells, results in the accumulation of protein in the ER and decreased secretion, suggesting that C-mannosylation may be required for export from the ER via cargo receptor binding and transport vesicle packaging [125]. Thus both N-glycosylation and C-mannosylation are required for mucin dimerization, folding and for export from ER to Golgi. Mucin products at this point are presumed to have a globular, random coil conformation with molecular weights of 250–500 kDa for disulfide linked monomers and 0.5–1 MDA for dimers and multimers [158].

4.5. ER to Golgi transport

Continued synthesis of correctly folded proteins proceeds by packaging into COP II (coat protein complex) transport vesicles with the assistance of cargo receptors VIP36 (Vesicular Integral Protein 36 kDa) and ERGIC-53p (ER to Golgi Intermediate Compartment Protein 53 kDa) and transported to the cis-Golgi on microtubules [120,136,177].

4.6. O-glycosylation

Once in the Golgi, mucin proteins acquire their most prominent and characteristic salient feature, namely extensive O-glycosylation, which can constitute 50–90% of the final mucin molecular mass. In contrast to N-glycosylation which transfers, en bloc, a large preformed oligosaccharide from a lipid precursor, O-glycosylation occurs by the sequential addition of single monosaccharides from nucleotide sugar precursors. O-glycosylation is accomplished in three principal steps, initiation, elongation and termination [23]. The first step of initiation, which occurs in the cis- and medial-Golgi (reviewed in [137]), occurs by the addition of a single GalNac from a UDP-GalNAc precursor, from its reducing end C1 to the hydroxyl side chains of serine (Ser) and threonine (Thr) residues, by the action of enzyme UDP-N-acetylgalactosamine:polypeptide galactosaminyl transferases (GalNAc-Tase) to yield GalNAc-α1-O-Ser/Thr [159]. There are 10–20 GalNAc-Tase type II membrane proteins [156] which are conserved from fungi to mammals. Some of these initiating GalNAc-Tases (GalNAc T1-T6 and T10-T14) transfer the initial GalNAc to unglycosylated protein substrates, whereas other GalNAc-Tases (T7-T9) enzymes containing a carboxy-terminal rich in lecin (Gal/GalNAc binding) domain that is required for binding to substrate, complete the dense O-glycosylation of clustered Ser/Thr residues [170]. After addition of the initial GalNAc, mucin molecules have a molecular weight of 0.5–2 MDA and would be slightly less flexible and more extended in the STP-tandem repeat regions due to steric exclusion of the carbohydrates [145].

All mucin O-linked oligosaccharide structures are built on a limited number of eight common “Core” structures, based on the addition of monosaccharides Gal or GlcNAc directly to different positions on the initial GalNAc to form linear or branched Core structures. Many different Core structures can be present on a single mucin molecule [23,131,132]. Elongation of the basic core structures continues by stepwise sequential addition of monosaccharides from nucleotide precursors by alternate action of various GlcNAc and Gal transferases. Alternating Gal-GlcNAc disaccharides either beta 1-3 or beta 1-4 lactosamines are common. The elongated structures may be either linear or branched. The specific structures that are formed are determined by the cell or tissue specific expression patterns and substrate specificities of the glycosyltransferases. Thus it is possible for the same mucin peptide to have different glycosylation structures when expressed in different cells or tissues. For example, for cervical mucin glycosylation, the specific combination of glycosyltransferases, and their levels are hormonally regulated during the menstrual cycle [4]. More than 100 different O-linked oligosaccharide structures have been identified on a single colonic MUC2 protein [98].

Termination of O-linked oligosaccharide chain growth occurs in the medial- to trans-Golgi compartments by the competitive enzymatic addition of fucose, GalNAc or NeuNAc (Sialic Acid) mainly as the α-anomers to the periphery of the growing oligosaccharide chains. These terminal structures can include blood group ABO, Lewis ABXY and H secretor antigens [135]. Terminal sugars can also be modified with sulfates. Of the eight core structures, only cores 1–4 and 6 are substrates for the sulfotransferases [22]. It should be noted that the N-linked oligosaccharides also undergo trimming, elongation and modification in the Golgi to obtain high mannose, complex and hybrid type structures. The addition of the terminal sialic acid (NeuNAc) and sulfates contribute large amounts of negative charges to the mucin oligosaccharides. This causes charge and steric repulsion between the oligosaccharide chains and an extension and stiffening of the mucin molecule to an extended rod-like configuration with a “bottle brush” arrangement of oligosaccharides around the apomucin core, and with molecular weights from 2 to 10 MDa and occupation of a large hydrated volume in solution.

4.7. Granule condensation

As oligosaccharide construction is completed, mucins in the trans-Golgi network (TGN) are packaged into vesicles that bud off by means of the cargo receptor VIP36 (vesicular integral protein 36 kDa). Mucin packaged in vesicles undergo maturation to secretory granules (SG) by condensation by the formation higher order mucin multimers by disulfide association of amino-terminal vWFD domains, to form molecules that are 10–50 MDa extended rods with linear dimensions 1–10 μm [66,122], which are packaged into vesicles of <1 μm. Verdugo [164] applied the ideas of a large volume gel phase transition [101, 153] in charge polymer gels to mucins to both explain and demonstrate how this packaging is possible (for a recent review see [166]). The many repulsive negative charges (from sulfates and sialic acids) on the extended mucin molecules can be neutralized, shielded and crosslinked/complexed with positive counter ions such as H+, Na+, Ca2+, resulting in charge shielding, allowing mucin gels to undergo a 100 to 200 fold reversible volume collapse phase transition. This was demonstrated with mucins by increasing the extracellular solution concentration of H+ and Ca2+ to those concentrations found in secretory granules and observing shrinkage of the mucin gels. It has been shown that progression through the secretory pathway results in luminal compartments with increasing H+ and Ca2+ concentrations [46] with ER at pH 6.8, cis Golgi at pH 6.7, medial-Golgi at pH 6.3, trans-Golgi at pH 6.0 and secretory granules at pH 5.5. This is accomplished by an increasing density of a membrane bound vesicular
The intragranular proton pump that transports H⁺ into the Golgi lumen and by a decrease in leakage of H⁺ from the lumen [46,175]. Similarly, intragranular Ca²⁺ concentrations (23 mmol/kg) are found to be higher than intracellular or other compartment Ca²⁺ concentrations (6 mmol/kg) [77,93,117]. Once mucin’s charge repulsion has been neutralized by H⁺ and Ca²⁺, hydrophobic interactions between mucin molecules and lipids can dominate causing mucin condensation within the secretory granules.

4.8. Granule packaging

The intragranular condensed state of the mucin secretory granule matrix has been elegantly investigated by Perez-Vilar using fluorescent confocal microscopy and FRAP (fluorescence recovery after photobleaching) of recombinant GFP-mucin domain constructs that are packaged into secretory granules. Results indicate that the diffusion constant of GFP is greater in the ER or extracellularly (1.42 μm²/s) than in the secretory granule (0.014 μm²/s) demonstrating more restricted movement and a more condensed state in the granules than ER. This also indicates the existence of pores or spaces within the granule matrix that allow movement of small molecules which is important for exocytosis [126].

4.9. Secretion and exocytosis

After secretory granule budding from the TGN, condensation and maturation, mature secretory granules (MSG) move toward the peripheral apical areas of the cell for storage and secretion. Four broad steps and many substeps and mediators are involved in the secretory process (see Fig. 3): 1) transport of MSG from the TGN to the apical inner cell plasma membrane, 2) docking and tethering of MSG to the cell membrane, 3) pore formation by granule membrane to plasma membrane fusion, and 4) exocytosis of MSG contents into the extracellular space. More details can be found in reviews by Davis et al. [43], Evans et al. [53], Rogers [133], and Williams et al. [173].

4.10. Mucin granules

Mucin secretory cells showing granules have been imaged using fluorescence microscopy [126] as well as with non-fluorescent confocal light absorption and scattering spectroscopy (CLASS) [56].
compared CLASS images to fluorescently labeled granules from the same cell line and showed improved resolution from this label-free technique. An image of a mucus producing gastric cell (AGS cell) obtained by CLASS showing mucin granules ranging in size from 0.5 to 2 μm is shown in Fig. 4A.

4.11. Exocytosis

MSG and plasma membrane fusion causes the formation of an open pore having an “omega” structure ‘Ω’ [116]. The pore allows the interior of the MSG to be exposed to the extracellular environment. Verdugo applied the gel phase transition model to explain the mechanism by which mucins leave the secretory granule through the pore [165]. Adler et al. [1] describe the proteins involved in the fusion of vesicle with granules to the cell membrane and the signaling factors involved in activation of Ca\(^{2+}\) release leading to the secretion of mucin (Fig. 4B). As described above for mucin condensation, the MSG contains a condensed porous gel network consisting of collapsed negatively charged mucins complexed with high concentrations of H\(^+\) and Ca\(^{2+}\) counter ions which neutralize and crosslink mucin negative charges. When the MSG interior is exposed to the extracellular milieu, which has lower Ca\(^{2+}\) and H\(^+\) concentrations, the MSG Ca\(^{2+}\) and H\(^+\) rapidly diffuse out of the MSG faster than the large mucin molecules. This results in de-shielding of the mucin anions, yielding a net mucin negative charge and electrostatic repulsion of the mucin from its sialic acids and sulfates. This cationic diffusion also results in the formation of a net positive charge in the area immediately surrounding the extracellular regions of the pore opening. This diffusion driven charge separation defines a Donnan electric potential with mucin serving as the semipermeable membrane containing slowly diffusible negative charges [48,152]. The MSG mucin gel network undergoes a rapid volume expansion phase transition [167] driven by a combination of Donnan potential, mutual electrostatic repulsion of the deshielded mucin anions and by osmotic hydration of mucin saccharides by water influx into the MSG. The mucin molecules expand and spread away from the open fusion pore, covering the external apical surface of the cell.

Experimental verification of this “jack-in-the-box” explosive exocytosis process was demonstrated by the prevention of mucin expansion.

Fig. 4. Exocytosis of mucin from secretory granules. A. Image of gastric cell with granules. Gastric cell (AGS cell) imaged with a non-fluorescent optical technique, confocal light absorption and scattering spectroscopy (CLASS), showing mucin granules ranging in size from 0.5 to 2 μm. The scale bar is 2 μm. Note, the image is pseudo-colored for illustration purposes; the cells were not labeled with any chromophore or fluorescent dye. B. Exocytosis mechanism. Mucin exocytosis is depicted as (a) mucin is packaged in secretory vesicles in a condensed state by complexation with calcium Ca\(^{2+}\). The vesicles fuse with the plasma membrane via interaction with proteins, such as the Rab proteins and unidentified effectors. Activation of various receptors and proteins leads to generation of signaling molecules which activate release of Ca\(^{2+}\), destabilizing the binding of the negative charges on mucin. The diffusion of Ca\(^{2+}\) out of the fusion pore and increased repulsion due to negatively charged mucin, results in extrusion of a highly expanded mucin network. C. Expansion of mucin granules. Schematic summary of electron micrographs of isolated mucin granules in various stages of expansion [87]. (Image in panel A was provided by Dr. L. Qiu. Image in panel B was reproduced from Adler et al. [1]: Creative Commons Attribution License.) (Image in panel C was reproduced from Kesimer et al. [87] by obtaining license to reuse from the American Physiological Society via Rightslink.) Fig. 4C is reproduced from Kesimer et al. [87] by license provided by American Physiological Society and Copyright Clearance Center.
by increasing the extracellular H\(^+\) and Ca\(^{2+}\) concentrations to match those of the MSG interior. This eliminates the MSG interior to extracellular diffusion gradient and also eliminates development of the Donnan potential. Decreasing the external pH from 7.4 to 6.5 and increasing the external Ca\(^{2+}\) from 1 to 4 mM causes a measurable increase in the mucin swelling times and decrease in the diffusion constants from 2.25 \times 10^{-7} \text{ cm/s} to 1.11 \times 10^{-8} \text{ cm/s} [52]. The released mucin then remains in an un-expanded, condensed state and does not undergo the rapid volume expansion phase transition “jack-in-the-box” exocytosis [52,167,168]. Recently, this process has been experimentally observed using electron microscopy of isolated mucin granules in various stages of exocytosis (Fig. 4C) [87].

4.12. Mucus and mucin rheology and mucoadhesion

Although the main focus of this review is on mucus structure, composition and synthesis, we briefly discuss the rheological properties of mucus and mucin, the molecule that gives rise to its viscoelastic properties, as well as its ubiquitous mucoadhesive properties. Rheology of mucus is a major factor in controlling the transport of food, drugs, particles including nanoparticles and larger micron scale particles, and infectious organisms, across mucosal linings, as well as controlling its hydration and lubrication properties. The rheology of mucus from many different animals/tissues/organs has been investigated for a long time (see for e.g., [44,90,95,142,162]). The viscosity of purified mucin, particularly lung, salivary and gastric mucin has also been well studied using bulk, or macro-scale methods [30,75,94,158,176] and at the local, micro-scale using microscopic particle tracking methods [8,61,103]. We refer the reader to a recent review from our group [8] that provides a simple explanation of the basic concepts of rheology (i.e. elastic and viscous properties) and describes the different techniques used. Briefly, mucin and mucin exhibit frequency dependent viscoelastic moduli and their viscosity decreases with increasing shear rate, i.e. they are shear thinning materials. Many of the gel forming mucins, such as MUC5AC from purified porcine gastric mucin exhibit elastic response as well [30]. Georgiadis et al. [61] showed that pH induced gelation occurs not only in porcine gastric MUC5AC but also in porcine duodenal mucin MUC2. The viscoelastic behavior of mucins depends on pH, ionic strength, and degree of hydration and thus is particularly relevant to the transport of particles through mucus [96]. For example, the viscosity of mucus or mucin has been shown to influence binding to small molecules such as nicotine [33] and other drugs [141], and the transport of sperm [50], bacteria [31,107], viruses [19,20] and nutrients [179] through purified mucin or mucus layers.

Finally we add a cautionary note concerning the effect on viscoelastic properties related to differences in the methods for purification of mucin. The methods differ in many ways such as fractionation techniques, and treatment by digestive enzymes which can lead to degradation into subunits [92], or chaotropic agents like guanidium chloride GuHCl which remove associated proteins. These differences in methodology can lead to products differing in the fraction of soluble and insoluble mucins, varying in molecular weight and charge distribution, and the degree to which associated DNA and proteins are removed. Similarly the use of different chromatographic techniques for separation could also account for differences in the properties of the mucin. Recent studies have shown that the use of different methods influences rheology and gelation of the purified mucin [61].

Mucus is highly adhesive to many different substrates owing to the propensity of molecular interactions. Its glycan coat can bind to both neutral and charged surfaces by non-covalent and electrostatic interactions. It also exhibits hydrophobic binding most likely related to the non-glycosylated regions of the peptide. There is considerable interest in controlling mucoadhesivity and coating with polymers, ionic or other materials in designing drug delivery systems capable of transporting through mucosal lining or for topical applications on mucosal surfaces. We refer the reader to two recent reviews on mucoadhesivity [148,180] for this fascinating topic. Another topic of considerable current interest, that we have not discussed concerns the transport of nanoparticles through mucus (see for e.g. [51,59,68,127,140,171]).

5. Future outlook and conclusions

In summary, we have reviewed the basic structure, composition, synthesis and organization of mucus. Research on mucin and purified mucin is currently seeing a significant interest in the context of diseases, particularly cancer and infectious diseases caused by agents that breach the mucosal barrier, as well as in the context of delivering drugs and nutrients across the mucosal barrier. Understanding both the physical and chemical/biochemical interaction of this complex highly heterogeneous soft material is crucial for progress in this area. One of the major obstacles in the progress of this work has been the availability of well characterized and standardized preparation of mucus and purified mucin. An alternative to using purified mucus/mucins for applications where large quantities of material are required is to use synthetic formulations, or even mucin from other sources such as hagfish. However, these will not be identical to mucus, and thus results would have to be interpreted with caution. Another area of research which is just beginning to develop is novel methods for in vitro and in vivo imaging and other techniques to investigate mucus structure, rheology, adhesive properties and transport of nutrients, drugs, nanoparticles, bacteria and viruses through mucus. At the biochemical and molecular biological end novel immune-fluorescent and single molecule spectroscopy techniques are pushing the frontier forward to investigating molecular details of mucosal surfaces and mucus secretions. Biochemical and molecular modifications of the glycan coat may lead to development of new vaccines and drugs.

Note Added in Proof

The reference below confirms that hydrophobic interactions are involved in mucin gel formation and low pH viscoelasticity increase. The surfactant (very mild detergent) 1,2 hexandiol causes a reduction in mucin viscoelasticity.


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References


