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Recombinant mucin biotechnology and engineering

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ABSTRACT

Mucins represent a largely untapped class of polymeric building block for biomaterials, therapeutics, and other biotechnology. Because the mucin polymer backbone is genetically encoded, sequence-specific mucins with defined physical and biochemical properties can be fabricated using recombinant technologies. The pendent O-glycans of mucins are increasingly implicated in immunomodulation, suppression of pathogen virulence, and other biochemical activities. Recent advances in engineered cell production systems are enabling the scalable synthesis of recombinant mucins with precisely tuned glycan side chains, offering exciting possibilities to tune the biological functionality of mucin-based products. New metabolic and chemoenzymatic strategies enable further tuning and functionalization of mucin O-glycans, opening new possibilities to expand the chemical diversity and functionality of mucin building blocks. In this review, we discuss these advances, and the opportunities for engineered mucins in biomedical applications ranging from *in vitro* models to therapeutics.

Contents

Abbreviations: ADC, Antibody-drug conjugate; cDNA, coding DNA; CBM40, Carbohydrate-binding module 40; CeGL, Chemoenzymatic glycan labelling; CGS, Custom gene synthesis; CHO, Chinese-hamster ovary; CMAH, Cytidine monophospho-N-acetylneuraminic acid hydroxylase; CMP, Cytosine monophosphate; CRISPR/Cas9, Clustered regularly interspaced short palindromic repeat/targeted Cas9 endonuclease; FTIR, Fourier transform infrared; GalNAc, N-acetylgalactosamine; GalNAzME, N-azidoalaninyl galactosamine; GBP, Glycan Binding Proteins; GGTA1, Glycoprotein α-galactosyltransferase 1; GlcNAc, N-acetylglucosamine; HEK, Human embryonic kidney; ISOGlyP, Isoform-Specific O-Glycosylation Prediction; KI/KO, Knockin/Knockout; Pro, Proline; PTS, Proline Threonine Serine-rich domain; UDP, Uridine diphosphate; MGE, Metabolic glycoengineering; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; PNA, Peanut agglutinin; ppGalNAcT, Polypeptide N-acetylgalactosaminyltransferase; SNA, Sambucus Nigra lectin; Ser, Serine; StcE, Secreted protease of C1 esterase inhibitor; Thr, Threonine; ZFN, Zinc-finger nuclease; TR, Tandem repeat.

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1. Introduction – History of mucin bioproducts

Mucins evolved in early Metazoan life as protective biopolymers for cell and tissue interfaces[1]. Molecularly, mucins are protein and sugar co-polymers defined by their unstructured polypeptide backbone and dense grafting with serine (Ser) and threonine (Thr) linked O-glycans[2,3]. The glycan side chains, which can constitute 50% or more of the glycoprotein molecular weight, are linear or branched and often negatively charged at neutral pH due to their capping with sialic acids or sulfate groups[4,5] (Fig. 1). The extraordinary physical properties of mucins emerge from their unique molecular and chemical structure. The arrayed O-glycans strongly interact with water molecules, enabling mucins to hydrate, lubricate, and protect biological interfaces [6]. Secreted and cell-surface mucins are chiefly responsible for protecting mucosal surfaces against dehydration, mechanical stresses, oxidative degradation, and infection from viruses and bacteria.

From an applied perspective, known human interest in mucins dates to antiquity, where ancient Greeks were said to apply snail mucus as anti-inflammatory and anti-aging balms [7]. Until recently, purified mucin products have primarily been considered for hydration and lubrication of tissues, include the eye, cartilage,

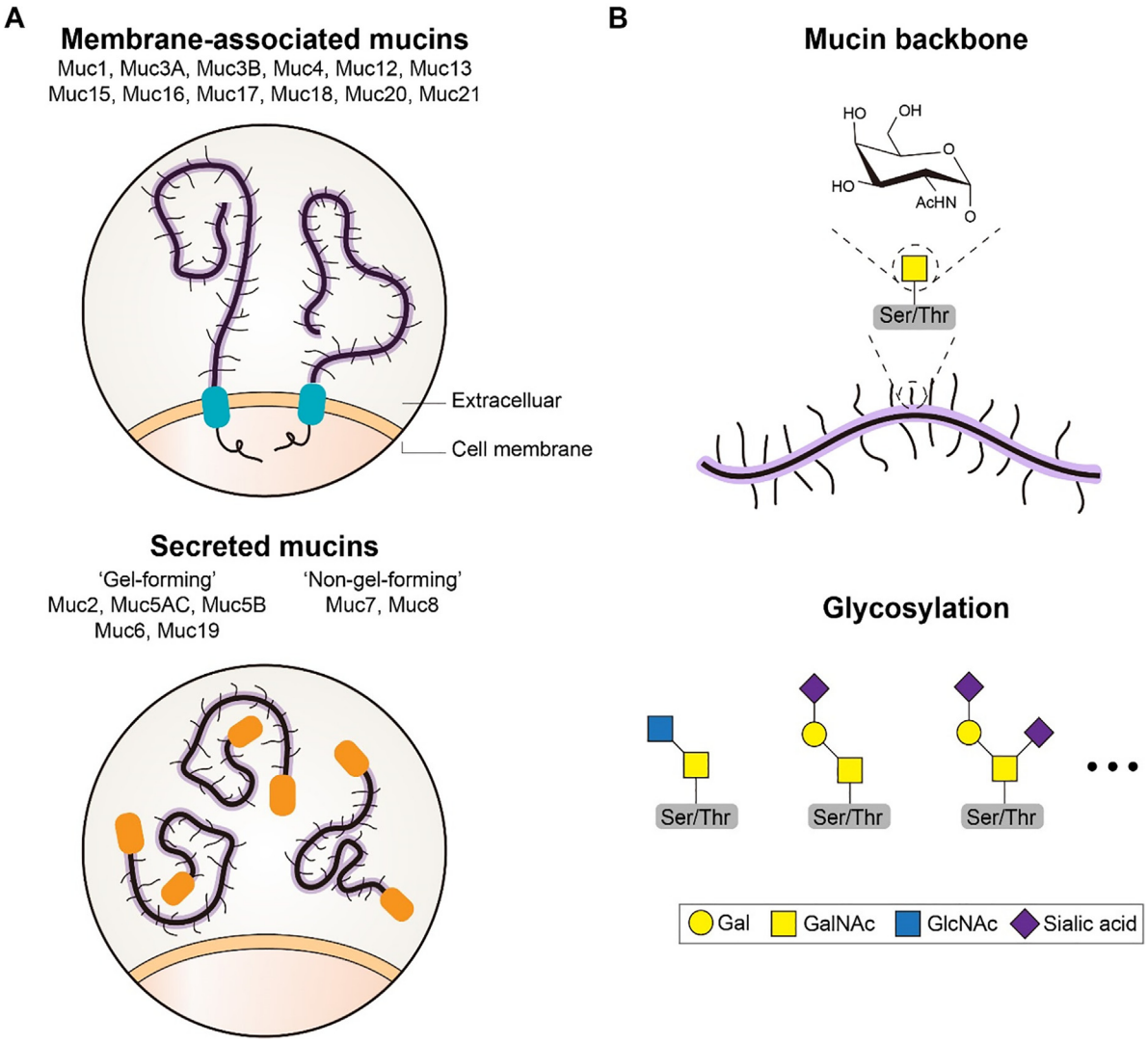


Fig. 1. Mucin backbone and glycosylation. (A) Mucins can be divided into secreted and membrane-associated family members. All mucins contain a serine, threonine, and proline rich region, often composed of variable number of tandem repeats (TRs), that are sites of O-glycosylation. In membrane-associated mucins, the heavily glycosylated domain is linked to a single pass transmembrane anchor and short cytoplasmic tail. Secreted mucins can be divided into gel-forming and non-gel forming subtypes. Gel-forming mucins contain N- and C-terminal cysteine-rich domains (orange, round) and contribute to the viscoelastic properties of mucus. (B) Mucins are defined by their densely grafted O-glycan structures on serine and threonine residues. O-glycosylation in mammals is initiated by transfer of N-acetylgalactosamine (GalNAc; shown) to the side chain of threonine or serine. Abbreviations: Gal, galactose; GlcNac, N-acetylglucosamine; GalNAc, N-glycolyl neuraminic acid; Ser, serine; Thr, threonine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tendons, mouth, and vocal cords. Interest in mucin biotechnology has surged in the past decade with increasing appreciation that mucins can have potent biological activity in addition to remarkable physical properties. Recent studies have implicated mucin O-glycans in the regulation of the immune system, as well as the phenotype of symbiotic and pathogenic microorganisms[8–13]. For instance, mucin O-glycans can suppress the virulence of *Pseudomonas aeruginosa*, an opportunistic pathogen that is a common source of hospital infections[14,15].

Shorter mucin fragments, including small mucin peptides for anti-tumor vaccines, can be constructed through direct glycopeptide synthesis or chemoenzymatic approaches[16]. Larger mucins are conventionally harvested from natural sources, including bovine submaxillary gland[17], pig gastric mucosa[18], chicken egg (ovomucin)[19], jellyfish bodies (qniumucin)[20], and snail pedal mucus[21]. These larger mucins are increasingly attracting attention for a wide array of applications that include anti-fouling coatings[22–25], anti-aging creams[26,27], wound healing agents[28,29], anti-microbials[30,31], saliva and tear film substitutes[32–35], lubricants[36–38], contact lens drops and coatings [39], advanced biomaterials [40,41], and drug delivery agents [42]. (Fig. 2)

Despite this potential, broader use of larger mucins in biomedical applications is being throttled by the current reliance on their harvesting from natural sources. Purifying large volumes of high-quality mucins from natural sources is challenging due to batch-to-batch variation, the limited volume of bodily secreted mucins from animal organs, reliance on collection procedures with poor scalability, risk of pathogen contamination, lack of established sterilization protocols, and differential mucin expression and gly-

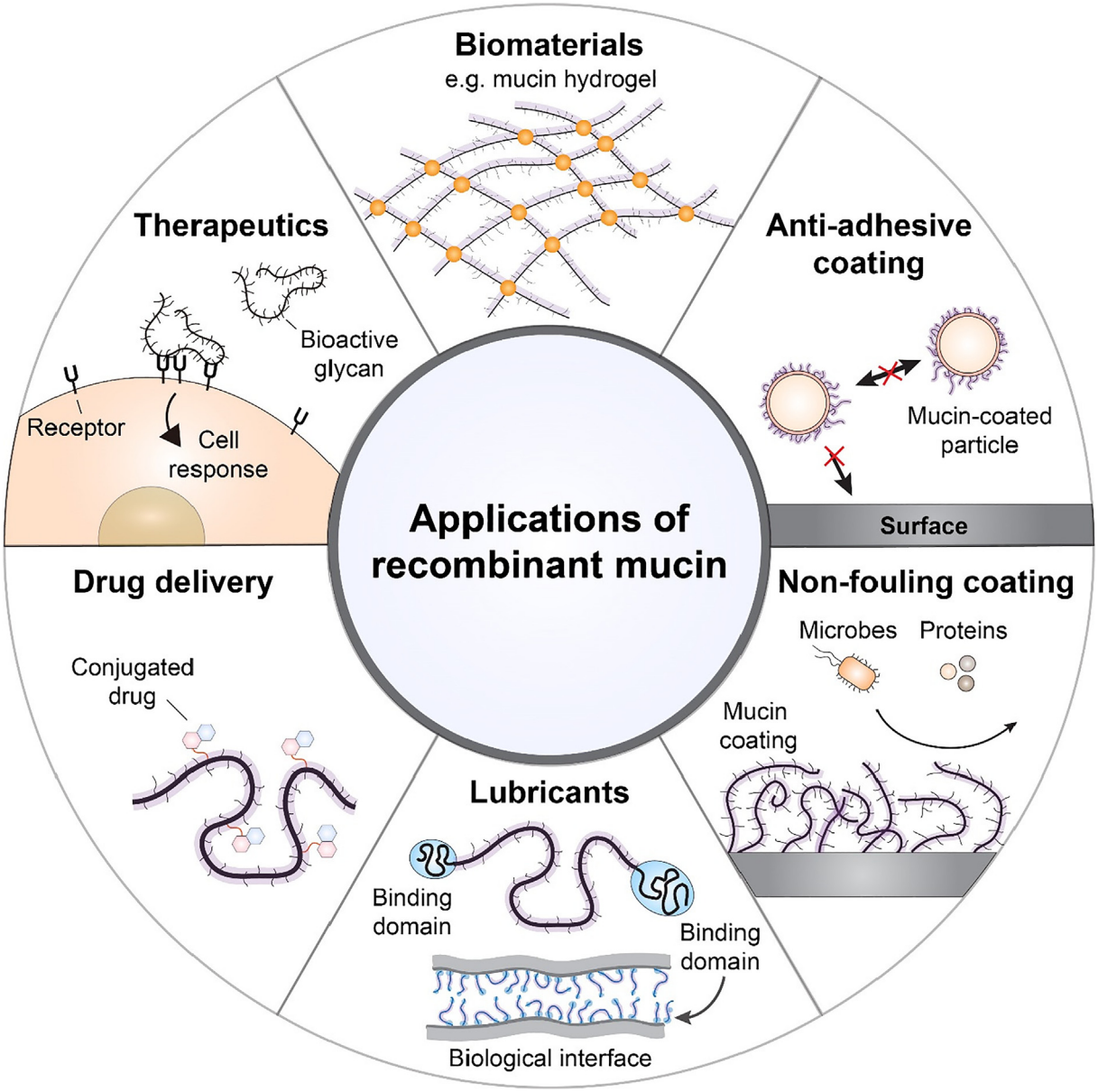


Fig. 2. Applications of recombinant mucins. Biomaterials: mucin biopolymers are under active investigation as building blocks for biocompatible materials and hydrogels. Therapeutics: mucin glycans have bioactivities that are being explored for immune modulation, attenuation of microbial virulence, and other applications. Drug delivery: mucins can be chemically functionalized to serve as carriers for drugs and other therapeutic agents. Lubrication: mucin-based lubricants like lubricin (shown) can hydrate, protect, and lubricate materials ranging from cartilage to contact lenses. Non-fouling coatings: mucin surface coatings can resist protein deposition and microbial interactions. Anti-adhesive coatings: mucins have potential as non-immunogenic alternatives to polyethylene glycol (PEG) and other synthetic polymers for surface coatings on liposomes and nanoparticles.

cosylation patterns when the animal organ is inflamed or diseased [43]. The use of naturally sourced bioproducts also is subject to increasing regulatory scrutiny following the 2007 contamination crisis with the sulfated polysaccharide, heparin. Typically sourced from pig intestines, heparin has been injected as an antithrombic medicine since the 1930s. In 2007, administration of contaminated heparin resulted in several deaths in the United States and hundreds of adverse reactions worldwide, drawing attention to the risks and supply chain vulnerability of animal-derived therapeutics[44].

As an alternative to natural sourcing, recombinant production provides an opportunity for stable and controlled biosynthesis of homogeneous mucin materials. Recent advances in host cell production systems have enabled the recombinant biosynthesis of mucins with more precise O-glycosylation patterns. Notable glycoengineering efforts include the bottom-up reconstruction of O-glycosylation pathways in bacteria, yeast, and plants, as well as the development of engineered mammalian production systems to generate desired O-glycan structures with minimal heterogeneity (See below). With these advances, recombinant technology presents an opportunity to fabricate designer mucins with precise biochemical and physical attributes that may be critical for advanced biomaterials, therapeutics, immunomodulatory agents, and drug delivery systems.

Production strategies for larger mucins are still in the early stages of research and development compared to processes for other biologics, such as antibodies. However, clinical demand for new biolubricants and biomaterials has motivated research into high-titer and cost-effective production of larger mucins. As a notable example, the mucin-like glycoprotein, lubricin, has long attracted commercial attention due to its remarkable ability to hydrate and lubricate tissue with its central mucin domain, as well as its ability to adhere to diverse tissue matrices through its N- and C-terminal globular domains [45]. Due to these abilities, lubricin has attracted keen interest as a biolubricant for diverse medical conditions, including as a treatment for post-traumatic osteoarthritis, dry mouth, dry eye disease, carpal tunnel syndrome, and surgical adhesions [46–48]. Full-length recombinant lubricin was first produced at small scales nearly two decades ago at Wyeth Corp[49]. Using strategies originally developed for recombinant antibody production, Greg Jay and colleagues later achieved higher titer production of human lubricin in Chinese Hamster Ovary (CHO) cells, suggesting that scalable production of therapeutic mucin products is feasible and commercially viable using industry-standard production platforms[50]. Recently, new customized and functional lubricin products have been produced using fully synthetic coding DNA (cDNA) sequences constructed through custom gene synthesis (CGS), suggesting how modern advances in DNA "printing" may be leveraged for the encoding of native and customized mucins for scalable manufacture [51,52].

In this review, we discuss the important technical advances that are leading to increased consideration of recombinant mucins as building blocks and scaffolds for biomaterials, drug delivery systems, and other biomedical applications, including *in vitro* model systems. The emphasis of the review is on larger mucins that cannot be synthesized at scale using alternative approaches other than natural sourcing. Progress in CGS combined with new algorithms for optimally encoding repetitive biopolymers have substantially advanced the speed at which new mucins can be designed and tested. Advances in mammalian cell production platforms and genome editing have made scalable biosynthesis of mucins with precisely tuned glycosylation patterns a practical reality. Comprehensive cellular resources have come online for manufacturing mucins with precisely tuned O-glycans[53,54]. Technologies for further refinement of mucin O-glycans through metabolic and chemoenzymatic methods can endow mucins with new functionalities and transform them into functional carriers of drugs and other bioactive payloads. Together, these advancements portend a future era of design and manufacture of customized mucins to solve pressing biomedical challenges.

2. Recombinant mucin production

2.1. Mucin encoding for recombinant manufacturing

Mucins are defined by their proline (Pro), Thr, and Ser rich polymeric regions (PTS domains) that are heavily O-glycosylated at Thr and Ser residues. Canonical mucin family members include the gel-forming mucins (Muc2, 5AC, 5B, 6, and 19), transmembrane mucins (Muc1, 3A, 3B, 4, 12, 13, 15, 16, 17, 18, 20, and 21), other secreted mucins (Muc7 and 8), and several family members that are not fully classified (Muc9, 14, and 22)[55]. In addition to the canonical family members, multiple glycoproteins contain densely O-glycosylated, PTS domains. In our definition of mucins, we include all canonical mucin family members, as well as additional glycoproteins that contain an O-glycosylated sequence of at least 100 contiguous amino acids with 5% or greater Pro content and 20% or greater Ser/Thr content. By this definition, lubricin and densely O-glycosylated transmembrane proteins, such as podocalyxin, are classified as mucins.

The PTS domains of mucins are often comprised of a variable number of tandem repeats (TR) that differ in sequence and length across the different mucin family members. For instance, the TR of Muc1 is a degenerate 60 base pair sequence with most human alleles comprising between 20 and 125 copies of the repeat (each allele can have a unique repeat number). Recombinant production of shorter mucin fragments with a limited number of TRs has generally been successful using standard approaches [56,57]. Partial PTS domains of a variety of human mucins including Muc1, Muc2, Muc3A/B, Muc5AC, Muc5B, Muc6, Muc7, Muc9, Muc13, Muc16, Muc17, Muc19, Muc20, Muc21 and Muc22 have been recombinantly expressed. [51,52,54,58–60]

For larger mucins with complete PTS domains, the highly repetitive nature of the repeats, as well as the high GC content of some TRs, can pose significant technical challenges in cloning, sequencing, and recombinant biosynthesis. These challenges are underscored by the fact that only recently have the full sequences of all human mucins been revealed[61]. Specialized cloning methods that deal with repetitive DNA sequences, including overlap elongation Polymerase Chain Reaction (PCR)[62], overlap extension rolling circle amplification[63], and recursive directional ligation methods[64–68], often generate heterogeneous products of different sizes and involve iterative procedures that are tedious to optimize. Highly repetitive DNA sequences also are prone to recombination during propagation of plasmids and viral vectors. Moreover, the fidelity of nearly all DNA processing steps, including replication, can be compromised in host production systems by slippage and other errors linked to repetitive sequences[69]. Thus, genomic stability of repetitive mucin cDNAs during extended cell cultivation may be of greater concern for manufacturing of mucins compared to other bioproducts. For instance, likely recombination of TRs has been reported during vector propagation and cell line development for production of full-length recombinant Muc1 and lubricin using native cDNAs[51,56,57].

2.1.1. Genetic strategies for encoding and stable expression of larger mucins

The emergence of rapid and cost-effective *de novo* DNA synthesis affords new opportunities to avert the inherent pitfalls of working with repetitive, native mucin cDNAs [70]. CGS has revolutionized protein engineering and synthetic biology by

enabling low-cost "printing" of non-repetitive DNA sequences. In typical CGS workflows, complete genes are assembled from short, overlapping DNA oligonucleotides that are chemically synthesized. Highly repetitive DNA sequences are not amenable to efficient CGS due to the inability to design the unique sequence overlaps that guide precise assembly. As a powerful workaround, *de novo* genes for repetitive polypeptide sequences can be optimized for CGS using codon-scrambling algorithms that identify the least-repetitive synonymous coding sequences for the desired polypeptide repeats[52]. Originally developed by Tang and colleagues for elastin-like polymers, this strategy exploits codon-redundancy to minimize nucleotide repetition in the design of recombinant cDNA sequences while conserving the native amino-acid sequence of the desired proteins [71]. Optimizing recombinant mucin cDNAs through codon-scrambling has the potential to improve the stability of mucin gene sequences, as it tactfully evades genomic instabilities of highly repetitive nucleotide sequences that are innate to native mucin TR domains. Consistent with this idea, codon-scrambled, synonymous cDNAs can faithfully produce full-length recombinant MUC1 and lubricin in suspension-adapted human embryonic kidney (HEK293-F) cells that are difficult to achieve with the corresponding native cDNAs [51]. As a notable example, a lubricin-like glycoprotein with 59 repeats of the consensus repeat, KEPAPTTP, has been stably produced for over 2 months in continuous culture using a fully synthetic, codon-scrambled gene [51].

Mucin genes can be stably integrated into the genomes of standard mammalian production systems through selection of cells following transient transfection with vectors containing a suitable selection marker[54]. For more efficient genomic integration, commercial transposase systems have been reliable for stable integration of large mucin cDNAs. The piggyBac™ system for genome editing is particularly well-suited for delivering mucin cDNAs due to its large cargo-carrying capacity of over 200 kilobases[72]. Notably, no overt signs of recombination in long mucin TRs have been observed in stable cell lines generated using the piggyBac transposase [52,73–75]. Viral systems for stable cell line generation should be used with caution. In addition to the concerns of viral contamination in the recombinant products, highly repetitive mucin cDNAs are susceptible to homologous recombination in retroviruses and lentiviruses [76]. For example, attempts at lentiviral-mediated integration of full length Muc1 cDNAs have resulted in expression of highly truncated products, consistent with recombination of the repetitive TRs [73]. By comparison, stable lines made with the piggyBac transposase for the same cDNA generated homogeneous products of high molecular weight [73]. Additional genetic strategies have proven useful in the development of high-productivity cell lines for recombinant mucins. Epigenetic regulators that mediate high and consistent expression of recombinant proteins [77] have been reported to enhance the recombinant production of full-length lubricin [50].

2.1.2. de novo mucin production

Can we design *de novo* mucin sequences with unique glycosylation patterns? Several high molecular weight, fully synthetic mucins have been rationally designed and constructed using CGS combined with codon-scrambling algorithms. For instance, new mucins have been constructed with TRs designed based on statistical analysis of mucin O-glycosylation sites (PPASTSAPG) or analysis of N-acetylgaslactosamine (GalNAc) transfer efficiency (DAATPAP and DAATPAPP)[52,78]. These approaches have also been applied for recombinant production of Muc1 variants with dozens and hundreds of mutations to its Ser and Thr glycosylation sites to create mucin variants with distinct glycosylation patterns [52]. The codon scrambler algorithm does not attempt to optimize codon frequency usage for a particular host organism, and, therefore, may not provide the optimal sequence for host cell productivity [71]. For lower TR numbers, standard codon optimization routines that minimize codon usage bias in a specific host can provide sufficient codon scrambling for CGS. For instance, Narimatsu and colleagues have applied standard codon optimization tools for successful CGS of over 20 mucin constructs with distinct TRs [54,79]. A list of synthetic mucin TR sequences that have been fabricated through CGS following encoding through either standard codon optimization or specialized codon scrambling approaches is presented in Table 1.

Scientists are starting to uncover the polypeptide sequence determinants for O-glycan initiation and extension, providing valuable insight for the rational design of new or engineered mucin sequences. Mucin O-glycosylation is initiated by a family of polypeptide n-acetylgalactosaminyltransferases (ppGalNAcT) that catalyze the transfer of GalNAc to a Ser or Thr residue on the protein substrate[80]. The ppGalNAcTs are type II transmembrane proteins that typically reside in the eukaryotic Golgi apparatus.

Table 1
Synthetic mucin tandem repeats (TR) fabricated through custom gene synthesis (CGS).

TR sequences	Mucin type	Number of repeats	Reference
PDTRPAPGSTAPPAHGVTSA	Muc1	10, 21, 42, 84	[46]
	Muc1	7	[58]
PDTRPAPGATAPPAHGVTSA	Muc1 (mutant)	21	[52]
PDTRPAPGATAPPAHGVTAA	Muc1 (mutant)	21	[52]
PDARPAPGATAPPAHGVTAA	Muc1 (mutant)	21	[52]
PSPPITTTTPPPTTT	Muc2	10	[58]
GTQTPTPTPITTTTTVTPTPTPT	Muc2	7	[58]
PLPVTDTSSASTGHAT	Muc3A/B	9	[58]
STTSAPTT	Muc5AC	18	[58]
TTAVPPTPSATTLDPSSASAPPE	Muc7	7	[58]
TSDIITASSPNDGLIT	Muc13	9	[58]
TSTPSEGSTPFTSMPVSTMPVVTSEAST	Muc17	5	[58]
SESSASSDGPHPVITPSRA	Muc20	8	[58]
SSGASTATNSESSTV	Muc21	10	[58]
SETTVTSTAG	Muc22	15	[58]
KEPAPTTP	Lubricin	59	[51]
	Lubricin	20	[52]
DAATPAP	Synthetic mucin	40, 80	[52]
DAATPAPP	Synthetic mucin	40, 80	[52]
PPASTSAPG	Synthetic mucin	40, 80	[52]

Each ppGalNAcT poses a luminal facing catalytic domain and a lectin domain that together determine substrate preference and enzyme specificity. Many of the 20 ppGalNAcTs in mammals possess some degree of overlap in sequence preference and, thus, can function with some redundancy in O-glycan initiation[81]. However, unique position-sensitive features have been uncovered for the ppGalNAcT isoforms, and some of the isoform structural differences that determine peptide substrate preference have been resolved [81–83]. Technological advancements are poised to further accelerate the understanding of glycosyltransferase specificities. For instance, rapid cell-free synthesis and mass spectrometry analysis have been combined to uncover peptide specificities of human glycosyltransferases, including ppGalNAcT-1 and ppGalNacT-2 [84]. Such understanding may be leveraged in the future to engineer transferase-specific glycosylation patterns.

Several powerful bioinformatics tools have been developed for O-glycosylation site prediction. The popular NetOGlyc4.0 server uses neural networks and O-glycoproteomics training data for prediction of mucin-type O-glycosylation in mammalian glycoproteins with an accuracy of approximately 75%[85,86]. Isoform-Specific O-Glycosylation Prediction (ISOGlyP) is a powerful algorithm for *de novo* prediction of O-glycosites in a contextual manner that can account for the specific ppGalNAcT isoforms that are expressed in the host cell[86]. ISOGlyP has a reported accuracy of 70% in glycosite prediction[86]. Context specific mining of the O-glycoproteome also can be conducted in the GlycoDomain Viewer, which incorporates data from multiple human and animal cell lines, as well as some organs and body fluids[86]. Together, better definition of the sequence determinants of mucin O-glycosylation, combined with predictive bioinformatics tools and advances in gene synthesis, are advancing the feasibility of fabricating *de novo* mucins.

2.2. Cellular host production systems for recombinant mucins

The repertoire of cellular O-glycosylation machinery is a primary criterion for selecting an appropriate host cell system for recombinant mucin production. The initiating mucin GalNAc, which is often referred to as the Tn antigen, is subsequently elongated into the four primary core structures through the sequential actions of enzymes that are organized throughout the Golgi stacks (Fig. 3). The most common extension is through the core 1 synthase (C1GalT-1) to form the T antigen, Galβ1-3GalNAc, which can be branched by C2GnT-1,3 to form the core 2 structure, Galβ1-3(GlcNACβ1-3)GalNAc. C1GalT-1 function is dependent on the presence of the essential chaperone COSMC[87]. Expression of the core 3 synthase, β3GnT-6, is largely restricted to the gastrointestinal and respiratory mucosa and appends N-acetylglucosamine (GlcNAc) to the Tn antigen to form the core 3 structure, GlcNAcβ1-3GalNAc. The core 3 structure subsequently can be branched by C2GnT-2 to form the core 4 structure. The primary core structures can be further elongated and branched by N-acetyllactosamine chains and/or terminated by blood group ABH-related structures, fucose, sialic acids, and sulfate groups (Fig. 3). Only mammalian cells natively express the full repertoire of glycosyltransferases that can recapitulate human O-glycosylation patterns. However, other eukaryotic cell platforms are under development in which human O-glycosylation pathways are being constructed from the ground up for the purpose of recombinant mucin production.

Yeast cells have a functional Golgi apparatus that can generate mucin-type O-glycans if the appropriate machinery is introduced through genetic engineering. Yeasts natively produce UDP-GlcNAc and UDP-Glc but lack an epimerase for conversion of the nucleotide sugar building blocks into UDP-GalNAc and UDP-Gal for construction of mucin O-glycans. Yeasts also do not contain

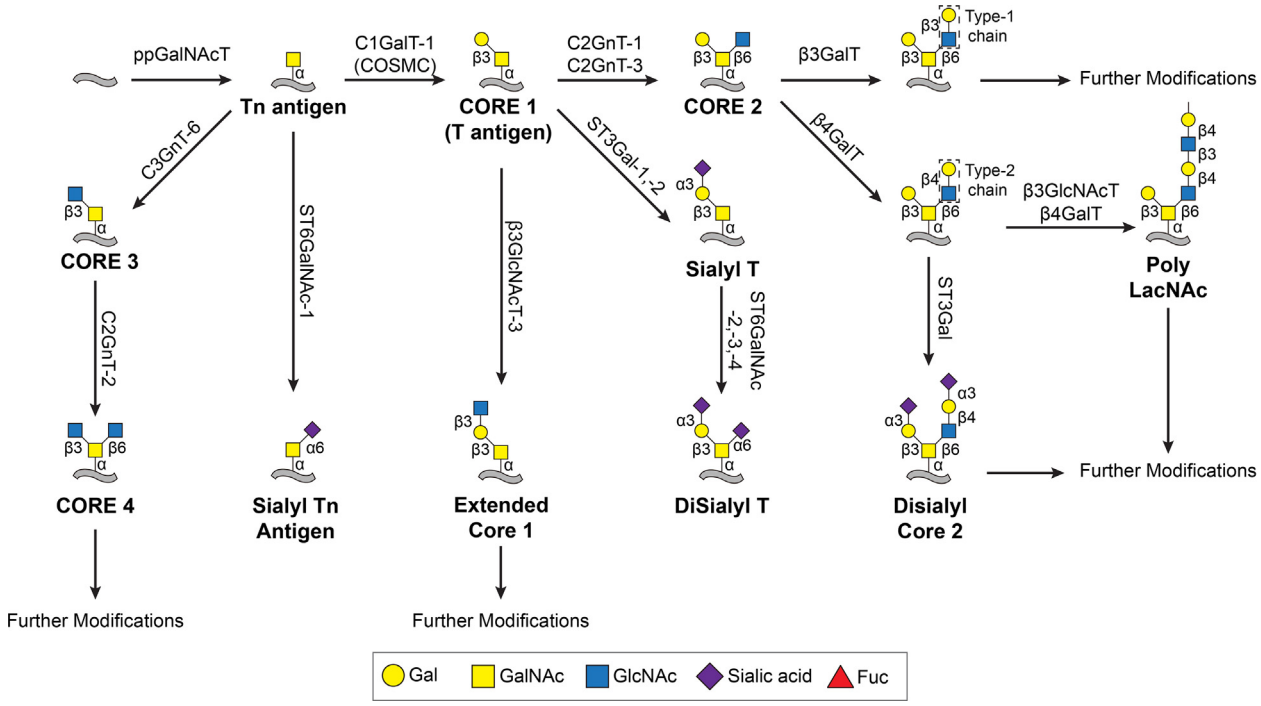


Fig. 3. Human O-glycosylation pathway map. Graphic depiction of O-glycosylation pathways with mucin-related glycosyltransferase genes. The basic O-GalNAc structure (Tn-antigen) is generated by a family of ppGalNAcT enzymes on serine or threonine residues primarily in the Golgi apparatus. The Tn-antigen can be extended to form one of the primary core structures (core 1–4) or capped with sialic acid to form the sialyl-Tn antigen. The core structures can subsequently be elongated or branched and capped through sialyltransferases, fucosyltransferases, sulfotransferases, and other enzymes. Main core structures and examples of their extended structures are shown in bold. Glycan symbols are drawn according to the Symbol Nomenclature for Glycans (SNFG) format.

genes homologous to the ppGalNAcT gene family that initiates O-glycosylation nor genes for core O-glycan extension. To create strains capable of producing mucin-type glycopeptides, genes encoding *Bacillus subtilis* UDP-Gal/GalNAc 4-epimerase, human UDP-Gal/GalNAc transporter, human ppGalNAcT-1, and *Drosophila melanogaster* core 1 β1–3 GalT have been introduced into *Saccharomyces cerevisiae*[88]. The engineered strain can produce mucin-type glycopeptides containing O-linked GalNAc and core 1 structures. In yeast, the endogenous O-mannosylation pathway initiated in the ER is likely to compete for substrate sites with the Golgi-localized GalNAc O-glycosylation[88,89]. Future engineering efforts may be needed to attenuate O-mannosylation of mucin peptides and mitigate concerns of immunogenicity and product heterogeneity. Nevertheless, the rapid cell growth, high protein yields, and commercial scalability of yeast expression systems make them an attractive candidate for production of mucins with simple O-glycans.

Plants are another non-animal platform that offers the potential to design and build the pathways for mucin O-glycosylation from the bottom up. Human O-glycosylation has been successfully reconstructed in plants through the introduction of *Pseudomonas aeruginosa* UDP-Gal/GalNAc C4-epimerase and a combination of human ppGalNAcTs[90]. Unlike yeast, plants do not have competing O-glycosylation pathways for Ser and Thr residues[91]. However, plants produce another type of protein O-glycosylation, in which Pro is converted to hydroxyproline and subsequently appended with various O-glycans[92]. Hydroxyproline-linked O-glycans have been found on recombinant IgA1 and Muc1 peptides produced in plant cells[93,94]. Since hydroxyproline conversion does not appear to be essential for plant cell growth and viability, removal of the hydroxyproline-linked glycosylation pathway through genetic engineering may be possible[95].

Insect cell lines have garnered attention as a lower-cost alternative to mammalian cells for recombinant protein production. Insect cell lines offer several manufacturing advantages, including minimal risk for human-specific viral contamination and straightforward scalability due to the availability of optimized media formulations, no CO2 requirement for growth, and lower temperature requirements. Advances in baculovirus technology have simplified high-yield transgene expression in insect cells[96]. The most common insect cell platforms are derived from the fall armyworm *Spodoptera frugiperda* (Sf9, Sf21), the cabbage looper *Trichoplusia ni* (High Five™), and the fly *Drosophila melanogaster* (S2) [97]. All insect cell lines investigated have multiple genes homologous to the human ppGalNAcT gene family and can natively generate mucin-type O-glycosylation[98]. Tn-antigens and core 1 type structures have been confirmed in insect cell lines, and extended core 1 and core 2 structures have been reported in *Drosophila* embryos, confirming the innate capacity of insect cells to construct complex O-glycans[99–101]. Analyses of mucin-type O-glycans released from recombinant PSGL-1 produced in Sf9 and High Five cells have revealed a large repertoire of complex O-glycan structures[102]. However, these structures include O-glycans containing hexuronic acid and phosphocholine substitutions, which are not found in humans and may be immunogenic. Insect cells also have very limited capacity to produce glycans with terminal sialic acid, and introduction of sialylation machinery is most likely required for abundant generation of mucin-type sialoglycans [102,103]. Thus, while insect cells are a promising platform for mucin production, careful glycoengineering is required for synthesis of sialylated human O-glycans without competing non-human glycan structures.

CHO cells have been the most widely used mammalian cell platform for recombinant protein production, including therapeutic antibodies. Advantages of CHO cells include their robust growth, ease of clonal selection, adaptability to suspension culture for increased yields, the capability for complex glycosylation, and extensive safety and regulatory history. While CHO is an excellent candidate platform for mucin biosynthesis, O-glycosylation in conventional CHO production systems differs from human in several important ways, some of which have the potential to illicit immunological responses in humans [104] (Fig. 1**B**). First, CMP-Neu5Ac hydroxylase is inactive in human cells but its activity in CHO cells allows for the glycosylation of proteins with Neu5Gc sialic acids, which can provoke antibody responses[105]. Interestingly, the ability to synthesize Neu5Gc enhances the sialylation of CHO-driven proteins compared to those from human HEK293 cells [106,107]. Second, CHO cells may also possess α1,3-galactosyltransferase activity, which is absent in humans, and generate protein products with Galα1,3-Gal residues (α-Gal) that are known to elicit adverse anaphylaxis reactions[108]. Although α-Gal is typically associated with N-glycans, α-Gal has been reported on Muc1 in cell lines that have α1,3-galactosyltransferase activity[109]. Third, several other glycosylation enzymes that are expressed in human are missing in CHO cells. For example, CHO cells lack α1,3/4-fucosyl transferase and galactose α2,6 sialyltransferase that are found in humans [104,110–112]. A concern of adverse immunological reaction of CHO-derived mucins is mitigated by their low expression of cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH) and glycoprotein α-galactosyltransferase 1 (GGTA1), which primarily govern CMP-Neu5Ac hydroxylase and α1,3-galacto syltransferase activity, respectively. Recent advances in gene editing technology also enable straightforward knockout (KO) of CMAH and GGTA1, as well as knockin (KI) of genes to humanize O-glycosylation in CHO (See below), which may further improve the safety profile of CHO-derived mucins[113].

Recombinant mucin production in human cell lines can circumvent these problems by avoiding the risk of immunogenic reactions from non-human glycans, as well as generating products that only bear native human glycans. Advances in cell expression systems and media formulations have allowed for substantially increased productivity with human cell lines. Biotherapeutic products produced from the HEK293 and fibrosarcoma HT-1080 cell lines are now approved by the US Food and Drug Administration (FDA) [114]. Additional biotherapeutic products produced in the PER. C6, HKB-11, CAP and HuH-7 human cell lines are currently being evaluated by the FDA[114]. Of the human host production systems, HEK293 has emerged as a leading candidate to become the standard bearer for mucin manufacturing. In-depth pathway understanding has led to remarkable glycoengineering efforts to create HEK293 progenies that generate precise and homogeneous O-glycan structures (See below)[53,54]. Several HEK293 cell lines, including 293-F, 293-H, and 293-6E, have been adapted to high-density suspension growth in serum-free medium, enabling high density cultivation in bioreactors[115–117]. At present, dozens of mucins and mucin TR sequences with expected glycosylation patterns have been successfully generated in HEK293 platforms [52,54]. Notably, recombinant mucins that have been produced in HEK293 and CHO cells display different glycosylation patterns. For instance, lubricin produced in 293-F cells displays a mix of core 1 and core 2 O-glycan structures, similar to the glycosylation patterns of native lubricin in humans[118,119], whereas recombinant lubricin from CHO cells predominantly displays core 1 O-glycan structures, some of which may be sulfated [50,119].

A possible disadvantage of using human cell lines is the potential for human-specific viral contamination, although this risk can be mitigated with viral inactivation and removal steps during downstream bioprocessing[120]. Compared to CHO cells, HEK293 cells have a higher tendency to clump during growth in suspension, ultimately limiting cell proliferation, viability, and productivity due to oxygen limitations. Interestingly, expression of recombinant cell-surface mucins can substantially mitigate

HEK293-F clumping in suspension[121]. Like CHO cells and other immortalized cell lines, HEK293 cells exhibit genomic instability characterized by chromosomal translocations, copy number alterations, and other events, raising concerns of karyotypic and phenotypic drift during prolonged cultivation[122–124]. The potential for drift in O-glycosylation patterns during long-term cultivation and subcloning in human cell lines must still be addressed. Since the HEK293 cell line originates from the kidney of an aborted human embryo [125], ethical and religious concerns related to its use as a manufacturing platform have been raised.

Fed-batch and perfusion cultures, in which additional media components and nutrients are added in small batches, semi-continuously, or in a continuous manner, are the typical standards for high-yield biomanufacturing[126]. The optimal development of these processes requires detailed knowledge of the production cell line and its specific metabolic requirements for a particular product. Metabolic waste products, such as ammonia, can interfere with the sialyation of O-glycan structures, highlighting the potential for generating different O-glycan structures depending on the specific metabolic conditions of the bioreactor[127]. Careful selection of media formulations, feeding schedules, and bioreactor operating conditions may be necessary to minimize generation of any unwanted glycan structures, including immunogenic glycans and those with undesired biochemical activity. Knowledge of how nutrient availability and cellular metabolic program influences O-glycosylation during commercial production is rudimentary compared to the corresponding knowledge for N-glycosylation, which has been investigated in detail in the context of antibody production. Seemingly minor but important downstream bioprocessing strategies, including chromatography operations, must still be developed for mucins. For instance, solutions containing large mucins may not be amendable to ultimate sterilization through dead-end filtration using 0.22 μm or smaller filters, an industry standard approach. Nevertheless, the overall advances in genetic tools and cell production systems have improved production quantity and quality of recombinant mucins, paving the way for commercially viable mucin biomanufacturing.

3. O-Glycoengineering in mammalian cells

There is a long history of genetic approaches to engineer the glycosylation machinery in mammalian cells for desired glycan phenotypes[128]. Earliest examples of these efforts come from the isolation of CHO glycosylation mutants by random mutagenesis and selection for resistance against cytotoxic plant lectins [129–131]. Careful characterizations of an impressive range of lectin-resistant CHO mutants have led to key discoveries of glycosylation genes and pathways that regulate glycan biosynthesis[130]. With advancing knowledge in the glycosylation machinery, focus has also been placed on engineering mammalian cells by overexpressing, silencing, or knocking out specific glycosylation genes to create products with improved functionality, pharmacokinetics, and safety[132,133]. Decades of cumulative understanding in glycan biosynthesis has also enabled the assembly of detailed glycosylation gene maps to guide rational designs of genetic engineering efforts in cells [133]. Combinatorial KI and KO strategies to globally engineer multiple glycosylation genes provide tremendous opportunities in generating stable cell lines with desired glycosylation capabilities[128]. As one of many glycoengineering examples, mammalian cells have been rationally engineered by KI of ST6GAL1 in the background of KO of B4GALNT3/4 to eliminate terminal GalNAc and achieve near complete α-2,6-linked sialylation of N-glycans on biologics for improved pharmacokinetics[134].

Precise and stable manipulation of glycosylation genes in mammalian cells can now be rapidly achieved at high efficiency through recent developments in nuclease-based gene editing tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat/targeted Cas9 endonuclease (CRISPR/Cas9) [128]. Untargeted manipulation of glycosylation genes for modulating protein glycosylation status remains a popular and powerful strategy for recombinant protein production[135,136]. However, the untargeted integration of ectopic glycosylation genes typically does not offer fine control over expression levels, and low knockdown efficiency using RNA interference can result in residual gene activities, both of which can lead to recombinant products with unintended glycosylation[137]. Precision KI and KO strategies avoid these pitfalls and can provide stable, homogeneous glycosylation patterns. An outstanding early example is the ZFN-targeted KO of COSMC in CHO cells to construct stable 'SimpleCells' capable of generating homogeneous O-GalNac glycans, marking a key advance towards precision glycoengineering [138]. The ZFN-mediated SimpleCell strategy has also been applied to HEK293 and 12 human cancer cell lines from different organs [85], indicating that such strategies are broadly applicable in mammalian systems.

CRISPR/Cas9 genome editing system has emerged as a highly versatile tool to perform precise gene targeting for glycoengineering, particularly for combinatorial KO of genes. A validated guide RNA library is available for highly efficient CRISPR/Cas9 targeting of the complete human glycosyltransferase genome[139]. Multigene targeting can be achieved with CRISPR/Cas9 through simultaneous introduction of multiple guide RNAs into cells along with the CRISPR/Cas9 machinery[140]. Multi-gene targeting can greatly shorten the process of higher-order mutant generation and screening, as evidenced by the large library of glycoengineered HEK293 cell lines that Narimatsu, Clausen, and colleagues have generated in recent years using these approaches [53,54,141]. An isogeneic library has been created for edited O-glycosylation pathways that includes engineered lines for relatively homogeneous construction of Tn, sialyl-Tn, T, sialyl-T, disialyl-T, and core 3 O-glycans, as well as increased O-glycan sulfation and core 2 extension[53,54,141]. Although these engineered systems have primarily been used for basic research, such as evaluation of lectin-glycan binding specificities, their potential application in mucin manufacturing is obvious.

Control over where glycans are initiated and elongated along the mucin backbone is still difficult to achieve through glycoengineering alone. Part of this challenge stems from the presumably redundant nature of the 20 ppGalNAcTs in initiating O-glycosylation. However, the realization that ppGalNAcT isoenzymes may have stronger than realized preferences for specific mucin substrate sequences raises the possibility of tuning glycan site occupancy through rational manipulation of ppGalNAcT expression [58,142,143]. O-glycans largely have been overlooked as a design parameter in biomanufacturing, as most of the attention has focused on N-glycosylation. The recent advances in precision O-glycan editing should usher a more complete understanding of the importance of specific O-glycan structures in the *in vivo* function of mucins and other biologics containing mucin domains, including how O-glycans may mediate desired or undesired interactions with the immune system. For instance, comparatively little is known about the biological importance of O-glycan sulfation or O-glycan heterogeneity on a single mucin backbone.

4. Quality control and evaluation of glycosylation

Evaluating glycosylation patterns on recombinant proteins is essential for developing the proteins in therapeutic applications. Mass spectroscopy (MS) analysis remains the gold standard for

comprehensive analysis of glycan structures and glycan site occupancy. In typical glycomics workflows, glycans are released from the polypeptide backbone and analyzed to generate detailed information about the glycan structures. Although structural details can be obtained, information about the occupancy of the glycans along the polypeptide backbone is inevitably lost in glycomics when the glycans are released. Since there is no single enzyme that can release all O-glycan structures, chemical methods are currently employed to harvest glycans. Under alkaline conditions, the O-glycosidic linkage between the reducing glycan sugar and Ser/Thr residues is labile and readily hydrolyzed[144]. The annotation of glycan structures in MS datasets has historically required high levels of human expertise. However, advances in software, construction of structural databases, and standardization of experimental workflows are increasingly simplifying MS analyses[145]. Standardized guidelines, such as the Minimum Information Required for A Glycomics Experiment (MIRAGE), should be followed to ensure rigorous and reproducible glycomics analyses[146].

MS analysis of O-glycopeptides can provide additional information regarding glycan site occupancy and heterogeneity. Historically, O-glycopeptides analysis of larger mucins has been challenging due to 1) the resistance of mucins to complete proteolytic digestion with conventional enzymes, 2) the large number of glycopeptides that can be generated from a single large mucin, and 3) the dozens of possible O-glycan structures that can potentially occupy any Ser or Thr site. Standard algorithms in commercial software packages are ill-equipped to handle the massive computational task of considering all theoretically possible glycopeptides for a large mucin. However, highly efficient algorithms recently have been developed that substantially decrease the amount of processing time required to confidently complete glycosite localizations and glycopeptide identifications for mucin-derived samples[147]. Glycosite identification can also be simplified through mucin synthesis in glycoengineered cell lines that generate homogeneous O-glycan structures[148]. A notable advancement in O-glycoproteomics is the identification of a growing number of bacterial O-glycan-specific proteases (O-glycoproteases) that can specifically degrade mucins. These enzymes include OgpA from *Akkermansia muciniphila* (OpeRATOR), secreted protease of C1 esterase inhibitor (StcE) from enterohaemorrhagic *Escherichia coli*, SmEnhancin (SmE) from *Serratia marcescens*, and Immunomodulating metalloprotease (IMPa) from *Pseudomonas aeruginosa*, which successfully have been applied for O-glycopeptide generation or enrichment in glycoproteomics workflows that are well-suited for characterization of recombinant mucins [149–153].

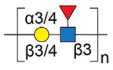
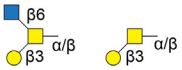
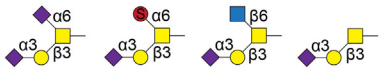
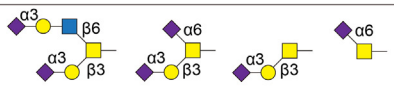
For more routine or targeted analyses, glycan binding proteins (GBPs), including lectins and antibodies, can serve as resources for quality control of glycosylation features in recombinant mucin production (Fig. 4). Printed glycan arrays from the Consortium for Functional Glycomics and new cell-based glycan arrays have provided more complete definition of lectin binding specificities [141,154] (Fig. 4). Naturally derived lectins that recognize the Tn-antigen include *Helix pomatia* agglutinin (HPA) and *Helix aspersa* agglutinin (HAA), which can also recognize terminal GlcNAc. Core O-glycans are specifically recognized by *Amaranthus caudatus* lectin (ACL), Peanut agglutinin (PNA), *Maclura pomifera* lectin (MPL), and *Artocarpus integrifolia* lectin (AIA, Jacalin). ACL recognizes the T (Galβ1-3 GalNAc), sialyl-T (α2-3-sialylated Galβ1-3 GalNAc), and disialyl-T (α2-3-sialylated Galβ1-3 [α2-6-sialylated] GalNac) structures, as well as core 2 O-glycans. Jacalin and MPA specifically bind the non-substituted, initiating GalNAc and various substituents at the 3′ position, including galactose (T structure) and GlcNAc (core 3). PNA recognizes the T structure and tolerates GlcNAc substitution at the 6-position of GalNac (core 2). The *Maackia amurensis* hemagglutinin (MAH/MAL-II) recognizes the α2-3-sialic acid of the sialyl-T and disialyl-T structure, as well as 3-O-sulfated galactose[155]. Sambucus Nigra lectin (SNA) and *Maackia amurensis* leucoagglutinin (MAL-I/MAA) probe α2,6 and α2,3-linked sialic acids, respectively, but they likely have a much stronger preference for N-glycans [154,155]. SNA has been reported to have minimal reactivity with the sialyl-Tn structure [79]. Natural lectins with selectivity for additional O-glycan epitopes, including N-acetyllactosamine chains, blood group ABH-related structures, and fucose, have been identified[154].

Although most lectins are derived from natural sources, several recombinant GBPs with affinity to O-glycans have been described in recent years. Compared to natural lectins, recombinant GBPs offer advantages that can include lower batch-to-batch variability, supply chain consistency, and opportunities for further engineering. The carbohydrate-binding module 40 (CBM40) from *Clostridium perfringens* NanI has high affinity to α2,3-linked sialosides (K_d of ~ 30 μM)[156]. The GBP has been engineered into a higher affinity divalent form, diCBM40[156]. Unlike MAL-II, diCBM40 specifically recognizes α2,3-linked sialosides with minimal cross-specificity for 3-O-sulfated epitopes. Siglec-like adhesins derived from *Streptococcus mitis* (10712_{BR}) and *Streptococcus gordonii* (Hsa_{BR}) have specific reactivity with sialylated O-glycans[53,157]. Hsa_{BR} specifically binds to the sialyl-T structure and may tolerate the core 2 branch[53,158]. The preferred binding epitope of 10712_{BR} is core 2 O-glycans with two terminal α2-3 sialic acid residues, making it one of the only GBPs or lectins available with a preference for core 2 structures[53]. The C-terminal glycan-binding domain of StcE, named X409, has general binding properties for mucins with a wide variety of O-glycan substitutions, as well as specific affinity for the disialyl-T structure [54,150,159]. Catalytically inactivated mucinases and glycan processing enzymes can also be used as mucin GBPs [150]. For instance, GBPs called Lectenz® have been developed from catalytically inactivated enzymes for detecting α2,3-linked sialosides and sialic acid in a linkage independent manner [79]. While the specificities of most commercial glycan antibodies against printed or cell-based glycan arrays are not available, several sialyl-Tn antibodies (B72.3, CC49, STn219) have been validated for specificity [160].

In addition to affinity probes for quality control of glycosylation patterns on target proteins, several biochemical and label-free approaches for glycan screening are applicable to mucins. Periodic acid-Schiff's reagent (PAS) staining has been widely used for analysis of carbohydrates and glycoconjugates. O-glycan constituents including GalNAc, GlcNAc, and sialic acid provide strong responses in standard colorimetric assays based on the PAS reagent[161]. Fourier transform infrared (FTIR) spectrometry has been developed for one-step, label-free evaluation of protein glycosylation. FTIR spectra provide unique signatures that are sensitive to minor changes in glycosylation and may be useful for rapid detection of changes in mucin glycosylation[162,163]. FTIR is also an excellent tool for monitoring metabolic incorporation of azido sugars into glycans (See below), since azide moieties have a strong FTIR peak at ~ 2120 cm^{-1} in the "bio-silent" region of the IR spectra[164]. Similarly, Raman spectroscopy can provide sensitive detection of alkyne functionalized glycans[165]. Together, the combination of such approaches with MS-based glycomics and affinity-probe analyses can provide the necessary characterization of glycosylation for process development, process monitoring, and final product assessment.

5. Chemical and enzymatic modification of recombinant mucins

Mucin biopolymers have complex chemistries that make them ideal building platforms for diverse chemical conjugations[40]. In

Probe	Type	Predominant binding motifs	Additional binding motifs	Reference
Amaranthus caudatus (ACA, ACL)	Natural lectin			[154]
Artocarpus integrifolia (AIA, Jacalin)	Natural lectin			[154]
Helix aspersa agglutiin (HAA)	Natural lectin			[154]
Helix pomatia agglutiin (HPA)	Natural lectin			[154]
Maclura pomifera (MPA)	Natural lectin			[154]
Peanut agglutinin (PNA)	Natural lectin			[154]
Maackia amurensis-II (MAL-II, MAH)	Natural lectin			[154]
HsaBR *Streptococcus gordonii*	Adhesin (recombinant)			[54]
NCTC10712BR *Streptococcus mitis*	Adhesin (recombinant)			[54]
diCBM40 *Clostridium perfringens* NanI	Engineered lectin (recombinant)			[156]
Pan-Lectenz	Lectin-like, enzyme derived (recombinant)			[79]
α2,3,-Lectenz	Lectin-like, enzyme derived (recombinant)			[79]
Sialosyl-Tn antibody (B72.3, CC49 , STn219)	Antibody (monoclonal)			[160]
X409 module EHEC StcE	Lectin domain (recombinant)	O-glycosylated mucin		[54]

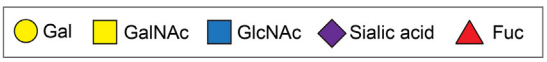


Fig. 4. Validated probes for evaluation of glycosylation. All indicated probes have been validated for specificity on printed or cell-based glycan arrays in the indicated references. Additional binding motifs are indicated, if known.

addition to a plethora of protein engineering approaches[134,166–169], O-glycosides that heavily decorate mucin backbones present tantalizing and as-of-yet mostly untapped opportunities for bioconjugation to increase mucin functionality. In this regard, for example, glycoengineering has become a critical tool for the bioconjugation of drug payloads and for introducing chemical handles to enable molecular assembly and targeted conjugation of bioactive materials[41,170,171]. GalNAc and sialic acids on mucins are ideally suited for chemical modification and subsequent bioconjugation reactions that are highly specific, easy to perform in aqueous solutions, and proceed at fast kinetics with little or no inoffensive by-products that typify bioorthogonal click chemistry [169]. In this section, we will focus on the enormous efforts in chemical glycoengineering and briefly discuss how these efforts may be applied to recombinant mucins (Fig. 5).

5.1. Glycan tagging by direct oxidation

Glycans are rich in natural monosaccharides displaying a vicinal diol that can be oxidatively cleaved by sodium periodate to generate an aldehyde handle[172,173], which can subsequently undergo imine/oxime ligation for conjugating molecules bearing hydrazine or aminooxy groups[174]. Periodate oxidation has emerged as a popular approach to directly modify glycans as the oxidation can be carefully tuned to selectively target sialic acids through mild conditions[175,176]. The practical applications of these

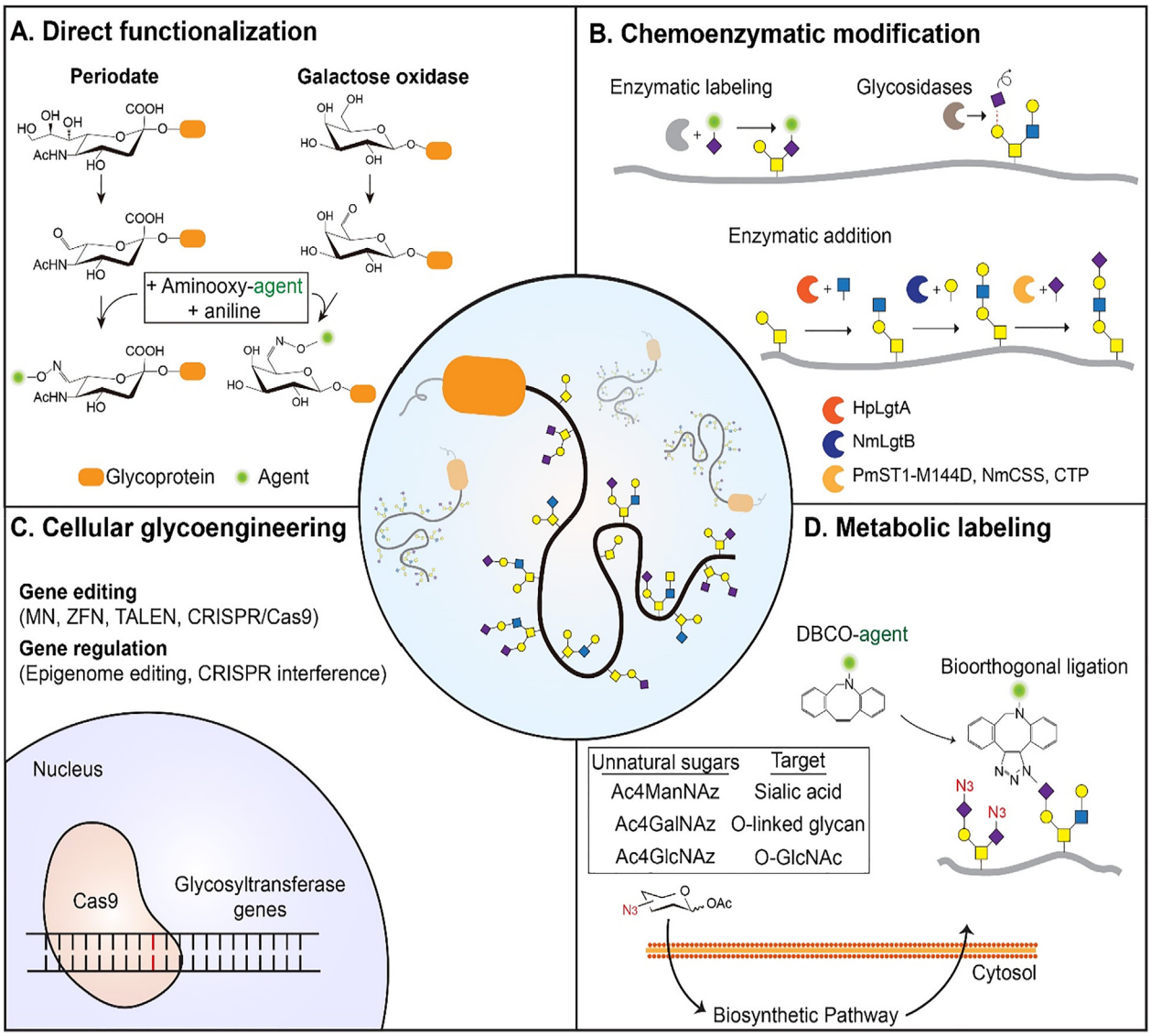


Fig. 5. Approaches for engineering and functionalization of mucin O-glycans. (A) Aldehydes are selectively introduced into sialic acids by periodate oxidation or into terminal galactose and N-acetylgalactosamine with galactose oxidase. The aldehydes can subsequently be coupled with aminooxy containing chemistries using aniline as a catalyst. (B) Glycan structures can be labelled, extended, and modified *in vitro* through chemoenzymatic approaches. (C) The cellular glycome and the glycosylation patterns on individual mucins can be tuned through individual or combinatorial knockout and knockin of glycosyltransferases (D) Metabolic glycoengineering exploits exogenous unnatural sugars, such as Ac4ManNAz, to functionalize glycan structures for subsequent bioconjugation with click chemistry.

approaches have been greatly propelled by nucleophilic catalysts, such as aniline, which allow imine/oxime ligation to proceed with fast kinetics at neutral pH to label biomolecules[177–179]. An important example is the development of Periodate oxidation and Aniline catalyzed oxime Ligation (PAL) that has permitted the glycan labelling of live cells without cytotoxicity[180,181]. Note that periodate indiscriminatory labels a variety of sialic acid containing structures on both N- and O-glycans[175], which may be useful to maximally derivatize mucins if the lack of conjugation site-specificity does not impede downstream applications[41].

Alternatively, enzymatic oxidation may be employed as a highly selective strategy. Terminal GalNAc monosaccharides, which are exclusively found on mucins O-glycans, can be enzymatically and selectively oxidized to generate aldehyde handles using a specific galactose oxidase[182]. Genetically engineered variants of galactose oxidases have been developed to specifically target a range of other monosaccharides[183]. Note that homogeneous decoration of mucins with terminal GalNAc can be achieved in SimpleCell platforms [138]. Enzymatic approaches may also sidestep potential off-target oxidation by sodium periodate on vicinal amino alcohols of terminal Ser and Thr [184,185], which may be alternatively circumvented by N-terminal protection strategies[186]. Note that imine/oxime ligations can suffer from reactions towards carbonyl groups that occur in complex biological system, but these groups should be absent on purified recombinant mucins unless introduced. The high efficiency and the minimal requirement of an intact live cell system make chemical and enzymatic oxidation, in combination with imine/oxime ligation, particularly attractive conjugation strategies of purified mucin products. With the fast-growing interests in using recombinant mucins as building materials for pharmaceutical and tissue engineering applications[187], it is highly conceivable that these approaches hold great potential for conjugating desirable functional groups and cargos to mucins for increased functionalities.

5.2. Glycoengineering with tagged monosaccharide analogs

Instead of direct modifications of natural monosaccharides already installed on glycans, chemical handles can also be introduced onto mucin glycans using modified unnatural monosaccharide building blocks that contain bioorthogonal functional group. Glycotransferases are known to tolerate structural promiscuity for substrate analogs that share high similarity with their natural counterparts[188,189]. Notably, studies in the late 1980s demonstrated that even large fluorophores attached to the C9 position of sialic acids can be tolerated by sialyltransferases[190]. Since then, unnatural monosaccharides with a variety of functional groups have been developed and successfully incorporated by glycotransferases onto glycans, including ketone, thiol, azide, alkyne, alkene, cyclopropane, isonitrile and cyclooctyne[191–193].

Unnatural monosaccharides bearing an azide or alkyne are among the most widely biorthogonal handles for chemical glycoengineering. While azides are fully abiotic and alkynes are mostly found outside of vertebrates[194], these two functional groups are both small in size and remain intact in biological systems until desired reaction as they are essentially inert to other biomolecules[169]. Azide and alkyne are complementary reaction partners and once tagged, the azide modified glycans can be stably conjugated to alkyne-containing molecules (and vice versa) through azide-alkyne cycloaddition, which can be copper-catalyzed or accelerated by strain-promoted cyclooctynes to avoid the complication of copper in living systems. The rapid growth of the field of chemical glycoengineering has vastly advanced the number of available unnatural monosaccharides and complementary bioorthogonal functional groups, and greatly improved reaction conditions for bioorthogonal click chemistry to enable fast and efficient bioconjugation[192]. In general, two main approaches have been employed to incorporate unnatural monosaccharides for chemical glycoengineering: metabolic glycoengineering (MGE) and chemoenzymatic glycan labelling (CeGL)

5.2.1. Metabolic glycoengineering

MGE relies on the presence of live cells for the uptake, processing, and incorporation of unnatural monosaccharides into glycans via cellular biosynthetic pathways[192]. This approach is particularly powerful as simple monosaccharide precursors for glycan biosynthesis are relatively easy to chemically synthesize and modify with desired functional groups. Once inside the cell, MGE exploits endogenous enzymes in glycan biosynthetic pathways to further process the precursor analogs for nucleotide activation and incorporation onto glycoconjugates by glycosyltransferases. MGE has expanded over the past three decades and emerged as a major tool for introducing chemical handles into a broad range of glycans in living systems for bioconjugation, using a variety of monosaccharide analogs and numerous ligation chemistries that are now readily available[195–199]. Notably, MGE has made use of unnatural GalNAc and analogs for N-acetylmannosamine (ManNAc)[193,200], which is the committed biosynthetic precursor of sialic acids. Thus, besides the impressive collection of toolkits, one major advantage of MGE is the potential to decorate recombinant mucins with useful functional groups during biosynthesis [195].

The main drawbacks of MGE lie in the variability of glycan labelling efficiency and specificity. The success of MGE depends on both the efficiency of incorporation of unnatural monosaccharides into glycans and the efficiency of the subsequent bioconjugation[201–204]. Monosaccharide analog design and the choice of host cell type can significantly impact the specificity, efficiency, and the rate of the metabolic incorporation[196,203,205–208]. Optimized ligation reactions can also drastically increase the efficiency of the subsequent bioconjugation[204]. These are thus important considerations for efficient and reliable mucin derivatization via MGE. Crosstalk between biosynthetic pathways can lead to the interconversion of monosaccharide analogs into unintended precursors and skew their incorporation into "off-target" glycan structures. An important example is the epimerization of GalNAc analogs, catalyzed by the UDP-GlcNAc/GalNAc epimerase (GALE), into the corresponding GlcNAc counterparts that are then incorporated into glycoproteins at high efficiency [209]. As mucins have the potential to carry a variety of glycan structures, these complications may pose significant challenges if homogeneous and chemically or structurally defined functionalized mucin products are sought after.

Tremendous efforts to unravel enzyme specificity in cellular biosynthetic pathways have led to improved metabolism and incorporation of unnatural monosaccharides[200,210–213]. Notably, the realization that native enzyme active sites may not optimally accommodate and catalyze unnatural monosaccharides has motivated the bump-and-hole strategy[210,212], where mutant enzymes are rationally designed and engineered to contain an enlarged active site (hole) for novel analogs bearing a bulky functional group (bump). These efforts have led to the development of a remarkable analog, N-(S)-azidoalaninyl galactosamine (GalNAzME), that is not epimerized into GlcNAc, and a mutant pyrophosphorylase structurally engineered to accommodate and metabolize GalNAzME to enable precision metabolic labelling of mucin O-glycans in live cells [214]. Additional metabolic incorporation of GalNAzME can be boosted by the expression of a rationally designed bump-and-hole GalNAc transferase. Once functionalized with GalNAzME, cell surface and secreted mucins can then undergo azide-alkyne cycloaddition for bioconjugation. Interestingly, bypassing metabolic bottlenecks by the introduction of custom designed biosynthetic enzymes may also dramatically improve the incorporation of poorly metabolized alkyne-bearing sugar analogs[215]. It is conceivable that similar approaches can likewise enrich mucins with specific chemical handles of choice. With the advent of powerful cell glycoengineering strategies for tuning specific mucin O-glycosylation patterns [53,54], these approaches should have great potential towards producing recombinant mucins with defined functionalization via MGE[128,216].

5.2.2. Chemoenzymatic glycan labelling

Complementary to MGE, CeGL employs cell-free recombinant glycosyltransferases to directly install modified monosaccharides from a suitable glycosyl donor onto mature glycoproteins [217]. This strategy circumvents the need for metabolic processing of cell-permeable monosaccharide analogs by live-cell biosynthetic pathways, which involve several enzymatic steps and metabolic bottlenecks. CeGL can therefore potentially accommodate unnatural sugars with bulkier functional groups to maximally exploit the promiscuous nature of glycosyltransferase enzymes for their substrates. On the other hand, the lack of an endogenous biosynthetic system also requires CeGL approaches to use activated sugar-nucleotide analogs, which can be challenging to synthesize and modify, for transfer onto a suitable glycan acceptor by glycosyltransferases. Although first described in 1979[218], CeGL has recently reemerged as a powerful technique to combine with bioorthogonal click chemistry for introducing chemical handles into glycoconjugates[217,219]. Like MGE, once tagged, glycoproteins such as mucins can undergo bioconjugation for further functionalization and derivatization via click reactions. However, the popular use of CeGL for glycoengineering has been largely hampered by the small number of available unnatural monosaccharides[217].

CeGL can be used to selectively functionalize mucin O-glycans by exploiting the inherent substrate specificity of known glycosyltransferases. One common approach has employed a mucin O-

glycan specific sialylatransferase, ST3Gal1, to selectively incorporate CMP-sialic acid analogs bearing either an azide or alkyne handle into mucin O-glycans for subsequent conjugations[220–222]. Efforts on expanding the CeGL toolbox have led to the development of novel nucleotide sugar derivatives of UDP-GalNAc and UDP-GlcNAc bearing a variety of functional groups[223]. Using these unnatural sugars, in combination with recombinant glycosyltransferases previously identified from bacterial and mammalian sources, specific glycan structures and linkages that are frequently found on mucins could be chemoenzymatically assembled to contain functional groups for subsequent bioconjugation. Examples of derivatized mucin glycans by this approach include the Tn and T antigens, and Neu5Acα2,3-Gal and Fucα1,2-Gal epitopes[224]. This highlights the utility of CeGL for the precise insertion of unnatural monosaccharides into desired glycan structures and the ability to target unique glycan linkages and higher order structures, which cannot be achieved by current MGE approaches due to the presence of overlapping glycosyltransferases in glycan biosynthetic pathways[217]. Recombinant glycosyltransferases and glycan editing enzymes, such as sialidases, can also be mixed-and-matched from different species to assemble specific glycan structures with desired functional groups. CeGL is thus able to tap into the vast amount of bacterial enzymes that are well characterized and easy to express for use[225].

It is worth noting that a cell-free modular enzymatic platform has recently come online capable of generating 83 common mucin O-glycan structures from the core glycans[226]. Engineered cell systems, such as SimpleCells, are already available to generate recombinant mucins endowed with glycans containing only the first GalNAc residue[85]. Combined with the production of recombinant mucins bearing defined glycans ready for elaboration through enzymatic platforms, with the expansion of novel bioorthogonal sugar nucleotide analogs, CeGL is poised to advance approaches for derivatizing recombinant mucins with specific functionalized glycans epitopes to confer new biochemical and biophysical properties in their application.

6. Applications and outlook

Current glycoengineering advances that target mucin O-glycans have largely focused on developing methodologies for the visualization and the selective delivery of molecules, such as therapeutics, that target the cell surface, or to enrich mucins for characterization[219,227–231]. However, the search for new biomaterials in biomedical engineering has sparked great interests in exploring mucin polymers as hydrogel backbones for constructing functional implants[40]. Some of these efforts have started to functionalize glycans for mucin assembly into robust hydrogels. For example, mild periodate oxidation has been employed to selectively functionalize sialic acids for crosslinking mucins[41]. These hydrogels exhibited different bioactivities, including susceptibility to protease degradation and macrophage response, as compared to hydrogels crosslinked via functionalized mucin protein backbone.

This points to the potential of tunable bioactivities of mucin hydrogels by functionalizing mucin glycans and especially highlights the utility of click chemistry for hydrogel assembly[232]. In addition, mucin hydrogels have been shown to modulate *in vivo* fibrotic and immune responses[233], possibly through sialic acid-bearing glycans[234], and to retain drug molecules for sustained delivery[235]. It is conceivable that these properties could be enhanced and customized by derivatizing mucin O-glycans. On a related note, MGE is becoming an attractive approach in tissue engineering to functionalize secreted extracellular matrix for bioconjugation through click chemistry[171,236–239]. It is also conceivable that mucin polymers with modified glycans bearing complementary clickable functional groups could be incorporated into these matrices to introduce new and tailored bioactive properties.

Mucin glycans are known to confer important properties of mucin polymers including structural stability, gelation, adsorption, lubricity, and specific bioactivities[43,240–242]. It is likely that glycoengineering can introduce functional groups to augment these properties. Potential applications of glycoengineering to increase mucin functions may be drawn from approaches that aim to enhance glycoproteins[243]. One important example is the use of glycan oxidation and CeGL to advance conjugation strategies for generating antibody-drug conjugates (ADC) [170,244–246]. These strategies include the GlycoConnect and GlyCLICK technologies for glycan remodeling and the incorporation of an azide-bearing GalNAc analog, enabling the site-specific and homogeneous antibody-conjugation of toxic payloads to enhance tumor killing and regression[247,248]. Similar glycoengineering strategies have also been employed for ADC radioimmunoconjugates, for example, to generate dual functionalized ADCs for simultaneous therapy and ImmunoPET[249]. Although these strategies have focused on engineering antibody N-glycans, they represent unexplored opportunities for mucin glycoengineering especially in light of mucins as potent anti-viral and anti-microbial agents [250–255], as delivery nanocarriers for drugs and imaging probes [187,256–259], as multi-layer surface coatings for on-demand drug release[257], as powerful biomedical lubricants and wear protectants[260,261], or as hydrogels for developing novel food materials[262]. Furthermore, advances in recombinant mucin production and engineering can be exploited for the development of better *in vitro* models of mucus and mucosal tissues. These and other applications are likely to greatly benefit from the scalable production of large, sequence-specific mucins with defined O-glycans.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [H.L.R. and M.J.P. have filed provisional patents related to some of the synthetic lubricin and mucin sequences referenced in this review.].

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