Bacterial glycoengineering: cell-based and cell-free routes for producing biopharmaceuticals with customized glycosylation

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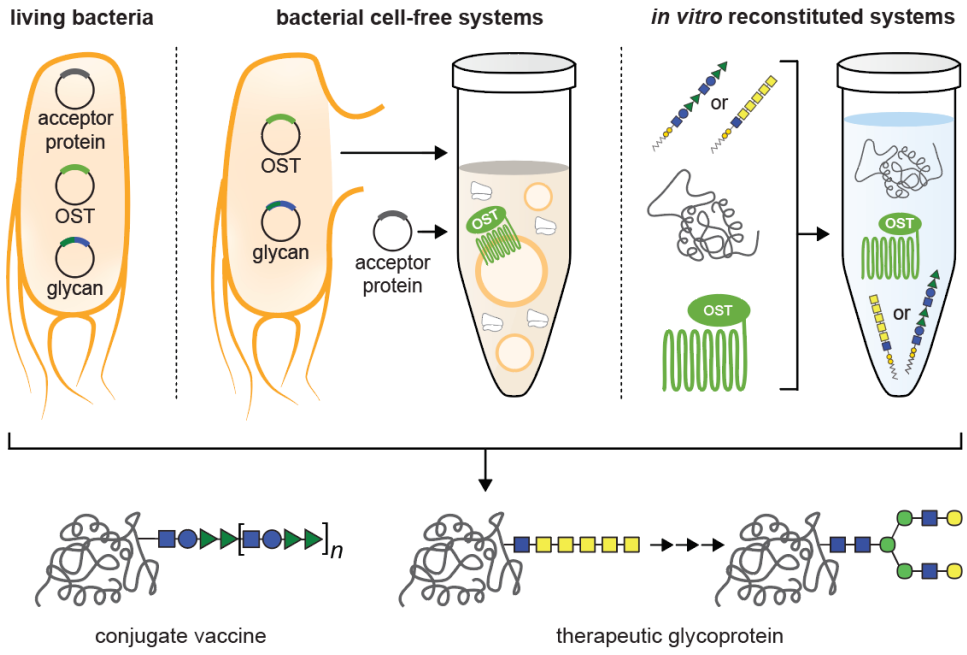
Graphical Abstract



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Abbreviations

ADCC – antibody-dependent cellular cytotoxicity
CDC – complement-dependent cytotoxicity
CFPS – cell-free protein synthesis
CFGpS – cell-free glycoprotein synthesis
CPS – capsular polysaccharide
CRM_{197} – cross-reactive material 197 from *Corynebacterium diphtheriae*
ENGase – endoglycosidase
Fc – fragment crystallizable
GalNAc – *N*-acetylgalactosamine
GlcNAc – *N*-acetylglucosamine
GH – glycoside hydrolase
GT – glycosyltransferase
IgG – immunoglobulin
LLO – lipid-linked oligosaccharide
mAb – monoclonal antibody
O-PS – O-polysaccharide
OST – oligosaccharyltransferase
PD – protein D from *Haemophilus influenzae*
PGCT – protein glycan coupling technology

Abstract

Glycosylation plays a pivotal role in tuning the folding and function of proteins. Because most human therapeutic proteins are glycosylated, understanding and controlling glycosylation is important for the design, optimization, and manufacture of biopharmaceuticals. Unfortunately, natural eukaryotic glycosylation pathways are complex and often produce heterogeneous glycan patterns, making the production of glycoproteins with chemically precise and homogeneous glycan structures difficult. To overcome these limitations, bacterial glycoengineering has emerged as a simple, cost-effective, and scalable approach to produce designer glycoprotein therapeutics and vaccines in which the glycan structures are engineered to reduce heterogeneity and improve biological and biophysical attributes of the protein. Here, we discuss recent advances in bacterial cell-based and cell-free glycoengineering that have enabled the production of biopharmaceutical glycoproteins with customized glycan structures.

Introduction

The attachment of complex sugars known as glycans to the side chains of protein amino acids, a process known as glycosylation, is a complex and abundant post-translational modification that occurs in all domains of life. Glycosylation shapes the functional landscape of proteins, with over 50% of the human proteome being glycosylated [1,2]. Glycans are structurally diverse and have been shown to play essential roles in the biological and biophysical properties of proteins, such as activity, stability, immunogenicity, serum half-life, and trafficking [3]. Accordingly, the strategic attachment of glycans is often used to enhance the therapeutic efficacy and pharmacological profiles of protein therapeutics and vaccines [4]. Given the association between distinct glycan structures and specific biological functions, heterogeneous glycosylation can compromise intended biological activity and evoke immunogenic responses, emphasizing the importance of homogeneous glycoprotein medicines.

The diversity and complexity of glycan structures, with more than 7,000 glycan determinants in the human glycome, stems from their non-template-based synthesis, which involves the spatial and temporal activity of enzymes known as glycosyltransferases (GTs). As such, widely used methods for genetic and protein engineering have limited applicability. Thus, the development of tools and platforms for efficient biosynthesis and modification of structurally diverse glycans on lipids and proteins is essential to enabling protein glycoengineering. Glycoengineering therapeutic proteins is achieved by a variety of approaches, with the ultimate goal of achieving site-specific attachment of homogeneous glycan structures. Mammalian-based expression systems are the current industry standard for producing therapeutic glycoproteins, with over 70% of approved recombinant glycoproteins being produced in mammalian cells such as Chinese hamster ovary (CHO) cells [5]. While strides are being made to improve glycoprotein expression in mammalian systems, for example, by using gene-editing technology to precisely tailor glycan structures [6,7], significant drawbacks such as high manufacturing costs, heterogeneous product formation, and contamination risks persist. Amidst rising global demand for protein biologics, exploring alternative strategies and production hosts that enable rapid, low-cost, and distributed biomanufacturing becomes imperative.

Bacterial glycoengineering is an emerging area of research that seeks to harness the genetic tractability of prokaryotic hosts together with detailed knowledge of glycosylation pathways and genes across phylogeny for the creation of novel glycomolecules including glycoprotein therapeutics and conjugate vaccines (recently reviewed in refs. [8-11]). In the earliest demonstration of this concept more than 20 years ago, the *N*-linked protein glycosylation pathway from *Campylobacter jejuni* was functionally transferred into *Escherichia coli* [12], paving the way for recombinant expression of glycoproteins in this simple, genetically tractable, and cost-effective host organism. Because *E. coli* cells lack endogenous glycosylation machinery, they offer a "blank canvas" for faithfully constructing virtually any glycan structure on acceptor proteins of interest. Indeed, the spectrum of glycoconjugates that are possible in *E. coli* seems limited only by imagination. On the one hand, laboratory strains of *E. coli* have been engineered to build capsular polysaccharide (CPS) and O-polysaccharide (O-PS) antigens from pathogenic bacteria and transfer these to carrier proteins [13,14], giving rise to conjugate vaccines that have proven effective in preventing infectious disease. On the other hand, human-type *N*- and *O*-linked glycosylation pathways have been assembled in laboratory strains of *E. coli*, bestowing these cells with the ability to produce human glycoproteins [15,16]. In addition to their use as glycoprotein factories, these glyco-competent *E. coli* have also been leveraged as chassis strains for sourcing cell-free extracts that co-activate *N*- and *O*-linked glycosylation reactions and enable biosynthesis of glycoprotein outside of living cells [15,17]. Inspired by these initial reports, a wide array of systems now exists that interface protein glycosylation with cell-free protein synthesis (CFPS), which we refer to as cell-free glycoprotein synthesis (CFGpS; recently reviewed in refs. [18-20]). To date, these systems have been used to produce conjugate vaccines [21-24] and therapeutic glycoproteins including human Fc domains [16,25,26], thus positioning CFGpS technology as an important new addition to the synthetic glycobiology toolbox for accelerating expression and biomanufacturing of glycoprotein products.

In this review, we discuss recent discoveries and approaches in bacterial glycoengineering that are paving the way for rapid, homogeneous, and scalable production of valuable glycoprotein biopharmaceuticals in laboratory strains of *E. coli* and

their cell-free extracts, with particular focus on two major product classes: conjugate vaccines and monoclonal antibodies (mAbs).

Biosynthesis of conjugate vaccines in glycoengineered bacterial cells

Conjugate vaccines are among the safest and most effective methods for preventing disease caused by bacterial pathogens [27-29] and are a significant advancement in vaccine technology. Conjugates overcome the challenge of eliciting a strong immune response against bacterial cell surface carbohydrate antigens by conjugating a pathogen-specific capsular polysaccharide (CPS) or O-antigen polysaccharide (O-PS) linked to an immunostimulatory protein carrier. Traditional conjugate vaccines are produced by extraction, fragmentation, and purification of pathogen-derived CPS or O-PS antigens, followed by chemical activation and random conjugation to the carrier protein (**Fig. 1a**). Unfortunately, this process can result in a number of undesirable outcomes including highly variable saccharide density per carrier protein, batch-to-batch product variability, and interruption of important B- and T-cell epitopes on the carrier protein [30]. Moreover, the multistep process is expensive and laborious, and is often met with low yields due to recovery losses at each of the successive steps.

An alternative approach is bioconjugation, also known as protein glycan coupling technology (PGCT), which leverages glyco-competent *E. coli* as cellular factories for total biosynthesis of designer conjugate vaccines against a wide array of different pathogenic bacteria (for recent reviews, see refs. [31,32]). The method involves simultaneous expression of the oligo- or polysaccharide antigen of interest, a glycan conjugating enzyme, and a carrier protein in laboratory strains of *E. coli*, resulting in a streamlined and low-cost process that overcomes many of the shortcomings associated with production of traditional conjugates (**Fig. 1b**). At the heart of bioconjugation technology are single-subunit transmembrane oligosaccharyltransferases (OSTs) from bacteria. These enzymes are capable of transferring diverse CPS and O-PS antigens from undecaprenyl pyrophosphate (Und-PP) onto either the amide group of asparagine residues in the case of *N*-glycosylation [13] or the hydroxyl group of serine or threonine residues in the case of *O*-glycosylation [33]. Most notable among these enzymes is the OST from *C. jejuni* named PglB (*Cj*PglB), which exhibits relaxed specificity towards the

glycan structure [13,14] and is capable of installing diverse oligo- and polysaccharides onto almost any recombinant protein that harbors a D/E-X_{-1}-N-X_{+1}-S/T ($X_{-1,+1} \neq$ P) acceptor site ("glyco-tag") either natively or engineered at internal or terminal locations in the carrier [25,34].

Based on this catalytic flexibility, *Cj*PglB has been widely used to produce antibacterial conjugate vaccines bearing O-PS, CPS, or other exopolysaccharide antigens that are pre-assembled as Und-PP-linked intermediates and ultimately transferred to suitable vaccine carrier proteins. In one of the earliest examples, *Cj*PglB was leveraged for the production of a conjugate vaccine composed of the *Shigella dysenteriae* serotype 1 O-PS (O1) glycan conjugated to exotoxin A from *Pseudomonas aeruginosa* (EPA) harboring two engineered glycosylation sites [35]. Importantly, this conjugate was tested in human clinical trials (NCT01069471) and found to be well tolerated both locally and systemically and elicited statistically significant immune responses against O1 polysaccharides at all time points in all groups [36].

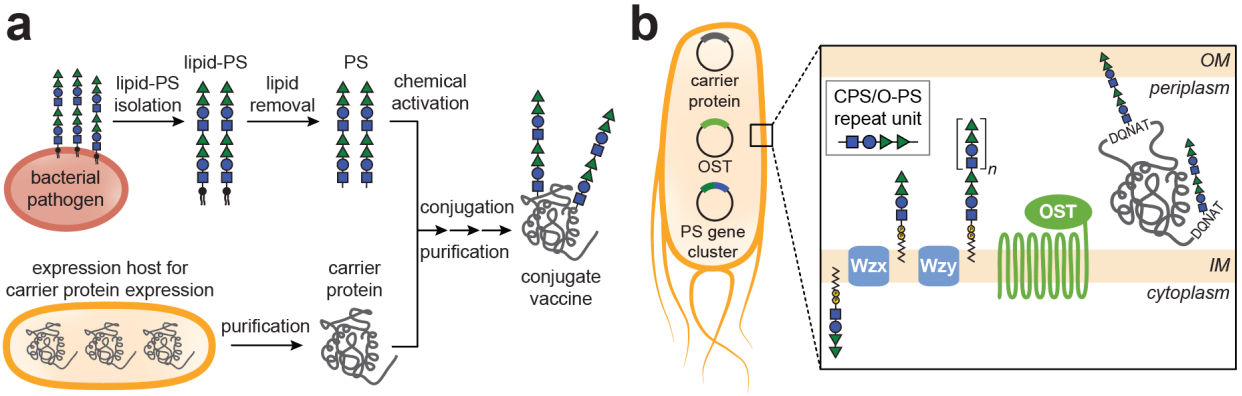

Figure 1. Traditional conjugate versus bioconjugate vaccine production. (a) The traditional chemical conjugation method for producing conjugate vaccines is a multistep process in which the polysaccharide (PS) antigen is purified from the pathogen of interest, separated from its lipid carrier, chemically activated, and randomly conjugated to a separately expressed and purified carrier protein. Following conjugation, several additional rounds of purification are required before administration. (b) The bioconjugation method involves engineering non-pathogenic *E. coli* with three components – glycan biosynthesis pathway, conjugating enzyme, and carrier protein – that enable a renewable supply of glycoprotein products. Glycan biosynthetic pathways are typically ~10-20 kb in length and encode most of the enzymes (~10-15) required for biosynthesis of the CPS or O-PS antigen of interest. The polysaccharides are assembled on a lipid carrier on the cytoplasmic side of the inner membrane (IM), translocated to the periplasmic side of the IM by the Wzx flippase, and extended by the Wzy polymerase. The OST recognizes the reducing-end of the pre-assembled polysaccharide and transfers it *en bloc* to a preferred acceptor sequence (e.g., DQNAT) in a periplasmically expressed carrier protein. While these components are commonly encoded in plasmids that are used to transform the bacterial host, stable integration of these components into the host genome has also been demonstrated. Overall, the process yields a glycoconjugate in which the polysaccharide antigen is site-specifically conjugated at one or more defined locations in the carrier protein.

A major advantage of bioconjugation technology is its modularity, offering a plug-and-play platform where any of the three main components – polysaccharide antigen, conjugating OST, and carrier protein – can be readily interchanged for producing an array of new conjugate designs. In the context of carrier proteins, EPA remains a popular choice because of its long-established compatibility with the bacterial glycosylation machinery [35]. However, conjugates involving licensed carrier proteins such as cross-reactive material 197 (CRM_{197}) from *Corynebacterium diphtheriae* and *Haemophilus influenzae* protein D (PD) have been produced in glyco-competent *E. coli* and are immunogenic and protective in mice [24]. Bioconjugation technology has also been used in conjunction with carrier proteins that are from the same pathogen as the glycan and are chosen based on their high conservation across serotypes, thus potentially providing broader vaccine coverage [37].

This modularity has also been exploited for customizing the polysaccharide component, thereby enabling conjugates against an array of bacterial pathogens including enterohemorrhagic *E. coli* (EHEC), *Francisella tularensis*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Shigella* sp., *Staphylococcus aureus*, and *Streptococcus pneumoniae*, among others (for a complete list of conjugate vaccines produced using bioconjugation technology that are in pre-clinical or clinical development, see ref. [32]). In the past three years alone, the repertoire of polysaccharides that have been successfully transferred by *Cj*PglB has expanded to include conjugate vaccines against enterotoxigenic *E. coli* (ETEC) serotypes O78 and O148 [24], extraintestinal pathogenic *E. coli* (ExPEC) serotype O25B [38], and Group A Streptococcus (Strep A or GAS) [39]. Interestingly, in the case of GAS, the reducing end of the native Group A carbohydrate (GAC) structure contains a rhamnose residue that is β1,4-linked to GlcNAc, which is known to be a poor substrate for *Cj*PglB [40]. To overcome this limitation, the authors created a hybrid GAC structure with a remodeled reducing end structure that was compatible with *Cj*PglB [39], highlighting the engineerability of the bioconjugation platform.

Despite the many successes to date, the biosynthesis of heterologous polysaccharides can be challenging for several reasons. First, the pathways are large, as exemplified by the 9-16-kb gene clusters (encoding 12-15 enzymes) involved in making

CPS antigens for several different *S. pneumoniae* serotypes [41]. Because of their large size, creation of plasmids that encode these clusters involves complicated cloning strategies for stitching together multi-gene pathways. Second, maintaining such large plasmids in the host can be difficult; hence, efforts have been made to stably integrate glycan biosynthesis pathways and/or the OST into the host genome [42-45]. Third, the host *E. coli* strain may natively express certain factors, which can be both productive and counterproductive. For example, the *E. coli* WecA enzyme natively primes the lipid carrier with *N*-acetylglucosamine (GlcNAc), which is advantageous for making heterologous polysaccharides that initiate with GlcNAc such as the O-PS from *S. dysenteriae* serotype 1 but is undesirable for those that initiate with alternative monosaccharides such as the O-PS from *S. flexneri* serotype 6 that primes off *N*-acetylgalactosamine (GalNAc). To address these issues, strain engineering to delete certain genes and overexpress others has proven to be an effective strategy for enhancing polysaccharide biosynthesis [46]. It should also be noted that conjugate vaccines have been produced by introducing the OST and carrier protein directly into the pathogenic bacterium, which bypasses some of the limitations of *E. coli* as a host and avoids the need for cloning and recombinant expression of the O-antigen gene cluster altogether [45,47].

Another challenge relates to the conjugating enzyme, *Cj*PglB, which despite its relaxed glycan substrate specificity exhibits poor transfer of polysaccharides lacking an acetamido group modification on their reducing-end sugar [14] or involving β1,4-linkage between the two sugars proximal to the lipid carrier [40]. One of the most successful strategies for overcoming these limitations of *Cj*PglB has been the use of alternative conjugating enzymes. Indeed, non-homologous bacterial OSTs have been identified that execute *O*-linked glycosylation of serine and threonine residues in distinct acceptor sequences and can transfer long-chain bacterial polysaccharide antigens to carrier proteins expressed in the *E. coli* periplasm. Among these, the *O*-linking OST named PglL from *Neisseria meningitidis* is particularly promiscuous with respect to the glycan structures it can transfer [33] and has been used to create an *O*-linking bioconjugation strategy [48], with the caveat that it requires an 8-amino acid glycosylation site flanked by long hydrophobic sequences. Recently, this system has been used to produce novel conjugate vaccine candidates against *Brucella abortus* [49], ExPEC serotypes O5 and

O7 [50], uropathogenic *E. coli* (UPEC) serotype O21 [51], and *Klebsiella pneumoniae* O1 [52]. PglS is another *O*-linking enzyme and the only OST shown thus far to transfer polysaccharides with glucose at the reducing end [53], allowing the bioconjugation of unique polysaccharide structures from *Streptococcus pneumoniae* [54] and multiple serotypes of *K. pneumoniae* including K1 and K2 [55,56]. The newest class of *O*-linking OSTs termed TfpM from *Moraxellaceae* bacteria can transfer diverse CPS and O-PS structures from a variety of bacteria including *Salmonella*, *S. pneumoniae*, *K. pneumoniae*, and Group B *Streptococcus* (GBS), with *M. osloensis* TfpM being used to create an immunogenic conjugate containing the type III CPS from GBS [57]. It is anticipated that bioconjugation technology will expand even further as the substrate preferences for these and other coupling enzymes are more deeply characterized, and their conjugating activity optimized.

Biosynthesis of conjugate vaccines in bacterial cell-free systems

An emerging alternative for making conjugate vaccines is cell-free glycoprotein synthesis (CFGpS) technology, which uses cell lysates rather than living cells to synthesize glycoproteins *in vitro* (for recent reviews, see refs. [18-20]) (**Fig. 2a**). CFGpS leverages glyco-competent *E. coli* strains, such as those described above, to source cell extracts that are selectively enriched with glycosylation components, namely lipid-linked oligosaccharides (LLOs) and OSTs (for a detailed methodology, see [58]). The resulting extracts enable seamless integration of transcription/translation with protein glycosylation in a one-pot reaction scheme for efficient and site-specific glycosylation, as we demonstrated in proof-of-concept studies using model glycosylation components [17,59]. Building on this earlier work, we recently adapted the method for biosynthesis of conjugate vaccines bearing O-PS antigens from highly virulent *F. tularensis* Schu S4, with the resulting conjugates completely protecting mice against lethal challenge with live vaccine strains (LVS) of *F. tularensis* [21]. Similarly, CFGpS was used to prepare a conjugate against UPEC serotype O7 and ETEC serotype O78 with the latter eliciting bactericidal antibodies against the pathogen [21,24], highlighting the modularity of the CFGpS platform that allows structurally diverse LLOs to be readily interchanged in a plug-and-play fashion. In addition to different LLOs, distinct OSTs including *Cj*PglB, PglB from

D. marinus and PglL from *Neisseria gonorrhoeae* have been shown to function in CFGpS reactions [22], potentially expanding the functionality of the technology. Importantly, cell-free platforms offer multiple advantages including that they: (1) are unconstrained by the potential toxicity arising from expression of the glycosylation components; (2) offer shortened vaccine development timelines; (3) enable distributed glycoprotein production; (4) can be freeze-dried for distribution at temperatures up to 50 °C and reconstituted by just adding water; and (5) can be produced inexpensively (~US$0.50 for a single conjugate vaccine dose) [21,23]. With these advantages, bacterial cell-free systems offer unique opportunities to accelerate development of glycosylated biologics and enable decentralized, cold chain-independent biomanufacturing.

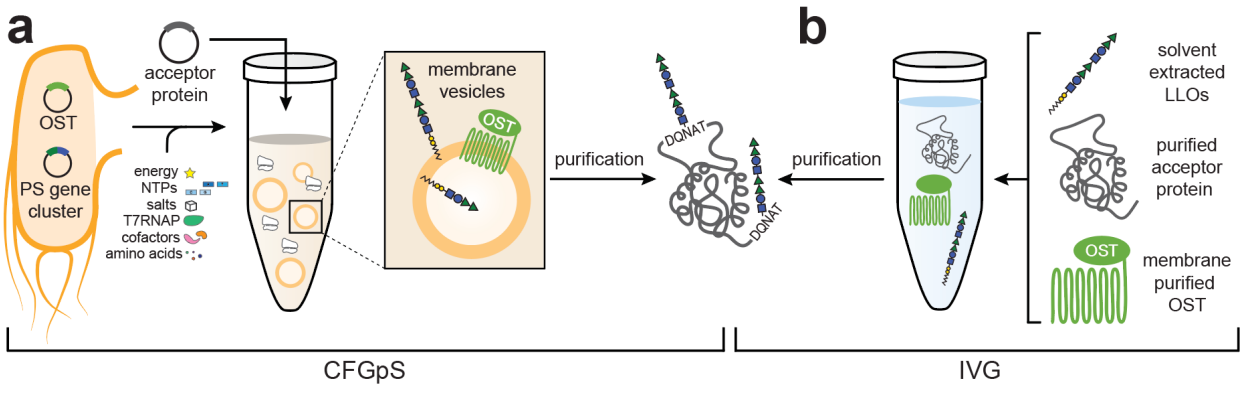

Figure 2. Cell-free approaches for making glycoproteins. (a) Cell-free glycoprotein synthesis (CFGpS) utilizes glyco-enriched extracts derived from a glycosylation competent *E. coli* chassis strain carrying plasmids encoding the glycan biosynthesis pathway and the OST. Lysates derived from this strain are supplemented with translation components (e.g., NTPs, T7 RNA polymerase, amino acids) and primed with the DNA encoding the protein of interest, such that transcription/translation and glycosylation are integrated in a single pot reaction. Glycosylation involves membrane vesicles that are enriched with the OST and LLOs. (b) *In vitro* glycosylation (IVG) involves mixing separate preparations of solvent-extracted LLOs, membrane-purified OST, and purified acceptor protein that is already folded. Each component is added in controllable ratios to permit glycoprotein production in a cell-free reaction.

Bacteria-enabled systems for making mAbs with structurally defined glycans

Therapeutic mAbs are an expanding class of immunotherapy that are widely used in the treatment of cancers, autoimmune diseases, inflammatory diseases, and bacterial and viral infections. Most therapeutic mAbs are of the immunoglobulin G (IgG) subclass, which are glycosylated at a conserved asparagine residue (Asn297) in the CH2 domain of the fragment crystallizable (Fc) region. *N*-linked glycosylation of IgG-Fc is vital for the structural and functional properties of mAb therapeutics, including stability,

pharmacokinetics, safety, and clinical efficacy [60]. IgG-Fc glycans are also essential for Fc receptor binding, and consequently, are key drivers of important antibody effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).

A significant challenge in mAb drug development is the fact that glycans attached at Asn297 are usually heterogeneous due to (1) variable addition and processing of outer-arm residues (e.g., sialic acid, galactose) and core fucose residues that occur on the biantennary heptasaccharide, $GlcNAc_2Man_3GlcNAc_2$ (designated G0) and (2) alterations that are dependent on the expression host and culture conditions. Because such glycoform heterogeneity can adversely affect important therapeutic properties, strategies for creating mAbs with only tailored glycoforms that exert specific effects are in high demand. For instance, absence of core fucose on IgG-Fc *N*-glycans increases mAb binding affinity to FcγRIIIa present on immune effector cells and promotes enhanced ADCC activity [61]. Additionally, increased galactosylation and sialylation on IgG Fc *N*-glycans enhances the anti-inflammatory and complement-dependent cytotoxicity (CDC) properties of mAbs [62]. To this end, glycoengineering has emerged as a promising approach for producing desired mAb glycoforms with improved efficacy (e.g., enhanced ADCC, CDC) and safety (e.g., decreased immunogenicity) (for recent reviews, see refs. [63,64]). Here, we focus on antibody Fc engineering strategies that leverage bacterial cell and cell-free expression systems.

One of the most user-friendly strategies for engineering *N*-glycan structures is chemoenzymatic synthesis in which glycosyltransferase (GT) enzymes are used in conjunction with appropriate *N*-glycan precursors and sugar donors to build desired glycoforms (for a recent review, see ref. [65]). Over the past several decades, our understanding of the *in vitro* activity of GTs has increased greatly, revealing a large collection of enzymes that are capable of remodeling *N*-glycans outside of living cells. Importantly, bacterial cells have played a major role in enabling chemoenzymatic glycan synthesis. For example, glyco-engineered *E. coli* that produce human-type $Man_3GlcNAc_2$ *N*-glycans [16] have been used for supplying lipid-linked *N*-glycan precursors that were elaborated *in vitro* to create hybrid-type and complex-type biantennary *N*-glycans using a panel of GTs (e.g., *Nicotiana tabacum* GnTI, *Homo sapiens* GnTII, and *Bos taurus* GnTIV

and β1,4-GalT) that were separately expressed and purified from *E. coli* cells [66]. A similar panel of enzymes, also expressed and purified from *E. coli*, was used to generate synthetic LLOs bearing hybrid-type and complex-type *N*-glycans [67]. The free reducing-end or lipid-linked *N*-glycans resulting from these chemoenzymatic approaches serve as well-defined starting materials for building glycoproteins via either transglycosylation using endoglycosidases (ENGases) [68] (discussed in more detail below) or *in vitro* glycosylation (IVG) using single-subunit OSTs [17,67] (**Fig. 2b**), respectively.

Chemoenzymatic synthesis strategies have also been developed for direct glycan editing on intact glycoproteins, enabling the conversion of undesired and/or heterogeneous *N*-glycan structures into more uniform and desirable glycoforms (**Fig. 3**). For example, remodeling the *N*-glycans on bovine RNAse B from high-mannose type to disialylated complex biantennary *N*-glycans was accomplished in both step-wise and one-pot reactions with a small panel of bacterial and mammalian glycoside hydrolases (GHs) and GTs that were recombinantly expressed and purified using *E. coli* cells [69]. While these authors obtained soluble expression by truncating the mammalian GTs to remove their transmembrane domains and fusing them to maltose-binding protein (MBP), this common *E. coli*-centric solubilization strategy is not always successful. To address this issue, we recently described a strategy for topologically converting membrane-bound GTs into water soluble biocatalysts, enabling high-level expression of nearly 100 difficult-to-express GTs, predominantly of human origin, in the *E. coli* cytoplasm with retention of biological activity [70]. Following purification from *E. coli*, a subset of these water-soluble enzymes was utilized for *in vitro* remodeling of both free and protein-linked *N*-glycans including those found on the therapeutic mAb trastuzumab, yielding homogeneous G0, G2, or G2S glycoforms. Along similar lines, an enzyme immobilization strategy was recently described whereby $Man_5GlcNAc_2$ *N*-glycans on a monomeric Fc fragment were remodeled to a mono-antennary human-like *N*-glycan using GTs that were expressed and biotinylated in *E. coli* and subsequently tethered to streptavidin-coated silica beads [71]. The immobilized system eliminates the need for GT and intermediate product purification and enables reuse of the enzymes, which should decrease the cost and simplify scalability of GT-driven remodeling reactions.

Alongside GTs, GH enzymes are an important component of the glycoengineering toolkit for redesigning therapeutic glycoproteins. Among these enzymes, endoglycosidases (ENGases) from bacteria stand out as excellent candidates for modifying the glycosylation patterns of heterogeneously glycosylated glycoproteins. ENGases can effectively trim branched *N*-linked glycans, yielding a single GlcNAc monomer on the glycoprotein. Furthermore, ENGases can also be utilized as glycosynthase enzymes through strategic mutations that eliminate their hydrolytic activity. Glycosynthase enzymes require activated glycosyl donors in the form of oxazolones or anomeric fluorides for *en bloc* transfer of pre-synthesized glycans to another acceptor glycan. Notably, this approach has been successfully exploited to generate homogeneously glycosylated glycoproteins including mAbs (for a recent review, see ref. [72]). Despite their shared ability to catalyze hydrolysis of the same glycosidic linkage (β1-4 of chitobiose core), various versions of ENGases exhibit distinct substrate requirements for complex, hybrid, and high-mannose glycans, as well as core fucosylation of the reducing-end GlcNAc. This structural specificity also extends to the glycosynthase mutants of ENGases. For instance, three diverse bacterial ENGases, namely Endo-S, Endo-F3 and Endo-S2, were used to orthogonally transglycosylate the Fab and Fc domains of the therapeutic mAb, cetuximab, with different glycoforms based on the substrate specificities of the enzymes [73].

The advent of ENGase technology has not only facilitated remodeling of *N*-glycans on mammalian cell-derived glycoproteins but has also opened avenues for using *E. coli* to source glycoproteins whose glycans can be humanized by ENGase-mediated remodeling (**Fig. 3**). Specifically, glyco-competent *E. coli* cells were used to install a linear $GalNAc_5GlcNAc$ *N*-glycan onto AcrA from *C. jejuni*, a model bacterial glycoprotein that harbors two glycosylation sites at Asn123 and Asn273 [26]. Subsequent digestion with exo-α-*N*-acetylgalactosaminidase (GalNAcase) led to the production of a pure AcrA glycoform carrying only single GlcNAc residues, which were converted to human-type $Man_3GlcNAc_2$ glycans by EndoA-mediated transglycosylation. We recently extended this approach to install complex human-type glycans at the conserved Asn297 residue in the Fc domain of human IgG1 (unpublished observations). Specifically, glycocompetent *E. coli* cells were used to produce human hinge-Fc fragments bearing $GalNAc_5GlcNAc$ *N*-

glycans that were trimmed with GalNAcase and subsequently converted to complex, human-type G2 *N*-glycans ($Gal_2GlcNAc_2Man_3GlcNAc_2$) using EndoS. Importantly, the resulting G2-hinge-Fc exhibited strong binding to human FcγRIIIa (CD16a), one of the most potent receptors for eliciting ADCC. Taken together, these studies open the door to using *E. coli* for the production and subsequent glycoengineering of human mAbs and fragments derived thereof.

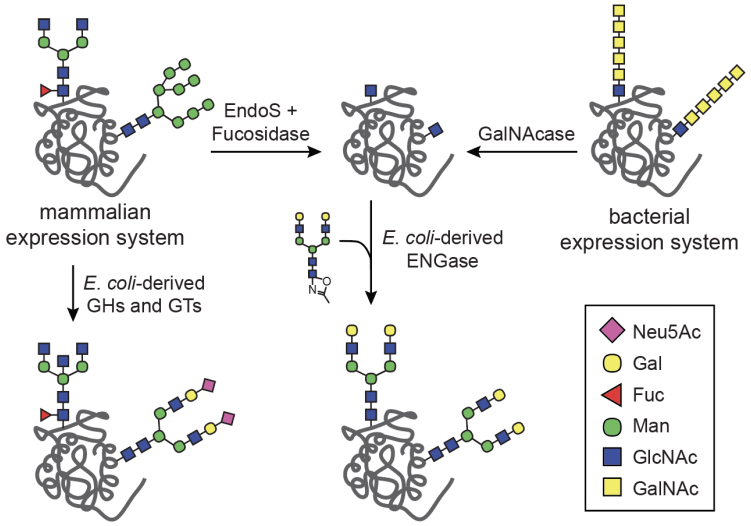

Figure 3. Chemoenzymatic approaches for producing homogeneous glycoproteins. Glycoprotein targets expressed in mammalian or glyco-competent bacterial host cells exhibit glycosylation profiles that are heterogenous or undesired, respectively. In both cases, the glycan moieties can be trimmed to single GlcNAc monomers by the action of endo- or exoglycosidases (top), and these trimmed structures can then serve as acceptors for extension into complex *N*-linked glycans via transglycosylation using bacterial endoglycosidases (ENGases) produced using *E. coli*. Alternatively, glycan remodeling of human-like glycans is performed using glycosyl hydrolase (GH) and glycosyltransferase (GT) enzymes, which can also be produced using *E. coli* (bottom left), to remove or introduce essential epitopes such as core fucose, bisecting GlcNAc, and terminal galactose or sialic acid.

Perspectives and conclusions

Ever since the discovery of *Cj*PglB and its functional transfer into laboratory strains of *E. coli* [12], great progress has been made in the development of bacterial cell and cell-free systems that leverage enzymes from all domains of life to biologically couple glycans to protein carriers. These efforts have resulted in a dramatic expansion of the bacterial glycoengineering toolkit that, in turn, is enabling the biosynthesis of a growing number of biopharmaceuticals with customized glycosylation. While conjugate vaccines and mAb-based products are among the most advanced targets to be addressed with bacterial glycoengineering, other important biopharmaceutical products including erythropoietin

(EPO) [17], interferon α-2b [74], MUC1 [15], and RNase A [16,75,76] have been glycosylated in *E. coli*, with many more on the horizon. It is also worth mentioning that while total biosynthesis of full-length mAbs bearing hybrid- or complex-type *N*-glycans has yet to be achieved in *E. coli* cells or cell-free extracts, the demonstration of hinge-Fc and IgG glycosylation with bacterial or paucimannose *N*-glycans in both cell-based and cell-free systems [16,26,77] provides precursor material for glycan remodeling as discussed above or a starting point for future efforts focused on direct OST-mediated installation of human-type *N*-glycans.

Increasingly, researchers are exploring ways to improve these systems by focusing on the three recombinantly produced components – OST, glycan, and acceptor protein – that all can be optimized in different ways for achieving efficient and controllable protein glycosylation. For example, the exploration of OSTs from various species beyond the well-characterized *Cj*PglB has enabled glycan transfer to an almost limitless number of minimal acceptor sequences including the native site in human IgG antibodies [77]. Insights gained from structural studies of these enzymes provide a basis for rational enzyme engineering, which can be used to tailor acceptor site specificity [76] or enhance glycosylation efficiency [78]. The ability to generate diverse glycan structures via recombinant expression of synthetic operons that are optimally tuned for glycan construction is itself an area of intensive research. These efforts are providing access to a growing number of natural and unnatural glycan structures, which is made possible by the creative mixing and matching of GT enzymes that processively assemble both *N*- and *O*-glycans directly on acceptor proteins or on lipid carriers followed by *en bloc* transfer to acceptor proteins [15,16,70,79]. At the same time, methods borrowed from synthetic biology and metabolic engineering such as combinatorial DNA assembly, promoter engineering, chassis strain engineering, and genome integration are emerging as powerful ways to improve glycosylation efficiency overall [42,43,45,46,80,81]. For cell-free platforms specifically, considerations of extract processing and formulation, which have already been shown to be important for extract stability, glycosylation efficiency, and overall system economics [21-23,58], are likely to take center stage as efforts to optimize these systems ramp up over the coming years. Finally, complementing all these efforts is

the development of high-throughput screening platforms that will be instrumental in expediting the design-build-test pipelines in glycoengineering [16,75,76,78,81-86].

With the maturation of bacterial glycoengineering techniques, the development of designer glycoprotein therapeutics and vaccines becomes increasingly accessible and controllable. Recent advances in both bacterial cell-based and cell-free systems have paved the way for efficient and cost-effective production of complex glycoproteins with tailored glycan structures. These breakthroughs offer promising solutions for making and studying structurally well-defined glycoproteins, but also hold potential for accelerating the translation of glycosylated biopharmaceuticals from bench to bedside.

Competing Interests Statement

M.P.D. and M.C.J. have financial interests in Gauntlet, Inc. and Resilience, Inc. M.P.D. also has financial interests in Glycobia, Inc., MacImmune, Inc., UbiquiTX, Inc., and Versatope Therapeutics, Inc. M.P.D.'s and M.C.J.'s interests are reviewed and managed by Cornell University and Stanford University, respectively, in accordance with their conflict-of-interest policies. All other authors declare no competing interests.

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Data availability

No data was used for the research described in the article.

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