Title

Cell-free systems for accelerating glycoprotein expression and biomanufacturing

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Abstract

Protein glycosylation, the enzymatic modification of amino acid sidechains with sugar moieties, plays critical roles in cellular function, human health, and biotechnology. However, studying and producing defined glycoproteins remains challenging. Cell-free glycoprotein synthesis systems, in which protein synthesis and glycosylation are performed in crude cell extracts, offer new approaches to address these challenges. Here, we review versatile, state-of-the-art systems for biomanufacturing glycoproteins in prokaryotic and eukaryotic cell-free systems with natural and synthetic *N*-linked glycosylation pathways. We address existing challenges and future opportunities in the use of cell-free systems for the manufacture, study, and design of glycoprotein biomedicines.

Introduction

Cell-free protein synthesis (CFPS) systems have emerged as promising platforms to accelerate protein design, biomanufacturing, and testing [1–8]. CFPS relies on the activation of transcriptional and translational machinery from crude cell extracts to produce proteins without

intact cells (**Fig. 1**). As CFPS technologies have matured, the cost and time required to prepare reactions have decreased, while protein yields have increased, in some cases, to grams of protein produced per liter of reaction [2, 9–12]. One reason for the continued development of cell-free systems is that CFPS provides distinct advantages over cellular expression for high-throughput experimentation. For example, in screening campaigns, CFPS reactions provide excellent speed and flexibility because they can produce protein from linear DNA templates in a matter of hours, avoiding rate-limiting transformation or transfection procedures [13]. Additionally, assembly of CFPS reactions can be automated and tuned using liquid-handling systems, increasing throughput for protein expression, optimization, and characterization [14–19].

Recently, CFPS systems that are tailored to produce proteins with post-translational modifications have been developed, opening the door to biomanufacturing therapeutically-relevant proteins. A key feature of these efforts has been developing strategies to leverage the open nature of CFPS, which affords rigorous control over the molecular environment of protein expression. This control allows users to study and optimize site-specific protein modifications that are often critical for proper folding and bioactivity of therapeutics and vaccines [2, 3, 20–24]. For

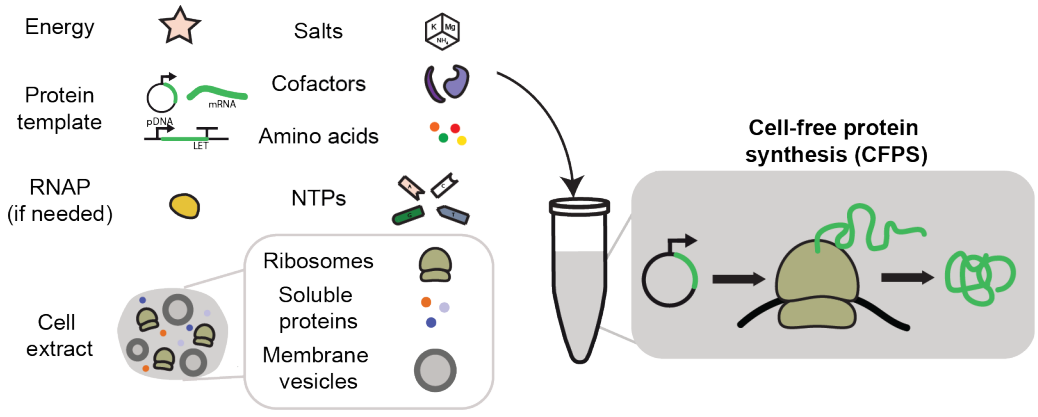

Fig. 1. Cell-free protein synthesis schematic. Cell-free protein synthesis is the activation of transcription and translation using crude cellular extracts instead of intact cells. Extracts are supplemented with exogenous resources, including amino acids, nucleotides, a secondary energy substrate, salts, and other necessary factors for protein synthesis. CFPS systems are modular with respect to the protein produced, requiring only changes in DNA or mRNA templates to produce different proteins.

example, CFPS offers flexibility to tune enzymatic protein modifications by varying the enzyme identity, concentration, and available substrates, thereby allowing control over parameters that can be confounding in living organisms [25–33]. Additionally, CFPS extracts can be prepared from an array of different culturable cell lines, allowing users to leverage strain-specific endogenous (or heterologous) biological machinery. As a result, CFPS systems are now capable of producing products such as antibodies, antibody fragments, multi-subunit enzymes, and conjugate vaccines that may require disulfide bonds and glycosylation for activity. CFPS also offers unique advantages for biomanufacturing, including simplified scalability from microliter to 100 liter reactions [34], and the ability to freeze-dry reactions that are in turn shelf-stable until rehydration at the point of use [15, 35–39].

The ability to produce and study glycoproteins is of great importance for engineering therapeutics and vaccines. Protein glycosylation, the covalent conjugation of sugars to amino acid sidechains, is one of the most prevalent and important protein modifications, occurring in all domains of life [40–44]. Glycosylation occurs on ~50% of eukaryotic proteins [45] and on the majority of preclinical and FDA-approved biologics [46], profoundly impacting protein folding [47], stability [48, 49], and immunogenicity [50, 51]. Unfortunately, building and testing defined glycoproteins in cells remains challenging for several reasons. These include glycoprotein heterogeneity [52–54], gaps in the methods and basic knowledge required to build defined glycoforms with desired pharmacological activities [55, 56], and the high costs and long times required to generate stable cell lines in mammalian cell culture. To address these issues, diverse CFPS platforms that allow user-defined glycosylation have been developed [56, 57].

Outline and scope

Here, we review CFPS systems for producing defined glycoproteins with asparagine-linked (*N*-linked) glycosylation (**Fig. 2**). A wide array of systems that interface glycosylation with cell-free protein synthesis, which we refer to as cell-free glycoprotein synthesis (CFGpS), have been

developed to date. While there are many other platforms that utilize chemical techniques [58–61] or enzymatic reactions with purified components to obtain defined glycoproteins [62, 63], here we restrict our scope to systems where (i) enzymatic glycosylation is used, and (ii) target glycoproteins are synthesized via CFPS in crude extracts. Studies using purified translation components, such as the protein synthesis using recombinant elements (PURE [64]) system are excluded, and systems using purified glycosylation components are excluded unless otherwise noted. More exhaustive reviews of CFPS [1–4, 6, 65, 66], and glycoengineering can be found elsewhere [56, 57, 67–72].

CFGpS platforms that use extracts from a variety of host organisms to install a diverse array of glycoforms have been developed. In this review, we classify CFGpS systems by topology and the origin of the glycosylation machinery (**Fig. 2**), which are key characteristics when determining

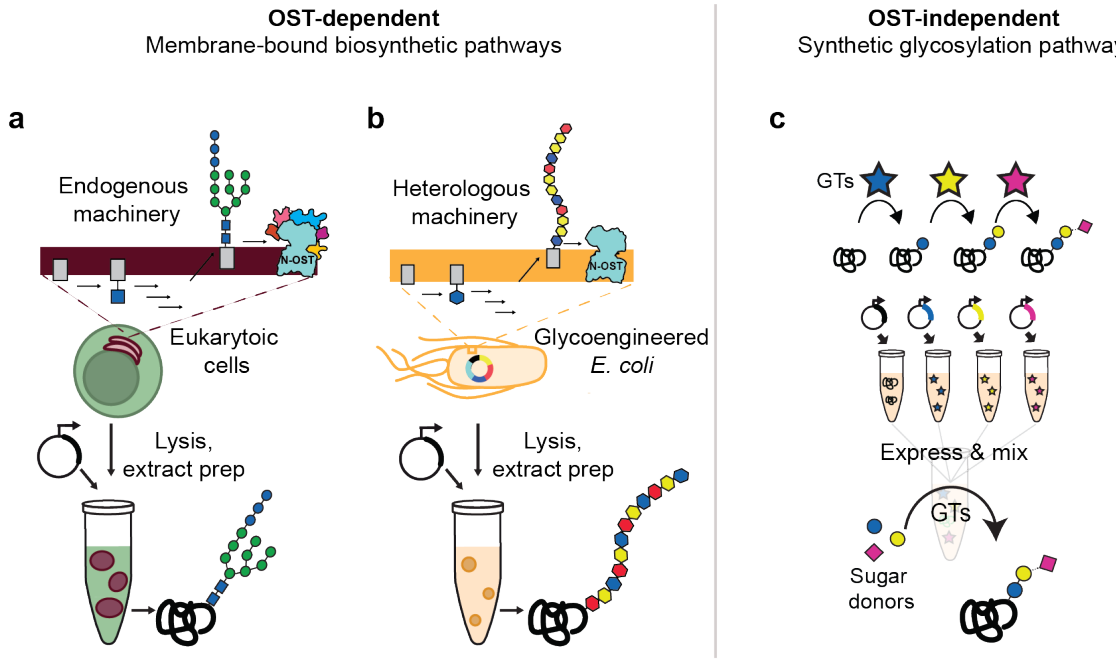

Fig. 2. OST-dependent and OST-independent cell-free glycoprotein synthesis systems. Membrane-bound OST-dependent glycosylation systems using endogenous **(a)** and heterologous **(b)** glycosylation machinery. **(a)** Cell-free glycoprotein synthesis systems derived from eukaryotic cell extracts enriched or supplemented with endoplasmic reticulum (ER) microsomes containing glycosylation components. **(b)** Cell-free glycoprotein synthesis systems derived from glycoengineered *E. coli* cells harboring heterologous, membrane-bound glycosylation machinery. **(c)** OST-independent glycosylation enzymes are mixed to make synthetic glycosylation pathways.

their best application areas. The topology of the glycosylation machinery is either membrane-bound and oligosaccharyltransferase (OST)-dependent, or soluble and OST-independent. Glycosylation machinery can be derived from endogenous expression in cells, heterologous expression in cells, or *in vitro* expression in CFPS. We order the sections from the most "natural systems" (containing endogenous enzymes naturally found in the host strain) to the most synthetic (containing heterologous and novel enzyme combinations, generating glycans that are not found in nature). This distinction illustrates how the use of synthetic biology principles in glycosylation systems has expanded their scope and provided access to new glycoforms [56].

In **Sections 1** and **2**, we review CFGpS systems that use OST-dependent *N*-linked glycosylation systems. All of these systems rely on membrane-bound OSTs to transfer complex, prebuilt glycans from lipid-linked oligosaccharide (LLO) donors to target proteins but vary in application depending on the glycan transferred. **Section 1** describes systems using endogenous glycosylation machinery from eukaryotic cells (**Fig. 2a**). **Section 2** describes *Escherichia coli*-based systems that contain heterologous glycosylation machinery (**Fig. 2b**). Thus far, the ability of eukaryotic systems to install human-like glycans points to greater utility in expression of functional therapeutics such as antibodies [73, 74]. *E. coli*-based systems have shown greater promise for expressing antimicrobial glycoconjugate vaccines that can be biomanufactured with low cost and in an on-demand format [75]. In **Section 3,** we discuss platforms that utilize CFPS to characterize, assemble, and prototype OST-independent pathways composed of soluble glycosyltransferase (GT) enzymes [17, 27]. In these systems, cell-free derived GTs sequentially decorate glycoproteins with minimal, synthetic glycans (**Fig. 2c**) [27, 76]. We anticipate that minimal glycosylation could enable fundamental understanding of the properties of isolated glycan motifs and open doors for the production on new bioactive proteins.

Main text

OST-dependent glycosylation systems

OST-dependent *N*-linked glycosylation consists of conserved steps, all taking place on or across lipid membranes (the periplasmic membrane in prokaryotes, and the endoplasmic reticulum (ER) in eukaryotes) [43, 52]. The first conserved step is the assembly of the LLO donor. LLO assembly occurs stepwise as biosynthetic enzymes incorporate monosaccharides to a growing chain which is then flipped across the lipid membrane into the periplasm or ER. Next, the oligosaccharide from the LLO donor is transferred to asparagine residues within an acceptor polypeptide by a membrane-bound OST. OSTs mediate glycosylation by their substrate specificity, glycosylating only when specific polypeptide acceptor sequences and LLO donor structures are recognized. While the general acceptor peptide motif (N-X-S/T) (or "sequon") is conserved, specific sequons and LLOs recognized by OSTs vary across organisms [52, 56]. In eukaryotes, OSTs are multi-subunit complexes assembled around a core catalytic subunit called STT3 [42, 77]. In prokaryotes, OSTs called PglB are single-subunit enzymes that bear homology to STT3, but are more tractable for heterologous expression [78]. Even the simplest OSTs, however, are large enzymes with ~13 transmembrane passes and require proper membrane embedding for activity [79]. Due to the complexity of OST-dependent glycosylation, the use of cellular extracts enriched with, or supplemented with, native cellular lipids and membrane-bound machinery has been the main strategy for obtaining active CFGpS systems [80].

Section 1: OST-dependent glycosylation with endogenous eukaryotic glycosylation machinery

The coactivation of CFPS and OST-dependent protein glycosylation was first observed in eukaryotic cell extracts supplemented with ER microsomes. CFGpS was reported in extracts from various origins (wheat germ cells [81], rabbit reticulocytes [82–86], and other higher eukaryotes [87]) supplemented with mammalian-derived microsomes. Additionally, homologous systems

were developed from yeast [88–90] and other fungal cells [91] by supplementing microsomes derived from the same strain as the extract. Taken together, these studies demonstrated that proteins fused to a proper microsome-targeting leader sequence could be produced and translocated into ER microsomes *in vitro*, and that *N*-linked glycosylation could occur on these microsome-targeted proteins.

While the original eukaryotic CFGpS systems were intended to study protein secretion and processing, the realization that glycosylation could be combined with the benefits of CFPS prompted the development of more robust, biomanufacturing-oriented systems. Other useful features of eukaryotic CFPS systems for manufacturing therapeutics include the presence of endogenous folding chaperones (e.g., protein disulfide isomerase) and a lack of endotoxins.

Recent advances in eukaryotic CFPS have enabled increases in protein titers, throughput, and glycosylation efficiency. Protein expression titers in commonly-used eukaryotic strains have now reached hundreds of μg/mL of model and non-model proteins by employing semi-continuous (where CFPS is run using dialysis) reaction conditions [92, 93]. Additionally, the implementation of internal ribosome mediated entry site (IRES) mediated translation has enabled programming of eukaryotic CFPS reactions with DNA templates instead of mRNA templates, increasing throughput by obviating the need to prepare mRNA templates [92, 94]. Processing methods have also been established for enriching extracts with intact microsomes, circumventing the need for tedious microsome purification/supplementation protocols. By using optimized lysis and extract preparation techniques, the ER is rearranged into well-defined microsomes that remain in the final extract and are active for glycosylation (**Fig. 2a**). Microsome-enriched extracts can now be made without the need for specialized cell disruption equipment or chromatography steps, simplifying extract preparation [95]. Moving forward, further improvement of batch eukaryotic CFPS systems to match the comparatively low costs and high CFPS titers of *E. coli*-based counterparts could make the technology more accessible [2, 3, 96].

Importantly for the production of defined glycoproteins, the cell lines – and thus, the diversity of glycosylation systems – available for preparing microsome-enriched extracts has expanded to include tobacco BY-2 [96], hybridoma [97], human [94, 97], insect [94, 95, 98–100], and Chinese hamster ovary (CHO) [93, 94, 101–103] cells. These systems have been used to produce a variety of complex, active glycoprotein targets. **Table 1** shows representative yields of proteins and glycoproteins synthesized in selected CFGpS systems. Notably, expression of active, multi-subunit glycoproteins stabilized by disulfide bonds, such as antibodies [73] and glucose oxidase, [96] is achievable in eukaryotic systems. Toward advanced biomanufacturing applications, the well-developed CHO and insect platforms have been interfaced with non-canonical amino acid (ncAA) incorporation [104–106], enabling glycosylation and incorporation of site-specific ncAAs [73, 100]. These advances enable, for the first time, high-throughput screening of valuable, chemically-defined glycoprotein therapeutics [100] and antibody-drug conjugates [73].

Table 1. Cell-free glycoprotein synthesis titers with OST-dependent glycosylation systems.

Extract	Protein	~Yield*	Quantitation method	Glycosylation machinery†	Notes	Reference
Tobacco	Luc	270	Luminescence	E	Not glycosylated	[96]
	GOx	7 U/mL	Colorimetric GOx activity assay	E	Multi-subunit, DSB required for activity	[96]
Insect	Luc	45	Luminescence	N/A	Not glycosylated	Promega TNT T7 kit
	gp120	25	WB densitometry	E		[98]
	EPO	5	Radioactive counting, autoradiography	E		[100]
CHO	Luc	50	Luminescence	E	Not glycosylated	[101]
	IgG	1, 9 (SC)	Radioactive counting, autoradiography	E	Multi-subunit, DSB required for activity	[73]
	EPO	5	Radioactive counting, autoradiography	E		[102]
	EPO	120 (SC)	ELISA	Supplemented via CHO-derived ER/Golgi microsomes		[93]

E. coli	sfGFP	600	Fluorescence	*Cj*LLO, *Cj*OST	Not glycosylated	[107]
	sfGFP-glyco	120	Fluorescence, WB densitometry	*Cj*LLO, *Cj*OST		[107]
	EPO	10	ELISA	*Cj*LLO, *Cj*OST	Non-native sequons	[108]
	MBP	20	Radioactive counting, WB densitometry	*Ft*LLO, *Cj*OST		[75]

*All units are in μg/mL unless indicated. U/mL refers to active enzyme units per mL CFGpS reaction. All reactions were conducted in batch, unless denoted with 'SC' for semicontinuous reaction conditions.

†E denotes when extracts were enriched with the endogenous glycosylation machinery from the host strain. For *E. coli* systems, the OST and LLO recombinantly expressed in the strain prior to lysis are indicated.

Abbreviations are: active firefly luciferase (Luc), glucose oxidase (GOx), human epidermal growth factor receptor (EGFR), HIV-1 envelope glycoprotein (gp120), super folder green fluorescent protein (sfGFP) maltose binding protein (MBP), erythropoietin (EPO), *Francisella tularensis* (*Ft*), *Campylobacter jejuni* (*Cj*), disulfide bond (DSB), Western blot (WB), and enzyme linked immunosorbent assay (ELISA)

A compelling present application of eukaryotic CFGpS systems is producing and screening therapeutic proteins, whose activity and immunogenicity can be strongly affected by differences in glycan structures [49, 55, 60, 109]. Therefore, developing methods to produce glycoproteins with the defined, human-like glycans present on therapeutic proteins is critical. **Fig. 3a** summarizes the information inferred from glycan analysis in a variety of eukaryotic, OST-dependent CFGpS platforms. Glycosylation in eukaryotic CFGpS is typically confirmed using enzymatic deglycosylation with PNGase F (specific for all eukaryotic glycans with a $Man_3GlcNAc_2$ core without α1-3 fucosylation) and/or Endo H (specific for $Man_5GlcNAc_2$ hybrid and high mannose glycans). Knowledge of these minimum recognition motifs, and of the LLO specificity of eukaryotic OSTs, indicates that glycans are consistent with ER-dependent *N*-linked glycosylation involving the transfer of a glycan resembling ($Glc_3Man_9GlcNAc_2$) [110]. The extent to which these glycans are trimmed and elaborated—as they would be in the ER and Golgi apparatus in living cells—still requires further characterization. The implementation of higher-resolution assays, such as those recently performed on insect and CHO systems (**Fig. 3a**), is helping to clarify the diversity of glycans that can be produced by CFGpS [93, 100]. For example, a mass spectrometry (MS)

based analysis of erythropoietin (EPO) derived from insect CFGpS revealed that glycans were trimmed down to structures as minimal as $Man_5GlcNAc_2$ [100]. Lectin-based analysis of EPO derived from CHO CFGpS supplemented with ER and Golgi vesicles showed the presence of high mannose, fucosylated, and galactose-terminated structures, indicating that the activity of Golgi enzymes is possible when microsomes are prepared appropriately [93].

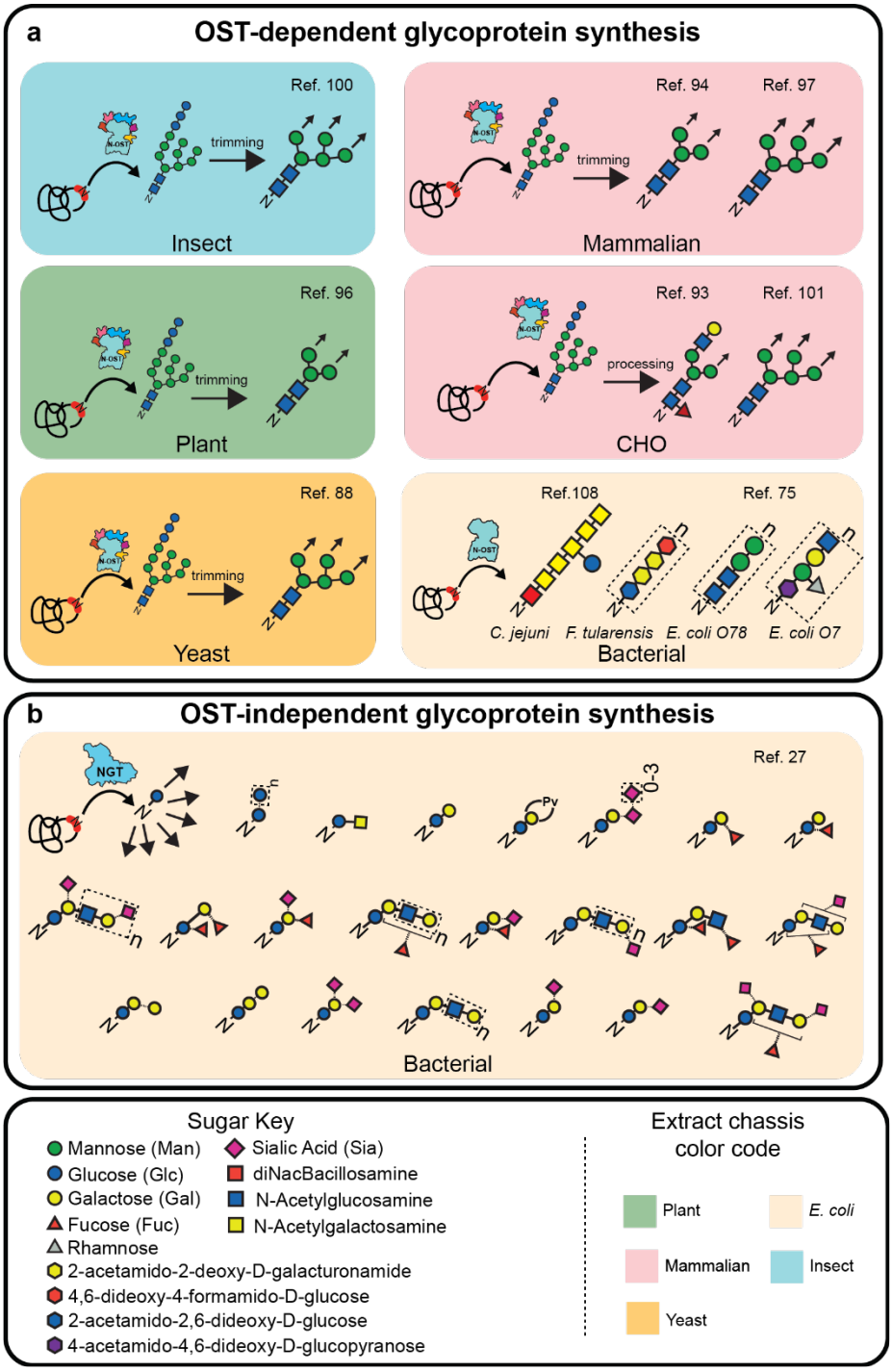

Fig. 3. Glycosylation in cell-free glycoprotein synthesis systems. (a) OST-dependent glycosylation in eukaryotic and *E. coli* extracts. **(b)** Bottom-up synthesis of glycoproteins in an OST-independent manner. The bottom panel shows a figure legend. When structural characterization was performed by deglycosylation studies, minimal recognition motifs (shown as structures with elaboration arrows) were inferred based on known glycosidase and OST specificities. References ('Ref.') for each structure are listed in the top right-hand corner of each section.

Further characterization of CFGpS products using high-resolution techniques such as MS and nuclear magnetic resonance spectroscopy are needed to define glycoforms in the future. These methods will be critical to develop strategies to control and remodel glycan structures. Promising avenues to achieve defined glycoforms include the use of glyco-engineered cell lines containing edited glycosylation pathways [111] and the supplementation of reactions with glycan-editing enzymes [112].

Section 2: OST-dependent glycosylation in glycoengineered *E. coli* extracts

E. coli-based platforms are the most robust and cost-effective CFPS systems currently available. With protein yields of thousands of μg/mL and costs at less than $5/mL [2], *E. coli* systems surpass eukaryotic systems in batch yields by an order of magnitude and are two orders of magnitude less expensive than commonly used CHO and insect systems [2, 3, 96]. Additionally, *E. coli* is faster and easier to grow than eukaryotic strains, enabling cell growth, harvest, and extract preparation to be completed in less than one day [113–115]. Furthermore, extracts from laboratory strains of *E. coli* provide a 'blank slate' for *N*-linked glycosylation because they contain no endogenous *N*-linked glycosylation machinery. Despite these advantages, laboratory *E. coli* strains could not be used to produce glycoproteins until the recent discovery of bacterial *N*-linked glycosylation systems [116, 117]. Efforts to harness these systems by transferring them into *E. coli* expression systems for engineering functional therapeutics, vaccines, and materials has spawned the new discipline of bacterial glycoengineering [118–120].

Of emerging interest for glycoengineering are single-subunit bacterial OSTs, which, despite having stringent sequon specificity [121–123], can be used as a tool to transfer diverse glycans to acceptor proteins engineered with proper sequons [118]. Since the functional transfer of the model *N*-linked glycosylation pathway from *Campylobacter jejuni* into *E. coli* [124], *E. coli* has been engineered with myriad OST-dependent glycosylation pathways. Glycan structures including the eukaryotic $Man_3GlcNAc_2$ core for mimicking eukaryotic glycosylation [125], microbial *O*-antigens for glycoconjugate vaccine development [126–128], Lewis structures for therapeutic

development [129], and exotic bacterial glycans [108] have been ported into living *E. coli*. In practice, recapitulating eukaryotic glycosylation in *E. coli* systems remains challenging, but the expression of bacterial glycosylation systems is relatively straightforward, enabling prototyping and biomanufacturing antimicrobial conjugate vaccines [69].

The first *E. coli* CFGpS system was developed via supplementation of purified *C. jejuni* OST (*Cj*OST) and LLO (*Cj*LLO) into CFPS reactions where the nascent acceptor protein was glycosylated via purified components [130]. This system provided a proof-of-principle that bacterial *N*-linked glycosylation is possible in the absence of intact cellular membranes. Toward a lower-cost, simpler system, the pathway from *C. jejuni* was recapitulated *in vitro* using crude *E. coli* extracts prepared from strains overexpressing the *Cj*OST and *Cj*LLO (**Fig. 2b**) [108]. The key idea is that OST and LLOs are overexpressed in the *E. coli* cells, then are subsequently enriched in the crude extract, rather than purified. Glycosylation components are present in *E. coli* extracts in nanoscale [107] membrane vesicles which serve the dual purpose of enabling (i) the activation of ATP regeneration through oxidative phosphorylation [131] and (ii) harboring active membrane-bound LLOs and OSTs [108]. Glycoengineered extracts of *E. coli* have been used to synthesize a variety of glycoproteins, such as EPO, with diverse glycan structures (**Table 1**).

Recently, the *E. coli* CFGpS platform has been combined with efforts in decentralized biomanufacturing to enable *in vitro* bioconjugate vaccine expression (iVAX) from freeze-dried, shelf stable reactions. iVAX is modular, allowing the transfer of diverse bacterial *O*-antigens to protein targets (**Fig. 3a**) and the expression of conjugate vaccine carriers including detoxified *Corynebacterium diphtheriae* toxin (known as CRM197) and the *Clostridium tetani* toxin [75]. Importantly, iVAX-derived vaccines against the pathogen *Francisella tularensis* have proven to be efficacious *in vivo*, protecting vaccinated mice from a lethal pathogen challenge [75]. iVAX glycoconjugate titers (**Table 1**) enable individual vaccine doses of 10 μg to be produced in one hour for ~$6 [75]. In efforts to drive biomanufacturing costs down further, *E. coli* CFGpS was

recently optimized to synthesize glycoprotein titers of >100 μg/mL in batch by increasing the concentrations of the LLO- and OST-harboring vesicles during extract preparation [107].

E. coli systems are a promising venue for producing therapeutic glycoproteins and on-demand vaccines. From a biomanufacturing perspective, concerns over producing disulfide bond proteins and the presence of endotoxin have recently been overcome [24, 132]. From the perspective of glycosylation, a major advantage of the *E. coli* system is the possible breadth of glycans that can be installed with OSTs, which will expand as new glycosylation pathways are engineered and characterized. Toward this goal, a class of bacterial OSTs termed *O*-OSTs (which carry out *O*-linked glycosylation on serine and threonine residues) that are structurally similar to bacterial *N*-OSTs, but with relaxed LLO specificities have recently been characterized [133, 134]. *O*-OSTs, which further expand the palette of glycoconjugate vaccines available for manufacture in *E. coli*, [67, 132] have recently been shown to be active in *E. coli* CFGpS [107].

A major challenge that remains for synthesizing therapeutic proteins, is the transfer of eukaryotic-type *N*-linked glycosylation in a high-yielding *E. coli* system. While advances have been made toward increasing eukaryotic LLO production in *E. coli* [137], and $Man_3GlcNAc_2$ has been installed both with purified *E.coli*-derived components *in vitro* [108] and in living *E. coli* cells [138], efforts have been limited by low transfer efficiency of $Man_3GlcNAc_2$ (**Fig. 3a)** by bacterial OSTs. Additionally, because the polypeptide substrates of well-expressing bacterial OSTs differ from eukaryotic OSTs, naturally-occurring sequons in therapeutic proteins must be replaced with synthetic sequons, changing the primary protein sequence [108, 138, 139]. To address these issues, characterization of natural [139] and engineered [140, 141] bacterial OSTs to improve conjugation efficiency of diverse glycans is of key importance for advancing *E. coli* CFGpS. To this end, the structure of a widely-used bacterial OST in complex with an LLO and an acceptor sequon was recently solved, providing high-resolution information for rational engineering of OSTs and providing new opportunities for glycoprotein biomanufacturing [141, 142].

OST-independent glycosylation systems

Section 3: *E. coli* cell-free systems for OST-independent glycoprotein synthesis

In addition to biomanufacturing, cell-free systems provide a flexible environment to construct and optimize new biosynthetic glycosylation pathways, and to interface glycosylation with high-throughput experimental workflows. A key determinant of the throughput accessible to study and engineer glycosylation systems in cell-free is the ability to utilize or synthesize glycosylation components outside of living cells, where they can be more easily varied, sampled, and controlled. The innovations described in **Sections 1** and **2** provide methodologies to utilize OST-dependent glycosylation components generated inside of living cells in a cell-free environment. However, taking full advantage of the cell-free paradigm for glycosyltransferase (GT) characterization, engineering, and biosynthetic pathway prototyping, requires the synthesis of glycosylation components outside of living cells.

A key challenge with OST-dependent *N*-linked glycosylation is that LLOs and OSTs are membrane-associated and are therefore more difficult to synthesize than soluble proteins. This challenge was partially overcome by supplementing *E. coli* CFPS reactions with lipid-protein nanodiscs to enable the synthesis of active OSTs at high titers *in vitro* [25], opening the door to high-throughput OST characterization. However, the *in vitro*, bottom-up synthesis of LLOs remains challenging [58] and the co-activation of LLO biosynthesis and CFGpS has not been demonstrated. Additionally, cell-free synthesis of eukaryotic OST complexes (e.g., STT3) has not yet been reported, limiting the diversity of OSTs that can be synthesized using current *in vitro* systems. Given the challenges associated with synthesizing OSTs and LLOs *in vitro*, recent efforts have sought to study and engineer OST-independent glycosylation pathways in a cell-free environment [17, 18, 27, 76]. The absence of membrane-associated enzymes or substrates in OST-independent glycosylation pathways make them easier to implement in cell-free and permits

the direct transfer of developed pathways into the bacterial cytoplasm. Furthermore, OST-independent systems promise to be more modular as they circumvent the specificities of OSTs for LLOs by sequentially installing monosaccharides onto proteins.

Thus far, OST-independent cell-free glycoengineering efforts have focused on a recently discovered class of cytoplasmic enzymes known as *N*-glycosyltransferases (NGTs) to transfer of a single glucose residue from a nucleotide-activated sugar (UDP-Glc) onto an acceptor asparagine within acceptor sequons that resemble the eukaryotic N-X-S/T glycosylation motif [143–146]. Because the acceptor sequence specificity of NGTs had not been rigorously characterized, and this information is required for site-specific modification of glycoproteins, initial efforts in this area used CFPS with a high-throughput experimentation platform for glycosylation sequence characterization and optimization by rapid expression and screening (GlycoSCORES) [17]. GlycoSCORES uses CFPS to produce a polypeptide-modifying glycosyltransferase of interest and self-assembled monolayers for matrix-assisted laser desorption/ionization mass spectrometry (SAMDI-MS) to determine its specificity [147]. This method was applied to determine the sugar donor and peptide acceptor sequence specificities of both *N*- and *O*-linked polypeptide modifying GTs from bacteria and humans using 3,480 unique peptides and 13,903 unique reaction conditions. This information was then used to redesign glycosylation sites within heterologous proteins (including the Fc region of human IgG) to increase their glycosylation efficiency by up to 5 fold in living *E. coli* and in a cell-free environment [17]. This method was later adapted to intact proteins, enabling the analysis of an 87-member protein library containing a single glycosylation site at all positions along the protein backbone. This assay provided insight into how the position of the acceptor sequon within a target protein can affect glycosylation [18], an approach called shotgun glycomutagenesis [148]. Another effort used the GlycoSCORES method to produce 41 putative NGT homologs in CFPS and rigorously characterize their acceptor sequence specificities. This campaign discovered four NGT variants with conditionally orthogonal

peptide acceptor specificities that were used to develop new workflows for sequential and site-specific glycosylation at up to four distinct locations within a single protein [76]. These works show how cell-free systems have been interfaced with OST-dependent glycosylation to accelerate glycoprotein design and testing.

Besides controlling the efficiency and position of glycan modifications, OST-independent cell-free systems have also enabled the bottom-up construction of multi-enzyme synthetic glycosylation pathways *in vitro* to generate proteins modified with a wide variety of glycan structures. A recent study reported the development and application of a modular, cell-free platform for glycosylation pathway assembly by rapid *in vitro* mixing and expression (GlycoPRIME) in which a target protein and GTs were synthesized in separate CFPS reactions and then combined to generate unique protein glycosylation pathways (**Fig. 2c**) [27]. The key idea is that cell-free biosynthesis "units" are made from crude cell lysates that are selectively enriched with pathway enzymes produced directly in lysates by cell-free protein synthesis. Then, these units are assembled modularly, in a mix-and-match fashion, to build and study biosynthetic pathways. Biosynthetic pathways yielding 23 unique glycosylation motifs were developed using this method (**Fig. 3b**). Once discovered *in vitro*, the pathways developed using GlycoPRIME were successfully transferred to living *E. coli* for cytoplasmic production of glycoproteins. These pathways were also shown to be functional in a one-pot format in which all plasmids for the target protein and GTs are combined in the CFPS reaction supplemented with activated sugar donors to generate glycoprotein in 24 hours. The use of OST-independent glycosylation systems has greatly expanded the diversity of number of glycosylation structures available for production in cell-free [17, 18, 27, 76] and cellular [17, 149–151] systems and they hold promise for applications including adjuvants and antigens for vaccines [149, 151–154], glycoprotein antitoxins [155, 156], biomaterials that promote cell growth or differentiation by interfacing with cellular lectins [157, 158], and stabilized therapeutics [159].

The ease of implementation of OST-independent CFGpS systems have enabled enzyme characterization and glycoprotein analysis at high throughput and may offer new paradigms for glycoprotein biomanufacturing methods. The glycans installed using these methods, however, are generally smaller than those installed by OSTs (**Fig. 3**) and do not occur in exactly the same form in nature. Further characterization of the functionality of minimal glycans is a critical pursuit to understanding and leveraging OST-independent glycoforms in the future. Several hurdles remain before human *N*-glycosylation can be precisely mimicked using OST-independent machinery. Specifically, NGTs discovered to date are unable to transfer GlcNAc, the reducing end sugar in all eukaryotic *N*-linked glycans [146]. Therefore, the discovery and engineering of NGTs capable of transferring GlcNAc remains an active area of research [17, 76, 160].

Conclusion

Cell-free systems hold great promise for expediting expression, testing, and biomanufacturing glycoproteins. Here, we reviewed natural and synthetic cell-free glycoprotein synthesis platforms that are useful for an array of applications including, but not limited to eukaryotic systems for expression of complex glycosylated therapeutics and engineered *E. coli* systems for glycoprotein expression and on-demand biomanufacturing. We also described works in the emerging field of bottom-up glycoprotein synthesis using synthetic glycosylation systems, highlighting how cell-free systems have expedited their development. We anticipate that the modularity and flexibility of cell-free glycosylation systems will continue to increase our understanding of glycosylation, advance applications in on-demand biomanufacturing, and accelerate glycoprotein research and development timelines.

Author contributions

The authors contributed to all aspects of the article.

Competing interests

M.C.J. has a financial interest in Design Pharmaceuticals Inc. and SwiftScale Biologics. M.C.J.'s interests are reviewed and managed by Northwestern University in accordance with their conflict of interest policies. All other authors declare no conflicts of interest.

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