nature protocols

PROTOCOL

https://doi.org/10.1038/s41596-022-00799-z

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Rapid biosynthesis of glycoprotein therapeutics and vaccines from freeze-dried bacterial cell lysates

Jessica C. Stark[1]✉, Thapakorn Jaroentomeechai[2], Katherine F. Warfel[3,4,5], Jasmine M. Hershewe[3,4,5], Matthew P. DeLisa[6]✉ and Michael C. Jewett[3,4,5,7,8,9]✉

The advent of distributed biomanufacturing platforms promises to increase agility in biologic production and expand access by reducing reliance on refrigerated supply chains. However, such platforms are not capable of robustly producing glycoproteins, which represent the majority of biologics approved or in development. To address this limitation, we developed cell-free technologies that enable rapid, modular production of glycoprotein therapeutics and vaccines from freeze-dried *Escherichia coli* cell lysates. Here, we describe a protocol for generation of cell-free lysates and freeze-dried reactions for on-demand synthesis of desired glycoproteins. The protocol includes construction and culture of the bacterial chassis strain, cell-free lysate production, assembly of freeze-dried reactions, cell-free glycoprotein synthesis, and glycoprotein characterization, all of which can be completed in one week or less. We anticipate that cell-free technologies, along with this comprehensive user manual, will help accelerate development and distribution of glycoprotein therapeutics and vaccines.

Introduction

Since the introduction of the tetanus and diphtheria toxoid vaccines in the 1920s (ref. [1]) and the approval of recombinant insulin in 1982 (ref. [2]), protein vaccines and therapeutics have transformed our ability to prevent and treat human disease. However, current biomanufacturing strategies are key contributors to the escalating cost to develop novel biologics (estimated at US$2.5 billion per new molecule in 2014)[3], due to the high costs (US$300–500 million) and long timescales (4–5 years) associated with building large-scale production facilities[4]. Additionally, with advancements in cell line engineering enabling order of magnitude increases in recombinant protein titers[5], as well as increasing demands for medicines tailored to biologically stratified patient populations[6], there is growing interest in scaled-down bioprocesses that can accommodate production of multiple biologic molecules. Finally, the current centralized biomanufacturing paradigm necessitates refrigerated supply chains for distribution of many protein vaccines and therapeutics. The need for cold-chain refrigeration presents substantial economic and logistical challenges for supplying life-saving biologics to regions with limited infrastructure, as well as in emergency situations[7–9].

As a result, novel biomanufacturing paradigms are emerging that enable decentralized and potentially portable production of protein therapeutics and vaccines at small scales[10–14]. A number of important protein products have been made using point-of-care production technologies, including recombinant interferon-α2b, human growth hormone, erythropoietin, granulocyte colony-stimulating factor, onconase, diphtheria toxoid and a panel of ten antimicrobial peptides, with some achieving purity, safety and efficacy in vitro and/or in vivo that was on par with marketed drug products[11,14,15]. However, so far, these technologies have been limited by their inability to produce complex protein biologics such as protein therapeutics with controllable and reproducible glycosylation.

[1]Department of Chemistry & Sarafan ChEM-H, Stanford University, Stanford, CA, USA. [2]Copenhagen Center for Glycomics, Departments of Cellular and Molecular Medicine, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark. [3]Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL, USA. [4]Center for Synthetic Biology, Northwestern University, Evanston, IL, USA. [5]Chemistry of Life Processes Institute, Northwestern University, Evanston, IL, USA. [6]Robert Frederick Smith School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY, USA. [7]Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL, USA. [8]Simpson-Querrey Institute, Northwestern University, Chicago, IL, USA. [9]Present address: Department of Bioengineering, Stanford University, Stanford, CA, USA. ✉e-mail: jcstark@stanford.edu; md255@cornell.edu; mjewett@stanford.edu

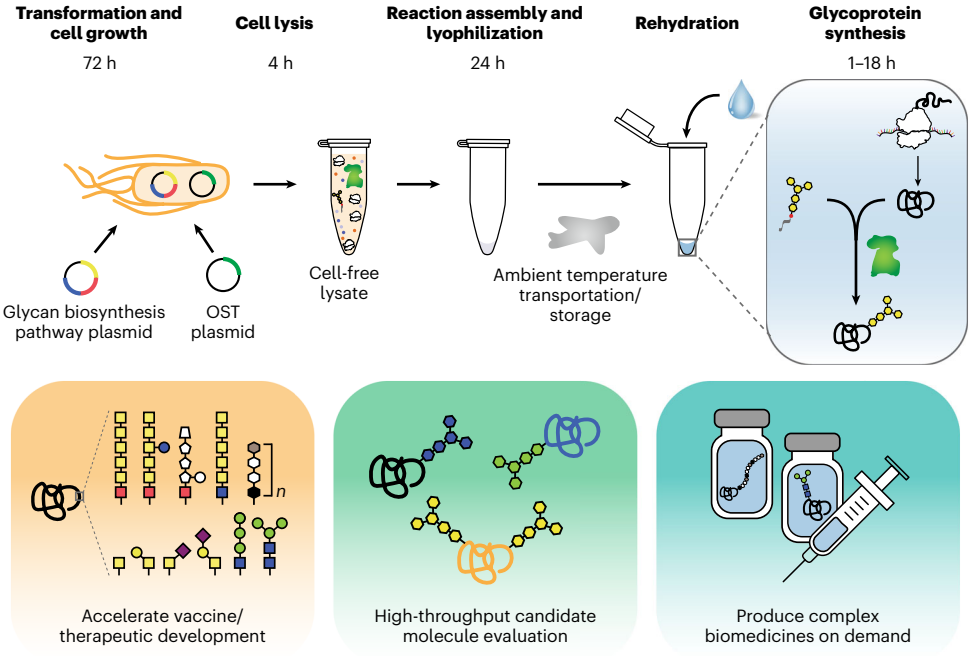

Fig. 1 | Cell-free systems accelerate glycoprotein production. Cell-free technology enables modular expression of user-defined glycoproteins in 5–7 d, which promises to accelerate development of glycoprotein therapeutics and vaccines. Further, cell-free reactions can be freeze-dried, enabling cold chain-independent distribution and on-demand glycoprotein production.

Glycosylation, the post-translational modification of amino acid side chains with oligosaccharides (glycans), is critically important for the production of recombinant protein therapeutics[16]. Approximately 70% of the >100 protein products approved by United States and European regulatory agencies and the ~500 candidates in clinical trials are glycosylated[17]. The majority of glycoprotein therapeutics contain oligosaccharides attached to asparagine residues (*N*-linked glycans) or serine/threonine residues (*O*-linked glycans), which are known to impact many therapeutically relevant protein properties including pharmacokinetics, immunogenicity and biological activity[18–21]. As a result of the important roles glycosylation plays in therapeutic efficacy, the ability to produce glycosylated proteins on demand represents an unmet biotechnological need.

Development of the protocol

To address this technological gap, we have recently developed cell-free expression platforms for on-demand biomanufacturing of glycosylated protein therapeutics and vaccines[22–30] (Fig. 1). Cell-free systems use cell lysates rather than living cells to synthesize proteins in vitro, which offers multiple advantages for glycoprotein production[31–33]. Cell-free platforms (i) enable decentralized glycoprotein production, as relevant amounts can be synthesized in vitro in as little as 1 h; (ii) can be freeze-dried for distribution at temperatures up to 50 °C and reconstituted by just adding water; and (iii) are inexpensive, with the ability to synthesize a conjugate vaccine dose for ~US$0.50 (refs. [22,30]). Importantly, glycoproteins produced using cell-free technologies are efficacious: we demonstrated that cell-free-derived conjugate vaccines completely protected mice against lethal challenge with the highly virulent bacterial pathogen *Francisella tularensis*[22], and that conjugates elicited bactericidal antibodies against the enterotoxigenic *E. coli* (ETEC) O78 O-polysaccharide[30,34]. Overall, cell-free systems offer a rapid method to synthesize glycoproteins for structural and functional interrogation, as well as therapeutic development and distribution.

Overview of the procedure

We describe a universal protocol for expression and characterization of glycosylated protein products from freeze-dried, cell-free reactions. We discuss procedures and guidelines for engineering and culturing chassis strains (i.e., strains used to generate cell lysates; Steps 1–24), generating glycosylation-competent cell-free lysates (Steps 25–36), preparing and performing cell-free reactions (Steps 37–41) and characterizing cell-free synthesized glycoproteins and controls (Steps 42–51).

Comparisons with alternative glycoprotein production platforms

For decades, eukaryotic cells have been the default host for cell-based glycoprotein production[5]. Eukaryotic cell-free systems, including those derived from insect[35], trypanosome[36] and mammalian cells[37–40], have been developed and used to produce glycoproteins. In response to growing interest in decentralized biomanufacturing platforms, lyophilized lysates from Chinese hamster ovary cells have been used to synthesize glycosylated erythropoietin on demand[14]. However, eukaryotic cell-free systems suffer from low yields[33] and are difficult to prepare, requiring supplementation with microsomes for glycosylation activity[14,40–42], which together present challenges for portable biomanufacturing. In addition, eukaryotic cell-based and cell-free systems for glycoprotein production rely on endogenous protein glycosylation machinery, limiting control over the glycan structures that can be installed. By contrast, bacterial platforms leverage bottom-up engineering of protein glycosylation pathways in *E. coli* strains that lack endogenous glycosylation machinery, uniquely enabling synthesis of proteins modified with user-specified glycan structures[16,22,23,29,33,43]. Leveraging such glycoengineered *E. coli* strains, we have developed cell-free technologies that enable production of protective conjugate vaccines bearing bacterial O antigens[22] and proteins decorated with nearly homogeneous trimannose core *N*-glycans or mucin-type *O*-glycans that serve as the foundational structures of all eukaryotic *N*-linked or *O*-linked glycans[23,29], respectively.

Production of these glycoprotein targets would not be possible in platforms with endogenous protein glycosylation machinery due to (i) the need to express heterologous biosynthetic pathways to synthesize and install chemically distinct bacterial glycans and (ii) increased glycan and glycoprotein structural heterogeneity that arises from the actions of essential endogenous glycosylation enzymes. With these advantages, bacterial cell-free systems offer unique opportunities to accelerate development of glycosylated biologics and enable decentralized, cold chain-independent biomanufacturing.

Applications

Diverse glycoprotein products have been synthesized using the cell-free approach described here. These include proteins decorated with model bacterial glycans such as the native *Campylobacter jejuni* glycan[23], conjugate vaccine antigens including the enterotoxigenic *E. coli* O78 (refs. [30,34]) and *Francisella tularensis* Schu S4 (ref. [22]) O antigens, and eukaryotic glycans, such as the trimmanose core *N*-glycan[23] and core 1 *O*-glycans[29]. In addition, the cell-free reaction environment can be readily adapted to facilitate biosynthesis of complex glycoproteins, including membrane and disulfide bond-containing proteins[22]. We have demonstrated cell-free biosynthesis and glycosylation of model glycoprotein targets such as sfGFP[23], in addition to therapeutically relevant targets including the licensed conjugate vaccine carrier proteins CRM197 (genetically detoxified *Corynebacterium diphtheriae* toxin) and PD (nonacylated *Haemophilus influenzae* protein D)[22], as well as human erythropoietin[23].

Limitations

Looking forward, there are multiple opportunities to further develop cell-free systems for glycoprotein production. One important goal will be to expand the diversity of glycan structures that can be synthesized. So far, cell-free systems have been used to produce proteins bearing more than 30 distinct glycan structures[22–30], including glycans of bacterial and eukaryotic origin attached via *N*-linked and *O*-linked glycosylation (Fig. 2, top left). Another area of ongoing research focuses on increasing glycoprotein yields. The original cell-free platform could synthesize 5–15 μg of defined glycoprotein per milliliter reaction[22,23]. While this was sufficient to produce multiple human doses of conjugate vaccines per milliliter of cell-free reaction, further optimization of the lysate preparation method resulted in glycoprotein yields greater than 100 μg/mL (ref. [24]), which promises to expand the types of biologics that can be synthesized using cell-free technology. With further adoption and development, cell-free systems hold potential as a rapid means to produce glycoproteins for fundamental or translational studies.

Experimental design

To construct lysates for cell-free glycoprotein synthesis, a user must first design the chassis strain for cell-free lysate (or crude cell extract) production and then select a lysate preparation method. Once lysates have been prepared, there are additional steps for cell-free reaction optimization to ensure maximum glycoprotein synthesis yields, and considerations for reaction formulation that impact cost and stability of lyophilized reactions. We describe each of these experimental design elements in detail below:

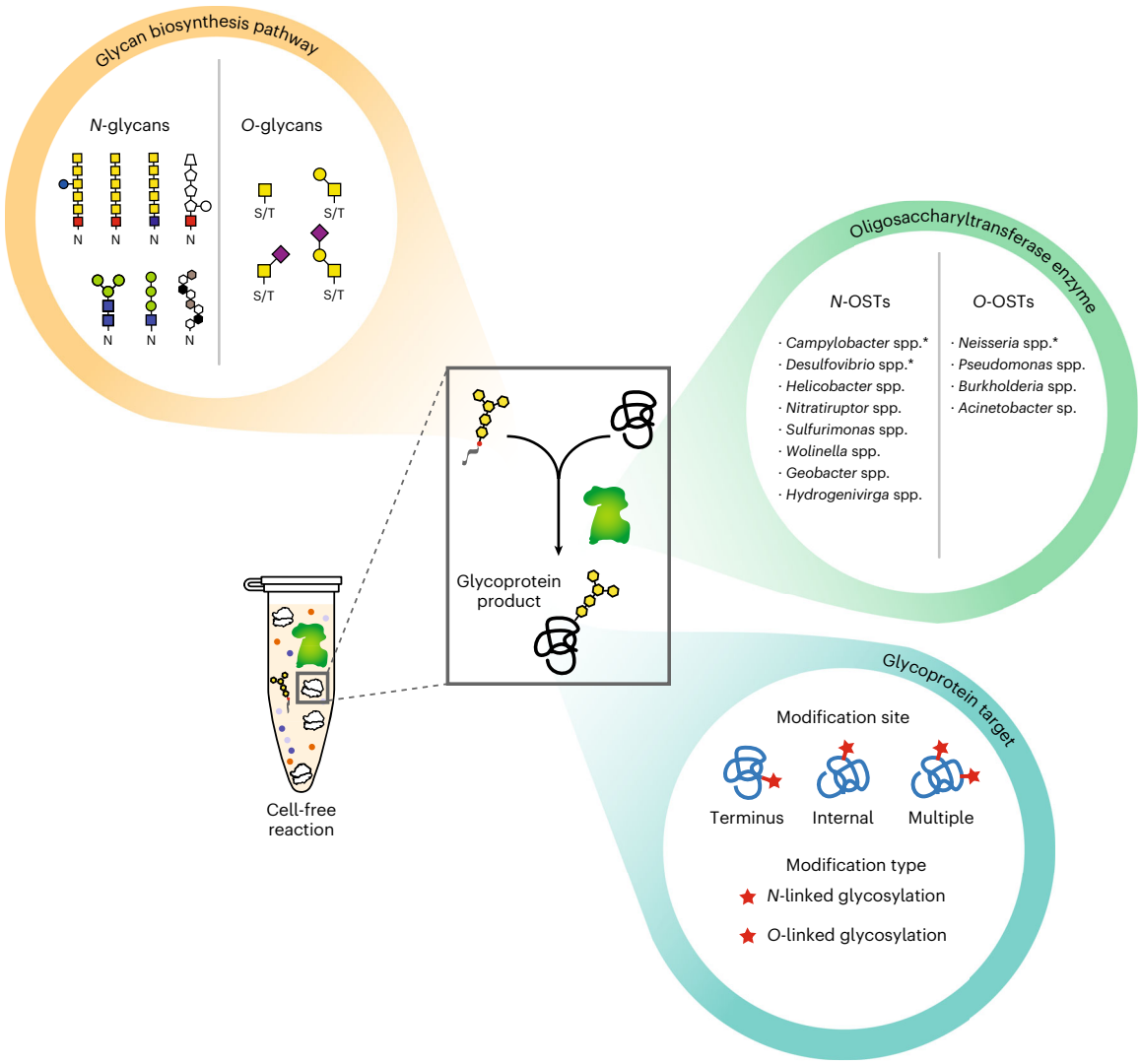

Fig. 2 | Constructing glycosylation pathways. A variety of glycoproteins bearing diverse glycan structures can be synthesized in cell-free systems through the assembly of synthetic glycosylation pathways. The glycosylation reaction is carried out using lipid-linked oligosaccharides (top, left) and an OST enzyme (top, right) that transfers the lipid-linked oligosaccharide to asparagine, serine or threonine residues on the glycoprotein target (bottom, right). Diverse natural and synthetic gene pathways for lipid-linked oligosaccharide biosynthesis have been utilized in cell-free systems (top, left). These include model bacterial glycans such as the native *C. jejuni* glycan[23], conjugate vaccine antigens including the *F. tularensis* Schu S4 O antigen[22] and eukaryotic glycans, such as the trimmanose core *N*-glycan[23] and core 1 *O*-glycans[29]. The desired glycan structure (examples shown), linkage and glycosylation sequon will determine the choice of OST enzyme (top, right). Bacterial OSTs that carry out asparagine-linked glycosylation (*N*-OSTs) as well as serine/threonine-linked glycosylation (*O*-OSTs) can be used in cell-free systems. OSTs denoted with * are the most well-characterized enzymes for cell-free glycoprotein synthesis[22–25,29]. Proteins can be engineered as substrates for cell-free glycosylation via inclusion of *N*- or *O*-linked glycosylation sequons at desired modification sites (bottom, right). In addition, the cell-free reaction environment can be readily adapted to facilitate biosynthesis of complex glycoproteins, including membrane proteins and proteins with other post-translational modifications such as disulfide bonds[22]. We have demonstrated cell-free biosynthesis and glycosylation of model glycoprotein targets such as sfGFP[23], in addition to therapeutically relevant targets including the licensed conjugate vaccine carrier proteins CRM197 (genetically detoxified *Corynebacterium diphtheriae* toxin) and PD (nonacylated *Haemophilus influenzae* protein D)[22], as well as human erythropoietin[23].

Selecting chassis strain and constructing synthetic protein glycosylation pathways

A chassis strain and glycosylation components must be selected to yield desired glycosylation activity in the cell-free reaction (Fig. 2).

Stage 1. Select chassis strain. The choice of chassis strain is dictated by the desired glycoprotein product and intended use. *E. coli* strain CLM24 was previously optimized for in vivo protein glycosylation[44] and can be used as a chassis for cell-free synthesis of glycoproteins bearing diverse

N-and *O*-glycans[23,24]. CLM25 is derived from strain CLM24 and contains an additional deletion of the *wecA* gene that encodes an endogenous phosphoglycosyltransferase. The addition of the *wecA* deletion eliminates formation of Und-PP-GlcNAc and should be considered if the reducing end monosaccharide of the desired glycan is not GlcNAc[29]. *E. coli* strain CLM24 Δ*lpxM* should be used to generate cell-free lysates for downstream assays or applications requiring low endotoxin levels[22].

Stage 2. Select oligosaccharyltransferase (OST) enzyme. This protocol exclusively describes en bloc protein glycosylation where a complete glycan structure is first assembled on the lipid substrate and is then transferred onto the target protein by an OST enzyme. Cell-free systems capable of sequential protein glycosylation, in which individual monosaccharides are added directly to asparagine residues or glycoproteins, have been described elsewhere[27,28,45]. The choice of OST will depend on the types of protein–glycan linkage and desired glycan structures. For *N*-linked protein glycosylation, *N*-OST enzymes from epsilonproteobacteria including *Campylobacter*, *Helicobacter*, *Sulfurimonas* and *Wolinella* genus, or those from deltaproteobacteria such as *Geobacter* and *Desulfovibrio*, should be selected[46]. For *O*-linked protein glycosylation, *O*-OST enzymes from *Neisseria* species such as *N. gonorrhoeae* and *N. meningitidis* or those from *Streptococcus* including *S. pneumoniae* can be utilized[47,48] (Fig. 2, top right).

Stage 3. Select glycan biosynthesis pathway. Glycan biosynthesis genes including monosaccharide synthetases/synthases and glycosyltransferases can be cloned from one or multiple organisms and assembled into a single operon. Care should be taken to ensure that:

1 Required nucleotide-activated monosaccharide substrates are synthesized endogenously in *E. coli* or necessary biosynthetic pathways are co-expressed.
2 The assembled glycan structure is an acceptable substrate for the selected OST. For example, the *N. meningitidis O*-OST is known to be able to transfer glycans containing reducing end galactose but not glucose residues. By contrast, the *S. pneumoniae O*-OST has the ability to transfer glycans containing glucose at their reducing end[48].
3 Glycan biosynthesis and OST plasmids have compatible antibiotic resistance genes and origins of replication for co-expression in *E. coli*.

Stage 4. Select target protein. Theoretically, any protein can be glycosylated using the cell-free system as long as the target protein contains at least one glycosylation sequon that is accessible to the OST. If needed, molecular cloning can be used to introduce one or more glycosylation sequon(s) into the target protein at the N- or C-terminal or at a desired internal site(s) (Fig. 2, bottom right). The glycosylation sequon is the shortest amino acid sequence that can be recognized and modified by specific OST enzymes. For example, the glycosylation sequon of the *C. jejuni N*-OST is D/E_{-2}-X_{-1}-N-X_1-S/T_2 where *X* is any amino acid but proline, and the number indicates position of the amino acid with respect to the asparagine residue (position 0)[49]. By contrast, *Neisseria* spp. *O*-OSTs recognize the WPAAASAP sequon preceded and succeeded by hydrophobic regions[47]. Note that the presence of the glycosylation sequon alone is not sufficient to guarantee successful glycan installation. The sequon must be accessible to the OST for efficient glycosylation. Optimization of sequon location can increase glycosylation efficiency for the target glycoprotein[50].

Choosing a cell-free lysate preparation method

There are multiple established methods to prepare *E. coli* cell-free lysates, but two are best suited for cell-free glycoprotein synthesis: homogenized S12 and S30 lysates. We have recently shown that S12 lysates have increased glycosylation activity using model *N*- and *O*-linked glycosylation pathways due to increased concentration of membrane vesicles[24] (Fig. 3). In addition, the S12 lysate preparation protocol may be more broadly accessible due to the elimination of high-speed centrifugation steps; all lysate clarification steps can be carried out using a standard refrigerated microcentrifuge[24]. However, our cell-free glycoprotein synthesis technologies were originally developed using S30 lysates and a broader array of glycoproteins have been synthesized using this approach[22,23,25,26]. Thus, here we outline protocols for both S12 and S30 lysate preparation as options for the end user.

Stage 1. Lysis. High-pressure homogenization is recommended for highly active, glycosylation-competent lysates. It should be noted that, while cell-free lysates can be prepared with either sonication or homogenization as the lysis method, we have found that glycosylation is less efficient in sonicated lysates. This is probably due to the fact that sonication results in lower numbers of membrane vesicles with more narrow size distributions formed during lysis[24] (Fig. 3b).

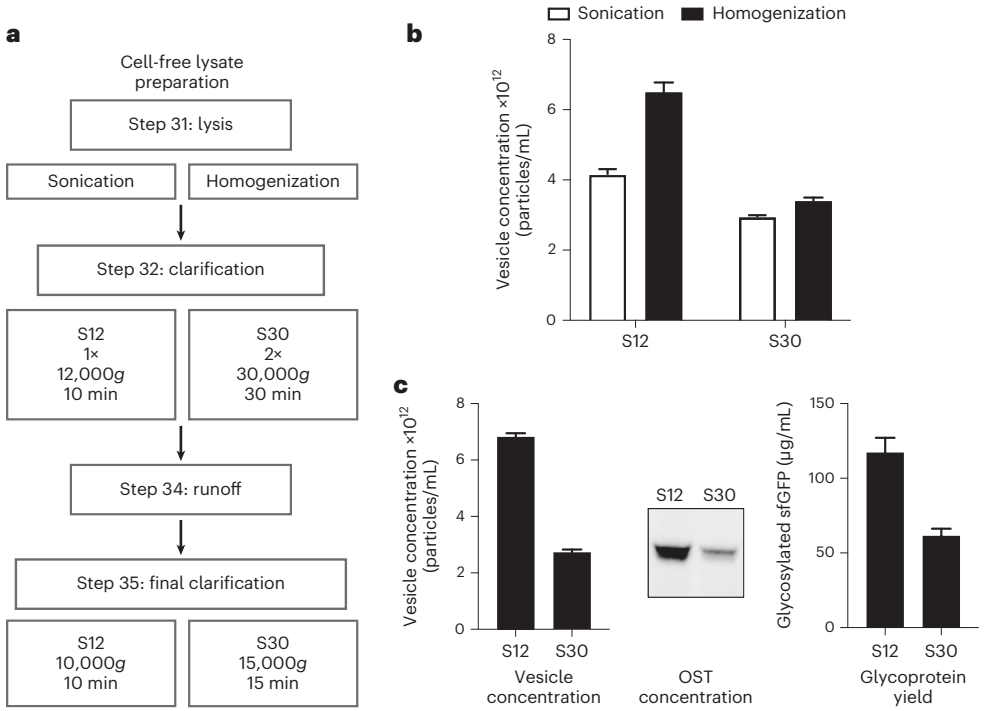

Fig. 3 | Cell-free lysate preparation methods. a, General workflow for cell-free lysate preparation, with options for lysis method and lysate clarification. The corresponding step is noted next to each key step of the workflow. **b**, Lysis and centrifugation methods impact the concentration of native membrane vesicles in cell-free lysates[24]. Vesicle concentration as measured by nanoparticle tracking analysis (NTA) is presented as the mean with error bars representing standard deviation of $n = 15$ replicates (three biologically independent CLM24 lysates that were each measured over five, 1 min NTA measurements)[24]. **c**, S12 clarification of homogenized lysates increases vesicle and OST concentration, as well as glycoprotein yields. Vesicle (left), OST (middle) and cell-free synthesized glycoprotein (right) concentration from homogenized lysates prepared with S12 or S30 clarification[24]. Vesicle concentration as measured by NTA is presented as the mean with error bars representing standard deviation of $n = 15$ replicates (three biologically independent lysates that were each measured over five, 1 min NTA measurements). Glycosylated sfGFP^{DQNAT} yields are presented as the mean with error bars representing standard deviation of $n = 3$ biological replicates. Western blot probed with anti-FLAG antibody to detect OST (PglB) is representative of $n = 3$ biologically independent lysates. Panels **b** and **c** adapted with permission from ref. [24], Springer Nature Ltd.

Stage 2. Clarification. Both S12 and S30 clarification methods result in highly active lysates for cell-free glycoprotein synthesis. The key difference between these methods is the time and speed of centrifugation. Impacts of these differences are listed below:

- The S12 preparation requires shorter centrifugation steps (10 min versus 1 h) at lower speeds (12,000*g* versus 30,000*g*) than the S30 method. Thus, S12 lysate is a good option if a high-speed centrifuge capable of reaching 30,000*g* is not available
- Homogenized S12 lysates contain roughly twice as many membrane vesicles (formed as the native membrane is disrupted during homogenization) than S30 lysates. Membrane-bound glycosylation components in lysates, including OSTs and lipid-linked oligosaccharides (LLOs), are localized in membrane vesicles. We have shown that the increased concentration of vesicles in the S12 lysate can result in increased glycosylation activity[24] (Fig. 3c)

Stage 3. Runoff reaction. The runoff reaction is designed to deplete endogenous mRNA transcripts from ribosomes in the cell lysate, thus increasing availability of ribosomes for cell-free synthesis of the target protein. We have shown that the runoff reaction improves lysate performance when using *E. coli* K strains such as those described in this protocol[51].

Optimizing cell-free reaction conditions

Reaction conditions can be adjusted to facilitate efficient biosynthesis, proper post-translational folding, and optimal glycosylation of desired target glycoproteins (Fig. 4a). Additionally, reaction conditions can

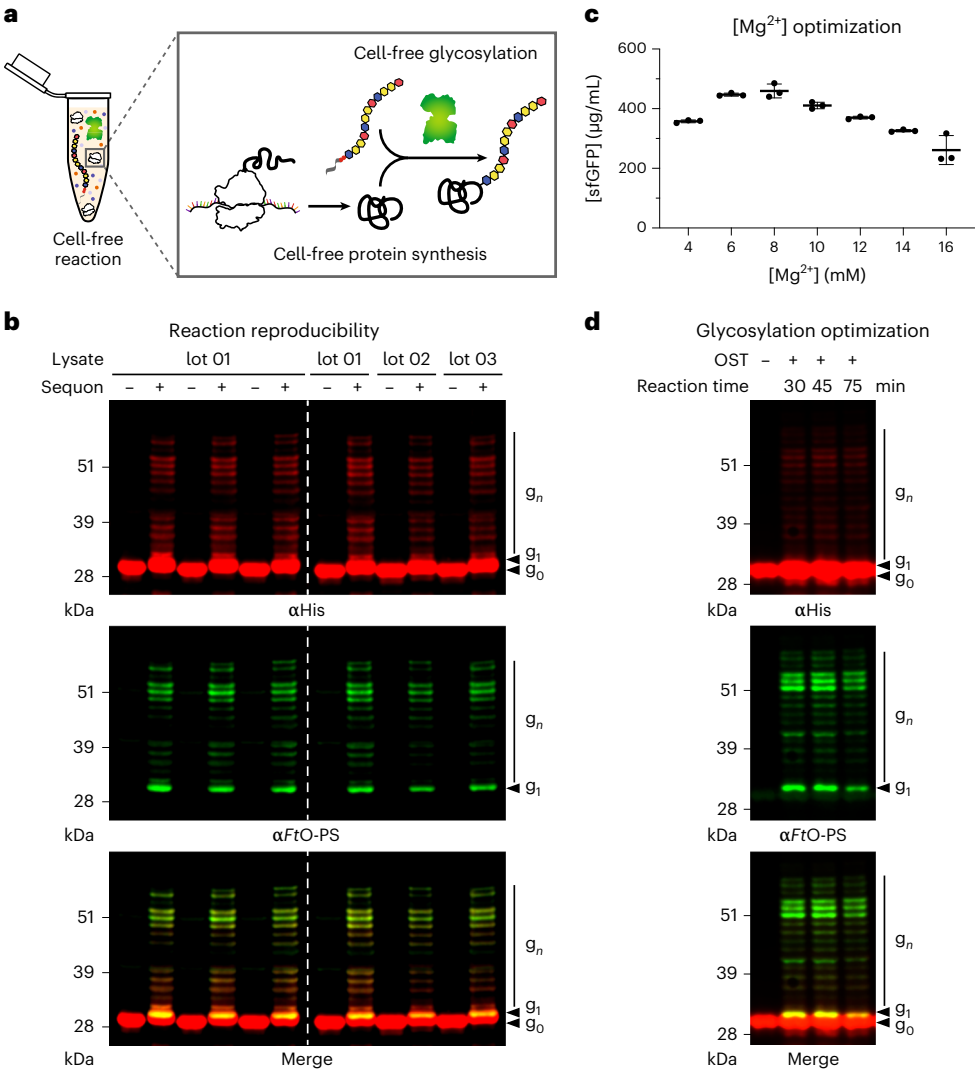

Fig. 4 | Reproducibility and optimization of cell-free glycoprotein synthesis. a, Cell-free glycoprotein synthesis proceeds via coordinated in vitro protein synthesis and glycosylation steps. Both steps can be optimized for reproducible and efficient glycoprotein production. **b**, Biological replicates ($n = 3$) of cell-free reactions producing sfGFP$^{217\text{-AQNAT}}$ (− sequon, aglycosylated control) or sfGFP$^{217\text{-DQNAT}}$ (+ sequon) using the same lot (left) or different lots (right) of cell-free lysate demonstrate reproducibility of reaction and lysate preparation[22]. **c**, Cell-free protein synthesis can be optimized by varying [Mg^{2+}] in the cell-free reaction. For the lysate tested here, cell-free synthesis of sfGFP$^{217\text{-DQNAT}}$ is maximized between 6 and 8 mM Mg^{2+}. Horizontal bars represent means and error bars show standard deviation of $n = 3$ biological replicates (unpublished data). **d**, Cell-free glycosylation can be optimized by varying the incubation time both before and after addition of glycosylation activation solution. In this experiment, three different secondary incubation times were tested. Western blot analysis shows that glycosylation of sfGFP$^{217\text{-DQNAT}}$ is maximized with a 45 min incubation after addition of glycosylation activation solution[22]. Blots in **b** and **d** were probed with anti-hexa-histidine antibody to detect the carrier protein (αHis) and FB11 antibody to detect the *F. tularensis* O antigen (α*Ft*O-PS). Bands that are cross-reactive with both αHis and α*Ft*O-PS probes are glycosylated. In all blots, aglycosylated proteins produced in cell-free reactions lacking the OST enzyme that performs protein glycosylation are shown as negative controls. All blots are representative of $n = 3$ biological replicates per timepoint. g_0, unmodified protein; g_1, glycosylated protein bearing one glycan; g_n, glycosylated protein bearing multiple (2, 3, ..., n) glycans. Panels **b** and **d** adapted with permission from ref. [22], AAAS.

be adjusted to decrease overall reaction cost and increase the thermostability of the lyophilized reaction[30]. We have shown that cell-free reactions reproducibly synthesize glycoproteins (Fig. 4b).

Stage 1. Optimizing Mg^{2+} concentration. Mg^{2+} concentration influences the protein biosynthesis process in numerous ways, such as impacting the ability of the 30S and 50S ribosomal subunits to assemble. As such, Mg^{2+} concentration should be optimized for each new cell-free lysate. Assemble cell-free reactions with varying amounts of magnesium glutamate added (6–18 mM final

concentrations are typical). Measure endpoint protein synthesis via western blot (e.g., if synthesizing vaccine carrier proteins) or fluorescence (e.g., if synthesizing GFP). The optimal Mg^{2+} concentration is that at which cell-free protein synthesis is maximized (Fig. 4c).

Stage 2. Optimizing in vitro glycosylation efficiency. Cell-free glycoprotein synthesis proceeds in a two-step process:

Cell-free protein synthesis

During an initial incubation period, the target protein is synthesized. The length of this initial incubation period can impact glycosylation efficiency by determining how much carrier protein is expressed before in vitro glycosylation. Importantly, maximum glycosylation efficiency differs for S12 and S30 lysates due to the concentration of membrane vesicles containing glycosylation machinery present in the lysate[24].

Cell-free glycosylation

After the initial incubation, glycosylation activation solution is added to cell-free reactions and glycosylation of target proteins proceeds. Stability of glycan structures and kinetics of glycosylation can vary for different glycans and will influence optimal secondary incubation times. To determine optimal incubation times, assemble replicate cell-free reactions with varying initial (10 min to 4 h) and secondary (45 min to 16 h) incubation times. Determine incubation times that maximize glycosylation efficiency via western blot (Fig. 4d).

Stage 3. Additional considerations for complex proteins. For expression of proteins containing disulfide bonds, reactions can be carried out under oxidizing conditions, as previously reported[52]. To achieve oxidizing conditions, pre-condition lysate with 750 μM iodoacetamide at room temperature (~21 °C) for 30 min to covalently bind free sulfhydryls (-SH), including the active site cysteines of the thioredoxin reductase (trxB) and glutathione reductase (gor) enzymes that represent the primary disulfide bond reducing enzymes in the *E. coli* cytoplasm. The cell-free reaction mix is then supplemented with 200 mM glutathione at a 4:1 ratio of oxidized and reduced forms and 10 μM recombinant *E. coli* DsbC. Protein databases such as UniProt and PDB can be used to determine whether a target protein contains disulfide bonds in its native state. If this information is not available a priori, prediction tools such as Disulfide by Design 2 (http://cptweb.cpt.wayne.edu/DbD2/) can be used.

For expression of membrane proteins, hydrophobic transmembrane domains can be stabilized by supplementing the cell-free reaction with nanodiscs or other membrane mimics. For example, we typically express the PorA protein using cell-free lysate that is supplemented with nanodiscs at 1 μg/mL (Cube Biotech). Importantly, an appropriately sized nanodisc is critical for successful cell-free expression of membrane proteins and, therefore, should be predetermined.

Stage 4. Additional considerations to increase thermostability of lyophilized reactions. To increase the thermostability of lyophilized reactions, maltodextrin can be added as a lyoprotectant at a final concentration of 60 mg/mL to the cell-free reaction. Optimal cell-free glycoprotein synthesis conditions should be evaluated after the desired storage conditions, as protein synthesis capabilities of the reaction can be impacted by storage time and temperature. Maltodextrin has been demonstrated to maintain activity of cell-free glycoprotein synthesis reactions after storage for 4 weeks at up to 50 °C (ref. [30]).

Stage 5. Additional considerations to lower reaction cost. If a lower-cost reaction is desired, maltodextrin with the supplementation of potassium phosphate dibasic can be used as an energy source instead of phosphoenolpyruvate (PEP). Further, nucleoside monophosphates (NMPs) can be used instead of nucleoside triphosphates (NTPs), and tRNA and CoA can be removed entirely from the reaction, while still maintaining cell-free protein synthesis yields required for robust cell-free glycoprotein synthesis[30,53,54]. With these modifications (Step 39), the most expensive components of the cell-free reactions are replaced or removed, substantially lowering the overall cost. Cell-free protein synthesis and glycosylation efficiency must be optimized separately for this modified formulation as endpoint yields or glycosylation efficiency can differ compared with the original formulation (Step 39). Mg^{2+} concentration must also be titrated in this formulation and may have a different optimum than the original formulation due to the presence of potassium phosphate dibasic in the reaction formulation. In this formulation, which is adapted to decrease reaction cost, maltodextrin also acts as a lyoprotectant, enabling storage at up to 50 °C for 4 weeks[30].

Controls

Reactions synthesizing aglycosylated target proteins should be prepared to (1) determine glycosylation efficiency and (2) serve as controls for downstream activity assays, as described in Step 39.

Materials

Biological materials

• See Table 1 for details of the bacterial strains and plasmids

Table 1 | Bacterial strains and plasmids

Reagent	Description	Source
Chassis strains		
CLM24	*E. coli* W3110 Δ*waaL* (for generating cell-free glycoprotein synthesis lysates in which the reducing end monosaccharide of the desired glycan is GlcNAc)	44
CLM24 Δ*lpxM*	*E. coli* W3110 Δ*waaL* Δ*lpxM* (for generating low-endotoxin lysates)	22
CLM25	*E. coli* W3110 Δ*waaL* Δ*wecA* (for generating cell-free glycoprotein synthesis lysates in which the reducing end monosaccharide of the desired glycan is not GlcNAc)	29
Lipid-linked oligosaccharide plasmids		
pGAB2	*F. tularensis* subsp. *tularensis* Schu S4 O antigen gene cluster in pLAFR1, Tc^R	56
pMW07-O78	*E. coli* O78 antigen gene cluster in pMW07, Cm^R	57
pJHCV32	*E. coli* O7 antigen gene cluster in pVK102, Tc^R	58
pMW07-PglΔB	*C. jejuni* heptasaccharide biosynthesis gene cluster in pMW07, Cm^R	59
OST plasmids		
pSF-*Cj*PglB	*C. jejuni* PglB with a C-terminal 1× FLAG epitope tag in pSF, Amp^R	23
pSF-*Cj*PglB-LpxE	*C. jejuni* PglB with a C-terminal 1× FLAG epitope tag and *F. tularensis* LpxE in pSF, Amp^R	Addgene 128389 (see also ref. [21])
pOG-T-NgPglO	Genes encoding *C. jejuni* Gne, *Acinetobacter baumannii* PglC, *E. coli* O104 *WbwC* and *N. gonorrhoeae* PglO in pMW07, Cm^R	29
pSF-NgPglO	*N. gonorrhoeae* PglO with a C-terminal 1× FLAG epitope tag in pSF, Amp^R	24
Target protein plasmids		
pJL1-sfGFP$^{217\text{-}DQNAT}$	Superfolder GFP variant modified after residue T216 with a 21 amino acid insertion containing the *C. jejuni* AcrA N123 glycosylation site but with a DQNAT glycosylation sequon and a C-terminal 6× His tag in pJL1, Kan^R	23
pJL1-sfGFP$^{217\text{-}AQNAT}$	Same as pJL1 sfGFP$^{217\text{-}DQNAT}$, but with an AQNAT glycosylation sequence that is not modified by *Cj*PglB in pJL1, Kan^R	23
pJL1-sfGFP^{DQNAT}	Superfolder GFP variant modified with a C-terminal optimal DQNAT glycosylation sequence and a C-terminal 6× His tag in pJL1, Kan^R	24
pJL1-sfGFP^{AQNAT}	Same as pJL1-sfGFP^{DQNAT}, but with an AQNAT glycosylation sequence that is not modified by *Cj*PglB in pJL1, Kan^R	24
pJL1-MBP$^{4\times DQNAT}$	*E. coli* maltose-binding protein (MBP) with a C-terminal 4× DQNAT glycosylation tag and a 6× His tag in pJL1, Kan^R	Addgene 128390 (see also ref. [21])
pJL1-PD$^{4\times DQNAT}$	*H. influenzae* protein D with a C-terminal 4× DQNAT glycosylation tag and a 6× His tag in pJL1, Kan^R	Addgene 128391 (see also ref. [21])
pJL1-PorA$^{4\times DQNAT}$	*N. meningitidis* PorA porin protein with a C-terminal 4× DQNAT glycosylation tag and a 6× His tag in pJL1, Kan^R	Addgene 128392 (see also ref. [21])
pJL1-TTc$^{4\times DQNAT}$	Fragment C domain of *Clostridium tetani* toxin with a C-terminal 4× DQNAT glycosylation tag and a 6× His tag in pJL1, Kan^R	Addgene 128393 (see also ref. [21])
pJL1-TTlight$^{4\times DQNAT}$	Light chain variant of *C. tetani* toxin containing an inactivating E234A mutation in the enzyme active site with a C-terminal 4× DQNAT glycosylation tag and a 6× His tag in pJL1, Kan^R	Addgene 128394 (see also ref. [21])
pJL1-CRM197$^{4\times DQNAT}$	*C. diphtheriae* toxin variant with an inactivating G52E mutation in the enzyme active site with a C-terminal 4× DQNAT glycosylation tag and a 6× His tag in pJL1, Kan^R	Addgene 128395 (see also ref. [21])
pJL1-TT$^{4\times DQNAT}$	*C. tetani* toxin variant containing an inactivating E234A mutation in the enzyme active site with a C-terminal 4xDQNAT glycosylation tag and a 6xHis tag in pJL1, Kan^R	Addgene 128396 (see also ref. [21])
pJL1-EPA$^{DNNNS\text{-}DQNRT}$	*P. aeruginosa* exotoxin A containing a DNNNS glycosylation site at residue 242 and a DQNRT glycosylation site at residue 384 and a C-terminal 6× His tag in pJL1, Kan^R	Addgene 128397 (see also ref. [21])
pJL1-scFv13- R4^{DQNAT}	Single chain Fv fragments against β-galactosidase modified with N34L and N77L mutation and a C-terminal DQNAT-6× His tag in pJL1, Kan^R	23
pJL1-hEPO$^{36\text{-}DQNAT\text{-}40}$	Human erythropoietin with native glycosylation motif around N38 mutated to DQN^{38}AT in pJL1, Kan^R	23
pJL1-MBP^{MOOR}	Gene encoding *E. coli* MBP with a C-terminal fusion bearing the 25-residue MOOR sequence in pJL1, Kan^R	29
pJL1-sfGFP^{MOOR}	Superfolder green fluorescent protein with a C-terminal fusion bearing the 25-residue minimal optimal *O*-linked recognition (MOOR) sequence and a C-terminal 6× His tag in pJL1, Kan^R	24
pJL1-sfGFP^{MOORmut}	Same as pJL1-sfGFP^{MOOR} but with a nonpermissible MOORmut sequence and a C-terminal 6× His tag in pJL1, Kan^R	24

Reagents

- Tryptone (Fisher, cat no. 211699)
- Yeast extract (Fisher, cat. no. 212720)
- Sodium chloride (NaCl; Sigma, cat. no. S3014)
- Potassium phosphate dibasic (K_2HPO_4; Sigma, cat. no. 60353)
- Potassium phosphate monobasic (KH_2PO_4; Sigma, cat. no. P9791)
- Sodium hydroxide (NaOH; Sigma, cat. no. S5881)
- Potassium hydroxide (KOH; Sigma, cat. no. P5958)
- Glacial acetic acid (Sigma, cat. no. A6283)
- Potassium chloride (KCl; Sigma, cat. no. P9541)
- Sodium phosphate dibasic (Na_2HPO_4; Sigma, cat. no. S3264)
- Tween-20 (Sigma, cat. no. P7949)
- Ampicillin sodium salt (Sigma, cat. no. A0166)
- Chloramphenicol (Sigma, cat. no. C1919)
- Tetracycline (Sigma, cat. no. T7660)
- L-Arabinose (Sigma, cat. no. A3256)
- Trizma base (TrisOAc; Sigma, cat. no. T6066)
- Magnesium acetate (MgOAc; Sigma, cat. no. M5661)
- Potassium acetate (KOAc; Sigma, cat. no. P1190)
- Magnesium glutamate ($Mg(Glu)_2$; Sigma, cat. no. 49605)
- Ammonium glutamate (NH_4Glu; Fisher, cat. no. MP21805951)
- Potassium glutamate (KGlu; Sigma, cat. no. G1501)
- Adenosine triphosphate (ATP; Sigma, cat. no. A2383)
- Guanosine triphosphate (GTP; Sigma, cat. no. G8877)
- Uridine triphosphate (UTP; Sigma, cat. no. U6625)
- Cytidine triphosphate (CTP; Sigma, cat. no. C1506)
- Folinic acid (Sigma, cat. no. 47612)
- *E. coli* tRNA (Roche, cat. no. 10109541001)
- Amino acids (Sigma, cat. no. LAA21-1KT)
- Phosphoenol-pyruvate (PEP; Roche, cat. no. 10108294001)
- Nicotinamide adenine dinucleotide (NAD; Sigma, cat. no. N8535-15VL)
- Coenzyme A (CoA; Sigma, cat. no. C3144)
- Oxalic acid (potassium oxalate monohydrate; Sigma, cat. no. P0963)
- Putrescine (Sigma, cat. no. P5780)
- Spermidine (Sigma, cat. no. S2626)
- T7 RNA polymerase (New England Biolabs, cat. no. M0251)
- Adenosine monophosphate (AMP; Sigma, cat. no. 01930)
- Guanosine monophosphate (GMP; Sigma, cat. no. G8377)
- Uridine monophosphate (UMP; Sigma, cat. no. U6375)
- Cytidine monophosphate (CMP; Sigma, cat. no. C1006)
- Maltodextrin-dextrose equivalent 4.0–7.0 (Sigma, cat. no. 419672)
- Iodoacetamide (Sigma, cat. no. I1149)
- L-Glutathione, oxidized (Sigma, cat. no. G4501)
- L-Glutathione, reduced (Sigma, cat. no. G4251)
- DsbC (Enzo Life Sciences, cat. no. ALX-201-268-C100)
- POPC Nanodiscs (Cube Biotech, cat. no. 26363)
- HEPES (Sigma, cat. no. H3375)
- Magnesium chloride (Sigma, cat. no. 63535)
- *n*-Dodecyl β-D-maltoside (DDM; Anatrace, cat. no. D310S)
- Sucrose (Fisher, cat. no. S25590)
- Plasmid prep kit (e.g., Omega Bio-Tek, cat. no. D6904-04)
- Bradford or bicinchoninic acid (BCA) assay kit (e.g., Bio-Rad, cat. no. 5000201)
- RNAseZap (Invitrogen or similar)
- Antibodies and western blot reagents (for details, see Table 2)
- Liquid nitrogen
- Intercept blocking buffer (LI-COR, cat. no. 927-70001)

Table 2 | Antibodies and reagents used for western blot characterization

Target	Source	Cat. no.	RRID	Dilution
Rabbit pAb to 6× His epitope tag	Abcam	ab1187	AB_298652	1:7,500
Mouse mAb FB11 to *F. tularensis* LPS	Fisher	MA121690	AB_302778	1:5,000
Rabbit pAb to *E. coli* O78 antigen	Abcam	ab78826	AB_1640456	1:2,500
Rabbit pAb to *C. diphtheriae* toxin	Abcam	ab151222	AB_2923071	1:2,000
Rabbit pAb to *C. tetani* toxin	Abcam	ab53829	AB_882903	1:2,000
Goat anti-rabbit IgG IR dye 680	LI-COR	926-68071	AB_10956166	1:15,000–1:10,000
Goat anti-rabbit IgG IR dye 800	LI-COR	926-32211	AB_621843	1:15,000–1:10,000
Goat anti-mouse IgG IR dye 800	LI-COR	926-32210	AB_621842	1:15,000–1:10,000
Biotinylated soybean agglutinin	Vector Laboratories	B-1015-5	NA	1:250
Biotinylated peanut agglutinin	Vector Laboratories	B-1075-5	NA	1:250
ExtrAvidin–peroxidase	Sigma	E2886	NA	1:2,000

Equipment

- Electroporator and cuvettes (e.g., Bio-Rad MicroPulser)
- pH meter and standards
- 0.22 μm sterile filter units (250 mL or larger)
- Bacterial growth flasks or fermenter
- Spectrophotometer for optical density (OD) measurement
- Refrigerated high-speed centrifuge (capable of 30,000*g*)
- Refrigerated tabletop centrifuge (capable of 7,000*g* and can accommodate 50 mL conical tubes)
- Sterile, ice-cold MilliQ water
- Sterile 1 L and 30 mL centrifuge bottles
- Sterilized spatula
- 50 mL conical tubes
- KimWipes
- EmulsiFlex B15 homogenizer (Avestin)
- Microcentrifuge tubes, PCR tubes and/or 15 mL conical tubes depending on desired scale of cell-free reactions
- Lyophilizer, e.g., VirTis BenchTop Pro lyophilizer (SP Scientific)
- Immobilon-P polyvinylidene difluoride membranes 0.45 μm (Sigma or similar)
- Semi-dry transfer cell (Bio-Rad or similar)
- Odyssey Fc imaging system (Li-COR or similar)
- Micropipettes+ tips and serological pipettes

Reagent setup

Luria–Bertani (LB) solid or liquid medium

To prepare 1.0 L liquid medium, dissolve 10.0 g NaCl, 10.0 g tryptone and 5.0 g yeast extract in 900.0 mL MilliQ water. Adjust pH to 7.2 using 5.0 N NaOH solution. Adjust final volume to 1.0 L using MilliQ water. To prepare solid medium, combine 500.0 mL LB liquid medium with 10.0 g bacteriological agar. Autoclave and store at room temperature for up to 2 weeks. **! CAUTION** 5.0 N NaOH is a strong basic solution and should be handled with caution. Wear appropriate personal protective equipment (PPE; gloves, laboratory coat and goggles) and handle concentrated solutions in a chemical fume hood.

2× YTP liquid medium

To prepare 1.0 L liquid medium, dissolve 5.0 g NaCl, 16.0 g tryptone, 10.0 g yeast extract, 7.0 g K_2HPO_4 and 3.0 g KH_2PO_4 in 900.0 mL MilliQ water. Adjust pH to 7.2 using 5.0 N KOH solution. Adjust final volume to 1.0 L using MilliQ water. Autoclave and store at room temperature for up to 1 week. **! CAUTION** 5.0 N KOH is a strong basic solution and should be handled with caution. Wear appropriate PPE (gloves, laboratory coat and goggles) and handle concentrated solutions in a chemical fume hood.

10% w/v L-arabinose stock solution
To prepare 10% w/v L-arabinose solution, add 10.0 g L-arabinose to 90.0 mL MilliQ water. Adjust final volume to 100 mL with MilliQ water. Sterilize using a 0.22 μm filter. Store at room temperature or 4 °C for up to 2 months.

1.0 M Tris acetate stock solution
To prepare 250 mL of stock solution, dissolve 30.5 g of Trizma base in 200 mL of MilliQ water at room temperature. Adjust pH to 8.2 using glacial acetic acid. Adjust volume to 250 mL with MilliQ water and sterilize using a 0.22 μm filter. Store at 4 °C for up to 2 months. **! CAUTION** Glacial acetic acid is a strong acidic solution and should be handled with caution. Wear appropriate PPE (gloves, lab coat and goggles) and handle concentrated solutions in a chemical fume hood.

1.4 M magnesium acetate stock solution
To prepare 250 mL of stock solution, dissolve 75.06 g of magnesium acetate tetrahydrate in 200 mL of MilliQ water. Gentle heat can be used to help dissolve salt. Adjust volume to 250 mL with MilliQ water and sterilize using a 0.22 μm filter. Store at 4 °C for up to 2 months.

6.0 M potassium acetate stock solution
To prepare 250 mL stock solution, dissolve 148 g of potassium acetate in 100 mL of MilliQ water at room temperature. Adjust volume to 250 mL with MilliQ water and sterilize using a 0.22 μm filter. Store at 4 °C for up to 2 months.

Lysis buffer
To prepare 250 mL lysis buffer, add 2.5 mL of 1.0 M Tris acetate stock solution, 2.5 mL of 1.4 M magnesium acetate stock solution and 2.5 mL of 6.0 M potassium acetate stock solution potassium acetate stock solution to 242.5 mL ice-cold MilliQ water. Prepare this buffer immediately before use.

1 M magnesium glutamate
Resuspend 19.43 g of L-glutamic acid hemimagnesium salt in a final volume of 50 mL nuclease-free water. Warm solution at 55 °C in a water bath to dissolve salt. Sterilize using a 0.22 μm filter, make 1 mL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

2.4 M ammonium glutamate
Resuspend 19.70 g L-glutamic acid ammonium salt in a final volume of 50 mL nuclease-free water. Warm solution at 55 °C in a water bath to dissolve salt. Sterilize using a 0.22 μm filter, make 1 mL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

4 M potassium glutamate
Resuspend 40.65 g L-glutamic acid potassium salt monohydrate in a final volume of 50 mL nuclease-free water. Warm solution at 55 °C in a water bath to dissolve salt. Sterilize using a 0.22 μm filter, make 1 mL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

Salt solution
Mix 0.313 mL 2.4 M ammonium glutamate and 2.438 mL 4 M potassium glutamate. Determine the optimal magnesium glutamate concentration for the lysate and add the appropriate amount of 1 M stock solution to make 5 mL of a 15× stock. Add nuclease-free water up to 5 mL final solution volume. Make 500 μL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

500 mM nucleoside triphosphate stocks (ATP, GTP, UTP and CTP)
Resuspend each NTP individually in ice-cold nuclease-free water: 5 g ATP in 18.4 mL, 1 g GTP in 3.8 mL, 1 g UTP in 3.5 mL and 1 g CTP in 3.8 mL. Slowly add each nucleotide to 80% of the desired final volume of ice-cold nuclease-free water. Keep solution on ice while dissolving (a small stir bar cleaned with RNAse Zap is recommended). After the nucleotide has dissolved, adjust pH through the gradual addition of 5 N KOH until the solution reaches pH 7.0–7.2. Add nuclease-free water up to the final desired volume and verify exact final concentration by measuring absorbance and using the extinction coefficient of the nucleotide. Make 1 mL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at

−80 °C for at least 1 year. ▲ CRITICAL Calculate the exact concentration of each solution using the measured absorbance and the extinction coefficient of each nucleotide as follows: absorbance/extinction coefficient [mM^{-1} cm^{-1}]/pathlength [cm] × 5,000 = concentration [mM]. Extinction coefficients for each nucleotide are: ATP: 15.4 mM^{-1} cm^{-1} at 259 nm; GTP: 13.7 mM^{-1} cm^{-1} at 253 nm; UTP: 10.0 mM^{-1} cm^{-1} at 262 nm; CTP: 9.0 mM^{-1} cm^{-1} at 271 nm. ▲ CRITICAL Be sure to clean the pH probe with RNAseZap followed by nuclease-free water before pH measurement to prevent RNAse contamination.

500 mM nucleoside monophosphate stocks (AMP, GMP, UMP and CMP)

Resuspend each NMP in ice-cold nuclease-free water: 5 g NMP in 25.6 mL, 1 g GMP in 4.9 mL, 1 g UMP in 5.4 mL and 1 g CMP in 5.4 mL. Slowly add each nucleotide to 80% of the desired final volume of ice-cold nuclease-free water. Keep each solution on ice while dissolving (a small stir bar treated with RNAse Zap is recommended). After the nucleotide has dissolved, adjust pH through the gradual addition of 20% glacial acetic acid until the solution reaches pH 7.0–7.2. Add water up to the final desired volume and verify the exact final concentration by measuring absorbance and using the extinction coefficient of the nucleotide. Make 1 mL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year. ▲ CRITICAL Calculate the exact concentration of each NMP solution using the measured absorbance and the extinction coefficient of each nucleotide as follows: absorbance/extinction coefficient [mM^{-1} cm^{-1}]/pathlength [cm] × 5,000 = concentration [mM]. Extinction coefficients for each nucleotide are: AMP: 15.4 mM^{-1} cm^{-1} at 259 nm; GMP: 13.7 mM^{-1} cm^{-1} at 253 nm; UMP: 10.0 mM^{-1} cm^{-1} at 262 nm; CMP: 9.0 mM^{-1} cm^{-1} at 271 nm. ▲ CRITICAL Be sure to clean the pH probe with RNAseZap followed by nuclease-free water before pH measurement to prevent RNAse contamination.

50 mg/mL tRNA

Dissolve 0.5 g of tRNA in ice-cold nuclease-free water to reach a final solution volume of 10 mL. Make 500 µL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

10.8 mg/mL folinic acid

Dissolve 0.1 g of folinic acid in nuclease-free water to reach a final solution volume of 9.26 mL. Make 500 µL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

Master mix

Add 1,023.60 µL of 50 mg/mL tRNA and 944.44 µL 10.80 mg/mL folinic acid to a 50 mL Falcon tube on ice. Determine the appropriate volumes of all NTPs for a final concentration of 18 mM ATP in the master mix and 12.75 mM GTP, UTP and CTP depending on their final stock concentrations (each stock concentration should be ~500 mM). Add nuclease-free water to reach a final volume of 20 mL. Vortex solution to mix. Remove a small aliquot and verify that the pH is ~7.2. Make 500 µL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year. ▲ CRITICAL For a low-cost formulation, replace NTPs with NMPs at the same final concentration in the master mix and replace tRNA with an equal volume of nuclease-free water. The low-cost version can reduce total protein synthesis yields by ~5–10%.

1 M PEP

Prepare 10 N KOH by resuspending 6 g KOH in 10 mL nuclease-free water. Resuspend 1 g of PEP in a 15 mL Falcon tube containing 3 mL ice-cold nuclease-free water and 1.1 mL 10 N KOH. Keep solution on ice. Titrate pH to 7.0 by adding 10 N KOH in 50 µL aliquots and reducing the volume as solution pH approaches 7.0. Make 500 µL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year. ▲ CRITICAL Be sure to clean the pH probe with RNAseZap followed by nuclease-free water before pH measurement to prevent RNAse contamination. ! CAUTION 10.0 N KOH is a strong basic solution and should be handled with caution. Wear appropriate personal protective equipment (PPE; gloves, laboratory coat and goggles) and handle concentrated solutions in a chemical fume hood.

300 mg/mL maltodextrin

In a 15 mL Falcon tube, resuspend 0.75 g of maltodextrin in nuclease-free water by vortexing to reach a final volume of 2.5 mL. ▲ CRITICAL Prepare maltodextrin fresh immediately before each experiment. If not prepared fresh, maltodextrin will precipitate out of solution during storage.

1.5 M potassium phosphate dibasic
Dissolve 13.06 g potassium phosphate dibasic in nuclease-free water to reach a final volume of 50 mL. Adjust pH to 7.2 using 20% glacial acetic acid. Sterilize using a 0.22 μm filter, make 1 mL aliquots and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

1 M oxalic acid
Resuspend 4 g potassium oxalate monohydrate in 21.7 mL nuclease-free water. Make 500 μL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

50 mM CoA
Resuspend 500 mg of CoA in ice-cold nuclease-free water to reach a final volume of 13.029 mL. Make 500 μL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

100 mM NAD
Add 754 μL of ice-cold nuclease-free water to each 50 mg vial of NAD to reach a final volume of 0.7537 mL. Mix resuspended vials (if multiple), make 500 μL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

1 M HEPES pH 7.2
Dissolve 7.15 g HEPES in 10 mL of nuclease-free water. Measure the pH of the HEPES solution and adjust pH to reach 7.2 by gradually adding 5 N KOH (~1.4 mL of 5 N KOH will be necessary). After pH is adjusted to 7.2, add nuclease-free water to a final volume of 30 mL. Check pH of solution to verify that it is still 7.2, then sterilize using a 0.22 μm filter. Make 1 mL aliquots and store at −80 °C. Solution is stable at −80 °C for at least 1 year. ▲ **CRITICAL** Be sure to clean the pH probe with RNAseZap followed by nuclease-free water before pH measurement to prevent RNAse contamination. ! **CAUTION** 5.0 N KOH is a strong basic solution and should be handled with caution. Wear appropriate personal protective equipment (PPE; gloves, laboratory coat and goggles) and handle concentrated solutions in a chemical fume hood.

250 mM spermidine
On ice, dissolve 1 g of spermidine in ice-cold nuclease-free water to reach a final volume of 27.5 mL. Make 1 mL aliquots and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

250 mM putrescine
Dissolve 1 g of putrescine dihydrochloride in ice-cold nuclease-free water to reach a final volume of 24.8 mL. Make 1 mL aliquots and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

50 mM 20 amino acid mix
Fill a 50 mL Falcon tube with 25 mL of nuclease-free water. Add amino acids in the following order, vortexing for at least 1 min, or until the solution is soluble after each addition: 0.234 g L-valine, 0.408 g L-tryptophan, 0.33 g L-phenylalanine, 0.262 g L-isoleucine, 0.262 g L-leucine, 0.242 g L-cysteine, 0.298 g L-methionine, 0.178 g L-alanine, 0.348 g L-arginine, 0.264 g asparagine, 0.266 g aspartic acid, 0.406 g L-glutamic acid, K salt, 0.150 g L-glycine, 0.292 g L-glutamine, 0.308 g L-histidine, 0.365 g lysine, 0.230 g L-proline, 0.210 L-serine, 0.238 g L-threonine, 0.362 L-tyrosine. Shake at 250 rpm for 10 min at 37 °C or until amino acids have dissolved (no more than 15 min) after the addition of L-isoleucine and after the addition of L-cysteine. After the addition of L-tyrosine, bring the final solution volume up to 40 mL by adding nuclease-free water and shake at 250 rpm for 15 min at 37 °C. Note that tyrosine will not completely dissolve. Make a small aliquot and check that the final pH of the solution is ~6.7. Make 500 μL aliquots, flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

Reagent mix for traditional cell-free reactions (Table 3)
To make 100 reactions worth of reagent mix, combine 60 μL 50 mM amino acids, 50 μL 1 M PEP, 6 μL 100 mM NAD, 8 μL 50 mM CoA, 6 μL 1 M oxalic acid, 6 μL 250 mM putrescine, 9 μL 250 mM spermidine, and 86 μL 1 M HEPES in a microcentrifuge tube on ice. Flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

Table 3 | Cell-free reaction assembly

Reagents	Stock concentration	Volume per reaction (μL)	Final concentration
Salt solution		1.00	
$Mg(Glu)_2$	180 mM		12 mM
$NH_4(Glu)$	150 mM		10 mM
K(Glu)	1,950 mM		130 mM
Master mix		1.00	
ATP	18 mM		1.2 mM
GTP	12.75 mM		0.850 mM
UTP	12.75 mM		0.850 mM
CTP	12.75 mM		0.850 mM
Folinic acid	0.51 mg/mL		0.034 mg/mL
tRNA	2.559 mg/mL		0.171 mg/mL
Reagent mix (see 'Reagent setup')		2.30	
20 amino acids	50 mM		2.00 mM
PEP	1,000 mM		33.33 mM
NAD	100 mM		0.4 mM
CoA	50 mM		0.27 mM
Oxalic acid	1,000 mM		4.00 mM
Putrescine	250 mM		1.00 mM
Spermidine	250 mM		1.50 mM
HEPES	1,000 mM		57.00 mM
T7 RNA polymerase	5 mg/mL	0.30	0.10 mg/mL
Lysate (Step 36)		4.00	
Nuclease-free water		6.40	
Total reaction volume		**15.00**	

Reagent mix for low-cost, thermostable cell-free reactions (Table 4)
To make 100 reactions worth of reagent mix, combine 60 μL 50 mM amino acids, 6 μL 100 mM NAD, 6 μL 1 M oxalic acid, 6 μL 250 mM putrescine, 9 μL 250 mM spermidine, 86 μL 1 M HEPES, and 75 μL 1.5 M postassium phosphate dibasic in a microcentrifuge tube on ice. Flash freeze with liquid nitrogen and store at −80 °C. Solution is stable at −80 °C for at least 1 year.

2 nM target glycoprotein plasmid
Purify using midi- or maxi-prep plasmid purification kit. Resuspend at 2 nM in nuclease-free water. ▲ **CRITICAL** Plasmid DNA purity can critically affect in vitro protein synthesis yields. Elute DNA following purification in nuclease-free water. We recommend measuring and documenting 260 nm/280 nm and 260 nm/230 nm absorbance ratios to assess DNA purity.

***N*-glycosylation activation solution**
Dissolve 0.247 g of manganese chloride tetrahydrate and 0.05 g *n*-dodecyl β-D-maltoside in 40 mL nuclease-free water. Mix well and adjust the final volume to 50 mL with nuclease-free water. This buffer is stable at 4 °C for up to 2 months. For longer-term storage, aliquot and store at −20 °C for up to 1 year. Minimize freeze–thaw cycles.

***O*-glycosylation activation solution**
Dissolve 1.71 g sucrose and 11.25 mg tetracycline in 40 mL nuclease-free water. Mix well and adjust the final volume to 50 mL with nuclease-free water. Aliquot and store at −20 °C for up to 1 year. Minimize freeze–thaw cycles. ▲ **CRITICAL** Note that 25 mM $MnCl_2$ (in the form of 0.247 g manganese chloride tetrahydrate) can be added to *O*-glycosylation activation solution instead of tetracycline if the *O*-OST requires a divalent metal ion cofactor for activity.

Table 4 | Cell-free reaction assembly adapted for low cost and high thermostability of lyophilized reactions

Reagents	Stock concentration	Volume per reaction (μL)	Final concentration
Salt solution		1.00	
$Mg(Glu)_2$	180 mM		12 mM
$NH_4(Glu)$	150 mM		10 mM
K(Glu)	1,950 mM		130 mM
Master mix		1.00	
AMP	18 mM		1.2 mM
GMP	12.75 mM		0.850 mM
UMP	12.75 mM		0.850 mM
CMP	12.75 mM		0.850 mM
Folinic acid	0.51 mg/mL		0.034 mg/mL
Reagent mix (see 'Reagent setup')		2.48	
20 amino acids	50 mM		2.00 mM
NAD	100 mM		0.4 mM
Oxalic acid	1,000 mM		4.00 mM
Putrescine	250 mM		1.00 mM
Spermidine	250 mM		1.50 mM
HEPES	1,000 mM		57.00 mM
K_2HPO_4	1,500 mM		75.00 mM
Maltodextrin	300 mg/mL	3.00	60 mg/mL
T7 RNA polymerase	5 mg/mL	0.30	0.10 mg/mL
Lysate (Step 36)		4.00	
Nuclease-free water		3.22	
Total reaction volume		**15.00**	

Phosphate-buffered saline (PBS)
To prepare 1.0 L PBS, dissolve 80.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 900 mL MilliQ water. Adjust pH to 7.4 with 37% HCl solution. Adjust final volume to 1.0 L with MilliQ water and sterilize using a 0.22 μm filter or by autoclaving. Store at room temperature for up to 1 year. **! CAUTION** HCl is a strong acidic solution and should be handled with caution. Wear appropriate PPE (gloves, laboratory coat and goggles) and handle concentrated solutions in a chemical fume hood.

Phosphate-buffered saline with Tween (PBST)
Dissolve 1.0 mL of Tween-20 in 0.999 L sterile PBS buffer. Store at room temperature for up to 1 year.

Procedure

Preparation of chassis strain, fermentation media, buffers: day 1 ● Timing: 1 d

1 Prepare media and buffers for chassis strain fermentation and extract preparation as described in 'Reagent setup'.
2 Select chassis strain and glycosylation pathway components (as described in 'Experimental design' and Fig. 2).
3 Inoculate 5 mL of LB medium with the selected chassis strain from a glycerol stock or LB agar plate and grow at 37 °C. Place at least 5 mL sterile MilliQ water on ice to cool to 4 °C.
4 When the culture reaches an exponential phase (OD_{600} 0.6–0.8), transfer 1.4 mL to a sterile microcentrifuge tube. Centrifuge at 10,000*g*, 4 °C for 1 min.
5 Discard supernatant and wash with 1 mL ice-cold sterile MilliQ water. Resuspend cell pellet in water by pipetting. Centrifuge at 10,000*g*, 4 °C for 1 min.
6 Repeat Step 5 two additional times for a total of three washes.
7 After the last wash, resuspend cells with 50 μL ice-cold sterile water by pipetting and proceed immediately to electroporation.
8 Transform cells with plasmid DNA encoding necessary biosynthetic machinery. For synthesis of glycosylated proteins (e.g., conjugate vaccines and glycoprotein therapeutics), transform cells with a

plasmid encoding OST and a plasmid encoding glycan biosynthetic pathway of interest. For synthesis of aglycosylated proteins (e.g., conjugate vaccine carrier proteins), transform with OST or glycan biosynthetic pathway plasmid only. We recommend transformation via electroporation using at least 50 ng of each plasmid to ensure efficient transformation of multiple plasmids.

▲ **CRITICAL STEP** The specific parameters for electroporation will depend on the user's system (e.g., cuvette size and electroporation instrument).

9 Allow transformed cells to recover in 1 mL LB medium for at least 1 h at 37 °C.

10 Plate on LB agar with appropriate antibiotic(s) (50 μg/mL ampicillin/carbenicillin, 10 μg/mL tetracycline and/or 34 μg/mL chloramphenicol). Incubate at 37 °C overnight.

? TROUBLESHOOTING

Inoculate chassis strain starter culture: day 2 ● Timing: 0.25 h

11 Inoculate 50 mL 2× YTP medium containing appropriate antibiotics (for recommended concentrations, see Step 10) with a single colony from the transformant plate from Step 10. Inoculate one 50 mL overnight culture per liter of expression culture. Grow at 37 °C with shaking at 250 rpm for at least 16 h.

E. coli chassis strain growth and harvest: day 3 ● Timing: 1 d

12 Add appropriate antibiotics (for recommended concentrations, see Step 10) to 1 L 2× YTP medium in a 2.5 L baffled flask.

▲ **CRITICAL STEP** Chassis strains for lysate preparation can be cultured at multiple scales, from 10 mL to 10 L (ref. [51]). We recommend starting with 1.0 L of culture, but users can choose to scale up or down as needed.

13 Measure the OD_{600} of the 50 mL overnight culture from Step 11 and use it to inoculate the 1 L fermentation (Step 12) such that the starting OD_{600} is ~0.08. Incubate at 37 °C with shaking at 250 rpm.

14 Monitor the OD_{600} every hour, and then more frequently as the culture approaches the OD_{600} for induction. When the OD_{600} is 0.9–1.0, induce expression of glycosylation pathway enzymes, if applicable, by adding L-arabinose to a final concentration of 0.02% w/v.

▲ **CRITICAL STEP** OD_{600} for induction and final concentration of L-arabinose inducer can critically affect the glycosylation efficiency of the lysate by influencing expression of glycosylation pathway enzymes. These parameters can be optimized for new strains and/or glycan biosynthetic pathway plasmids by measuring expressed protein (e.g., OST expression), or cell-free glycosylation efficiency by western blot. A protocol for assessing glycosylation efficiency via western blot is described below (Steps 42–51).

15 After induction, reduce the growth temperature to 30 °C and continue to incubate until the OD_{600} is 3.0. Culture pH can also be monitored to ensure that it remains in the optimal range for *E. coli* cell growth (6.9–7.2).

16 As the culture OD_{600} approaches 3.0, prepare all necessary materials for cell harvest as follows: prepare 250 mL lysis buffer per 1 L culture; ensure centrifuges, rotors and centrifuge bottles are chilled to 4 °C; ensure access to liquid nitrogen; weigh one 50 mL conical tube per 1 L of culture, record the weight and chill to 4 °C.

▲ **CRITICAL STEP** To preserve the activity of the lysate, it is important to maintain cells at 4 °C during washing and centrifugation steps.

17 When culture OD_{600} is 3.0, transfer the culture contents into chilled 1 L centrifuge bottles. Balance bottles and centrifuge for 15 min at 5,000*g*, 4 °C.

18 Discard supernatant. Using a sterile spatula, transfer the cell pellet into a weighed, chilled 50 mL conical tube and place on ice. Use one 50 mL conical tube for each pellet from 1 L of culture. A small amount (1–2 mL) of lysis buffer can be used to rinse any remaining cells out of the bottle, then pipette the cell/buffer mixture into the 50 mL conical tube.

19 Add 25 mL ice-cold lysis buffer to each 50 mL conical tube and vortex to resuspend to homogeneity. Vortex cells in 15–20 s bursts and rest on ice for at least 30 s in between pulses to keep samples cold, repeat for as many cycles as needed until pellets are fully resuspended.

20 Centrifuge resuspended cells for 10 min at 5,000*g*, 4 °C. Discard supernatant.

21 Repeat Steps 19 and 20 for two additional wash steps. After the third wash, centrifuge for 10 min at 7,000*g*, 4 °C.

22 Discard the supernatant, and wipe down the inside of the conical tube with a KimWipe to remove residual supernatant as much as possible, without disturbing the cell pellet.

23 Record the weight of tubes with the pellet and calculate wet cell pellet weight by subtracting the weight of the empty tube. Record the wet cell pellet weight.

24 Flash freeze cell pellets by submerging the tubes in liquid nitrogen. Store cell pellets at −80 °C or proceed immediately to lysate preparation.

PAUSE POINT Cell pellets can be safely stored at −80 °C for at least 1 year without measurable loss of activity.

Cell-free lysate preparation: day 4 ● Timing: 4 h

25 Select a cell-free lysate preparation method (S12 or S30; as described in 'Experimental design', Fig. 3).

26 Place fully frozen cell pellets from Step 24 on ice to thaw. Add 1.0 mL lysis buffer per 1.0 g wet cell pellet weight (calculated in Step 23) and incubate on ice for 40 min to 1 h.

27 Vortex to resuspend cell pellets to homogeneity. Vortex in 15–30 s bursts followed by 30 s breaks on ice to keep samples cold for as many cycles as needed until pellets are fully resuspended.

▲CRITICAL STEP The amount of bubbles in the resuspended cell/buffer mixture should be minimized to ensure efficient lysis via homogenization. Once cells have been resuspended, the mixture can be centrifuged briefly (1–2 min) at low speed (5,000*g* or lower) to remove bubbles, and then vortexed briefly to resuspend to homogeneity with fewer bubbles present.

28 Pressurize the EmulsiFlex B15 homogenizer to 20,000–25,000 psi.

! CAUTION Wear eye protection and proper personal protective equipment while using the homogenizer at high pressures.

29 Sterilize the chamber of the EmulsiFlex B15 homogenizer with 15 mL 0.1 N KOH, rinse with 15 mL MilliQ water, 15 mL 70% ethanol, a second 15 mL water rinse and prime chamber with 15 mL ice-cold lysis buffer.

30 Draw up to 15 mL of cell/buffer mixture from Step 27 into a syringe using an 18 gauge (or larger) needle. Safely remove the needle, then tap the syringe and eject any bubbles from the syringe before homogenization.

31 Disrupt cells by passing through the homogenizer once at 20,000–25,000 psi. Open the homogenizer chamber slowly and watch the pressure gauge to ensure a set chamber pressure is maintained throughout lysis.

▲CRITICAL STEP Detailed instructions on how to use the Avestin B15 homogenizer can be found at https://www.youtube.com/watch?v=4XnnppW6yts&ab_channel=aussiecomcarl.

32 Transfer cell lysate to prechilled, sterile 30 mL centrifuge bottles or to prechilled 1.5 mL microcentrifuge tubes for S30 or S12 lysates, respectively. Pellet cell debris for 30 min at 30,000*g*, 4 °C for S30 lysate or for 10 min at 12,000*g*, 4 °C for S12 lysate.

! CAUTION Follow safety precautions (e.g., balancing centrifuge bottles) when operating a high-speed centrifuge.

33 For S30 lysates only, transfer lysate supernatant to new chilled, sterile 30.0 mL centrifuge bottles. Centrifuge again for 30 min at 30,000*g* 4 °C to pellet any remaining cell debris.

34 Transfer lysate supernatant, after the second spin for S30 and after the first spin for S12, to prechilled 1.5 mL microcentrifuge tubes. Wrap tubes in aluminum foil and incubate at 37 °C with shaking at 250 rpm for 1 h to complete the runoff reaction.

35 For S30 lysate, centrifuge microcentrifuge tubes for 15 min at 15,000*g*, 4 °C. For S12 lysate, centrifuge microcentrifuge tubes for 15 min at 10,000*g*, 4 °C.

36 Transfer supernatant to clean, prechilled microcentrifuge tubes. Aliquot and flash freeze in liquid nitrogen. Lysate will be active for about three freeze–thaw cycles, so care should be taken to avoid repeated freeze–thaws.

▲CRITICAL STEP For quality control, the total protein concentration of the lysate should be ~40 mg/mL as determined by the Bradford or BCA assay, carried out according to the manufacturer's instructions (e.g., Bio-Rad, cat. no. 5000201).

PAUSE POINT Cell lysates can be safely stored at −80 °C for at least 3 years without measurable loss of activity.

Cell-free reaction assembly: day 5 ● Timing: 1–2 h

! CAUTION All reagents and assembled reactions should be kept on ice at all times.

37 Prepare necessary cell-free reagents used in the cell-free reaction assembly detailed in Tables 3 and 4 as described in 'Reagent setup'.

▲ **CRITICAL STEP** Reagent mix, master mix and salt solution can be pre-assembled in large quantities to speed reaction assembly and reduce error from pipetting small volumes.

▲ **CRITICAL STEP** If maltodextrin is being supplemented to the reaction as a lyoprotectant or energy source (as described in 'Experimental design'), maltodextrin stock solution must be prepared and used fresh directly before each reaction setup.

38 Determine options needed to optimize cell-free protein synthesis and glycosylation for each target glycoprotein of interest, as described in 'Experimental design' (Fig. 4).

39 Assemble reactions for in vitro glycoprotein expression as detailed in Table 3 for standard reactions or Table 4 for low-cost, thermostable reactions. Reactions are formulated on the basis of the previously described modified PANOx-SP cell-free system[30,55]. As described in 'Experimental design', maltodextrin at a final concentration of 60 mg/mL can be supplemented to reagents in Table 3 to increase thermostabilty of lyophilized reactions[30]. For expression of aglycosylated proteins (e.g., conjugate vaccine carrier proteins), lysate from the chassis strain lacking either the OST and/or the glycan biosynthetic pathway should be used. Conversely, for production of glycoproteins, lysate from the chassis strain expressing both OST and the glycan biosynthetic pathway should be used. Assemble reactions at 15 μL scale in 1.5 mL microcentrifuge tubes or PCR tubes, at 1 mL scale in 15 mL conical tubes, or at 5 mL scale in 50 mL conical tubes, volumes in Table 3 can be linearly scaled according to desired final reaction volume.

▲ **CRITICAL STEP** Note that variations in reaction volume or reaction vessel will change the surface area to volume ratio of the cell-free system, which can impact initial rates and total yields of cell-free protein synthesis. Cell-free protein synthesis yields and initial rates should be assessed for each reaction volume and vessel used, as described in 'Experimental design'.

Lyophilization of cell-free reactions: day 5 ● Timing: overnight

40 Once assembled in Step 39, flash-freeze reactions in liquid nitrogen. Poke holes in the lid of the tube or remove the lid and cover the tube with perforated foil to facilitate freeze-drying. Lyophilize reactions at 100 mTorr, −80 °C overnight or until fully freeze-dried.

■ **PAUSE POINT** For storage of freeze-dried reactions at ambient temperature, vacuum seal reactions using a commercial FoodSaver appliance with Dri-Card desiccant cards enclosed to prevent rehydration of the freeze-dried, cell-free pellets. Reactions are stable at ambient temperature under these conditions for at least 3 months. If maltodextrin is added to the formulation, either in addition to the reagents used in Table 3 or as a component of the modified formulation for cost and stability (Table 4), reactions are more thermostable. In the presence of maltodextrin, reactions are stable for at least 4 weeks at up to 50 °C (ref. [30]).

Cell-free (glyco)protein synthesis: day 6 ● Timing: 1-18 h

41 Cell free reactions can be used for aglycosylated protein synthesis (option A) or glycoprotein and conjugate vaccine synthesis (option B).

(A) **Cell-free aglycosylated protein synthesis: day 6 ● Timing: 1–17 h; can be done in parallel with glycoprotein/conjugate vaccine synthesis described below in Step 41B**

(i) Rehydrate freeze-dried reactions with plasmid encoding the protein of interest at 2 nM in the original reaction volume of nuclease-free water. Volume added will depend on the reaction scale (e.g., 15 μL, 1 mL or 5 mL), prepared in Step 39.

(ii) Incubate at 25–37 °C for 1–20 h. For best results, incubate reactions in a prewarmed heat block with water in the wells to ensure consistent incubation temperature and optimal heat transfer.

(iii) Centrifuge reactions for 10 min at 20,000*g*, 4 °C and transfer the supernatant to a clean microcentrifuge tube.

■ **PAUSE POINT** Reaction supernatants can be stored at −80 °C indefinitely. However, proceed immediately to purification if purified conjugate vaccines or aglycosylated carrier proteins are needed for downstream applications.

(B) **Cell-free glycoprotein and conjugate vaccine synthesis: day 6 ● Timing: 1–17 h; can be done in parallel with aglycosylated protein synthesis described above in Step 41A**

(i) Rehydrate freeze-dried reactions with plasmid encoding the protein of interest at 2 nM in the original reaction volume of nuclease-free water. Volume added will depend on the reaction scale (e.g., 15 μL, 1 mL or 5 mL), prepared in Step 39.

(ii) Incubate at 25–37 °C for target protein synthesis.

▲ **CRITICAL STEP** The length of this initial incubation period can impact glycosylation efficiencies and should be optimized for each glycoprotein of interest ('Experimental design'; Fig. 4d). Optimal initial incubation times typically range from 10 min to 4 h.

(iii) Add glycosylation activation solution, mix by pipetting and return samples to incubator for target protein glycosylation.

▲ **CRITICAL STEP** The length of the secondary incubation period can impact glycosylation efficiencies and should be optimized for each glycoprotein of interest ('Experimental design'; Fig. 4d). Optimal secondary incubation times typically range from 45 min to 16 h.

For *N*-glycoprotein and conjugate vaccine biosynthesis, add 1 μL *N*-glycosylation activation solution per 15 μL of cell-free reaction.

For *O*-glycoprotein biosynthesis, add 1 μL of *O*-glycosylation activation solution per 15 μL of cell-free reaction.

(iv) Centrifuge reactions for 10 min at 20,000*g*, 4 °C and transfer the supernatant to a clean microcentrifuge tube.

■ **PAUSE POINT** Soluble fractions can be stored at −80 °C indefinitely. However, proceed immediately to purification if purified conjugate vaccines or aglycosylated carrier proteins are needed for downstream applications.

Western blot analysis: day 7 ● Timing: 1 d

42 Run reaction soluble fraction from Step 41A(iii) or 41B(iv) on 4–12% Bis-Tris SDS–PAGE gels (Invitrogen or similar).

▲ **CRITICAL STEP** The volume of sample loaded should be optimized for each glycoprotein of interest. A few different volumes can be tested in parallel on the same western blot.

43 Transfer proteins to a polyvinylidene difluoride membrane using a semi-dry transfer cell or similar apparatus.

44 Wash membrane with PBS and incubate with shaking in Intercept Blocking Buffer or similar at room temperature for 1 h or overnight at 4 °C.

45 Remove blocking buffer and wash the membrane with PBST. Add enough PBST to cover the membrane and incubate at room temperature with shaking for 5 min. Repeat this step for a total of six washes.

46 Probe membrane with both an anti-6× His tag antibody and an antibody specific to the glycan of interest (if available) for at least 1 h at room temperature or overnight at 4 °C.

47 Remove antibody solution and wash the membrane with PBST. Add enough PBST to cover the membrane and incubate at room temperature with shaking for 5 min. Repeat this step for a total of six washes.

48 Probe with appropriate fluorescently labeled secondary antibodies for at least 1 h at room temperature.

49 Remove antibody solution and wash the membrane with PBST. Add enough PBST to cover the membrane and incubate at room temperature with shaking for 5 min. Repeat this step for a total of six washes.

50 Image using an Odyssey Fc imaging system or similar (for example results, see Fig. 5). CRM197 and tetanus toxin can also be detected with antibodies recognizing diphtheria or tetanus toxin, respectively. All antibodies and dilutions used are listed in 'Materials'.

? TROUBLESHOOTING

51 Cell-free synthesized glycoproteins can be further purified and characterized (Box 1).

Timing

Preparation of chassis strain, cell-free lysate, glycoprotein/aglycosylated protein synthesis reactions and western blot analysis can be completed in 7 d (Fig. 1).

Day 1 (Steps 1–10), preparation of chassis strain, fermentation media, buffers: 1 d

Day 2 (Step 11), inoculate chassis strain starter culture: 0.25 h

Day 3 (Steps 12–24), *E. coli* chassis strain growth and harvest: 1 d

Day 4 (Steps 25–36), cell-free lysate preparation: 4 h

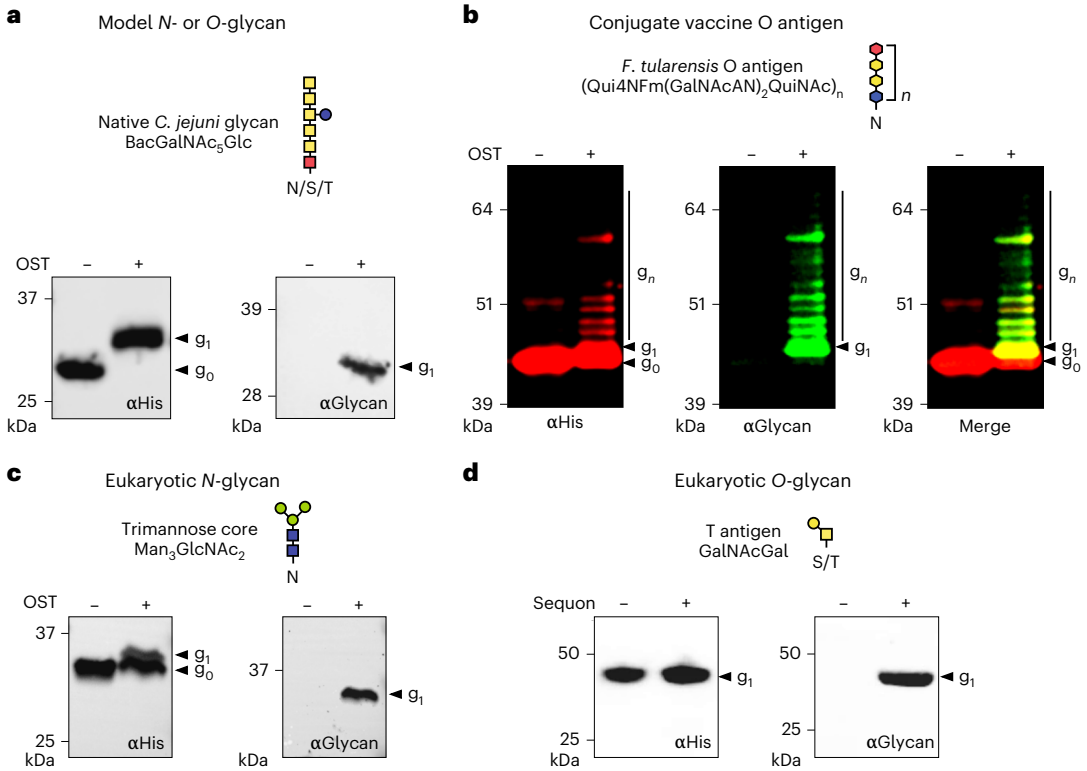

Fig. 5 | Cell-free systems synthesize diverse glycoproteins. a–d, Representative western blot characterization of cell-free synthesized proteins modified with the native *C. jejuni N*-glycan[23] (**a**), the *F. tularensis* Schu S4 O antigen[22] (**b**), the eukaryotic trimannose core *N*-glycan[23] (**c**) and the human core 1 *O*-glycan, also known as the T antigen[29] (**d**). Blots were probed with anti-hexa-histidine antibody to detect the target protein (αHis) and an anti-glycan affinity reagent to detect the glycan of interest (αGlycan). Bands that are cross-reactive with both αHis and αGlycan probes are glycosylated. Aglycosylated proteins produced in cell-free reactions lacking either the OST enzyme that performs protein glycosylation or the correct glycosylation sequon are shown as negative controls. In **a**, αGlycan: hr6 serum against *C. jejuni N*-glycan (note SBA lectin that binds to terminal or α-linked *N*-acetylgalactosamine residues can also be used to detect the *C. jejuni N*-glycan); in **b**, αGlycan: FB11 antibody against the *F. tularensis* O antigen; in **c**, αGlycan: conA lectin that binds internal and nonreducing terminal α-mannose residues in the trimannose core *N*-glycan; in **d**, αGlycan: PNA lectin that binds to the T antigen. Results are representative of at least $n = 3$ biological replicates. Bac, bacillosamine; GalNAc, *N*-acetylgalactosamine; Glc, glucose; Qui4NFm, 4,6-dideoxy-4-formamido-D-glucose; GalNAcAN, 2-acetamido-2-deoxy-D-galacturonamide; QuiNAc, 2-acetamido-2,6-dideoxy-D-glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine; Gal, galactose, g_0, unmodified protein; g_1, glycosylated protein bearing one glycan; g_n, glycosylated protein bearing multiple (2, 3, ..., *n*) glycans. Images adapted with permission from: **a** and **c**, ref. [23], Springer Nature Ltd.; **b**, ref. [22], AAAS; **d**, ref. [29], Springer Nature America, Inc.

Box 1 | Additional characterization of cell-free synthesized glycoproteins

We have described several additional assays that can be used for purification, quantification, glycan characterization and functional analysis of cell-free synthesized glycoproteins. Methods are described in our previous publications and noted below.

Affinity purification. Target glycoprotein constructs can be affinity purified via inclusion of a C-terminal 6× His tag[22].

ELISA. We have used ELISA assays to quantify and assess functional activity of cell-free synthesized glycoproteins, including antigen binding of single chain antibody fragments[23].

Mass spectrometry. Released glycan, digested glycopeptide or intact glycoproteins can be analyzed by mass spectrometry methods to determine glycan structure and glycan attachment site on cell-free synthesized glycoproteins[23,27].

Functional analyses. We have shown that cell-free synthesized conjugate vaccines elicit pathogen-specific IgG antibody responses[22,30,34] and confer protection to lethal pathogen challenge in mice[22]. We have also shown that recombinant human erythropoietin can induce proliferation of human TF-1 cells in vitro[23].

Day 5 (Steps 37–40), cell-free reaction assembly and lyophilization: 1–2 h, lyophilize overnight
Day 6 (Step 41), cell-free (glyco)protein synthesis: 1–18 h
Day 7 (Steps 42–51), western blot analysis: 1 d

Troubleshooting

Troubleshooting advice can be found in Table 5.

Table 5 | Troubleshooting table

Step	Problem	Possible reasons	Possible solutions
10	Low transformation efficiency when generating cell-free chassis strain	Incorrect antibiotics or concentrations used	Ensure the transformation plate has correct antibiotics and concentration(s) for selection of clones bearing desired glycosylation pathway plasmids
			Increase plasmid concentrations used for transformation or perform sequential transformations with one plasmid construct at a time
		Large plasmid size (especially glycan biosynthesis plasmid) reduces transformation efficiency	Use Super Optimal broth with Catabolite repression (SOC) instead of LB to recover cells to increase the number of viable colonies following transformation
50	Low cell-free protein synthesis yields	Incomplete cell lysis resulting in low protein concentration in cell-free lysates can cause low cell-free protein synthesis yields	Measure total protein concentration of cell-free lysates using Bradford/BCA assay and ensure that it is ~40 mg/mL
		Plasmid DNA impurities and/or RNAse contamination can reduce cell-free protein synthesis yields	Use midi- or maxi-prep kits to purify plasmid DNA used in cell-free reactions. Wear gloves when preparing and handling cell-free reactions and use RNAseZap to decontaminate surfaces or pipettes that are suspected to be contaminated with RNAses
		Nonoptimal Mg^{2+} concentration can reduce the efficiency of ribosomal subunit assembly and cell-free protein synthesis	Optimize Mg^{2+} concentration in cell-free reactions for maximized yield of cell-free synthesized protein (see Step 38, 'Experimental design'; Fig. 4c)
		Hydrolysis of phosphate groups from PEP can reduce the availability of the secondary energy source in cell-free reactions, reducing initial rates and overall yields of cell-free protein synthesis	Do not freeze PEP powder stock, prepare PEP solution as soon as possible after receipt of the reagent, and aliquot and store PEP solution at −20 °C to −80 °C
	Low cell-free glycosylation efficiency	Nonoptimal incubation timing resulting in low protein synthesis yields or glycosylation efficiency	Optimize timing of initial/secondary incubation times to optimize target protein synthesis and glycosylation efficiency ('Experimental design'; Fig. 4d). This will vary depending on lysate productivity/initial rate of protein synthesis
		Limited expression of lipid-linked oligosaccharide substrates for glycosylation or of the OST	Vary culture induction conditions for high-level expression of OST and/or glycan biosynthetic enzymes. Assess OST expression via western blot. Consider using S12 lysate preparation to ensure that a high concentration of membrane vesicles containing OSTs and lipid-linked oligosaccharides is present in the final lysate

Anticipated results

Following reaction optimization, S30 lysates routinely synthesize 5–15 μg/mL glycoprotein, while S12 lysates can yield up to 100 μg/mL glycoprotein. Example protein synthesis yields and western blot characterization from reaction optimization and synthesis of diverse glycoprotein targets are shown (Figs. 4 and 5).

Data availability

The data discussed in this manuscript were generated as part of our previously published work[22–24]. Source data are provided with this paper.

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Acknowledgements

J.C.S. acknowledges support from NIH/NCI F32 Postdoctoral Fellowship 1F32CA250324-01 and American Cancer Society Postdoctoral Fellowship PF-20-143-01-LIB. T.J. acknowledges support from the European Molecular Biology Organization Postdoctoral Fellowship 336-2021. K.F.W. acknowledges support from the National Defense Science and Engineering (NDSEG) Fellowship Program (ND-CEN-013-096). M.C.J. acknowledges support from the David and Lucile Packard Foundation, the Camille Dreyfus Teacher-Scholar Program, the Defense Threat Reduction Agency Grants HDTRA1-15-10052, HDTRA-12-11-0038 and HDTRA-12-01-0004, the Army Research Office Grants W911NF-20-1-0195, W911NF-18-1-0200 and W911NF-16-1-0372, the Army Contracting Command Contract W52P1J-21-9-3023 and DARPA Grant W911NF-23-2-0039.

Author contributions

J.C.S., T.J., K.F.W. and J.M.H. wrote and edited the manuscript. J.C.S., M.P.D. and M.C.J. conceptualized the manuscript. M.P.D. and M.C.J. directed the research and edited the manuscript.

Competing interests

M.C.J. is a cofounder of SwiftScale Biologics, Stemloop, Inc., Design Pharmaceuticals, and Pearl Bio. M.P.D. has interests in Glycobia Inc. and Versatope Inc. M.P.D. and M.C.J. have an interest in SwiftScale Biologics. M.C.J.'s and M.P.D.'s interests are reviewed and managed by Northwestern University and Cornell University, respectively, in accordance with their conflict of interest policies.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-022-00799-z.

Correspondence and requests for materials should be addressed to Jessica C. Stark, Matthew P. DeLisa, Michael C. Jewett.

Peer review information *Nature Protocols* thanks Nigel Forest Reuel and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Received: 11 March 2021; Accepted: 22 November 2022;
Published online: 16 June 2023

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