Improving cell-free glycoprotein synthesis by characterizing and enriching native membrane vesicles

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Cell-free gene expression (CFE) systems from crude cellular extracts have attracted much attention for biomanufacturing and synthetic biology. However, activating membrane-dependent functionality of cell-derived vesicles in bacterial CFE systems has been limited. Here, we address this limitation by characterizing native membrane vesicles in Escherichia coli-based CFE extracts and describing methods to enrich vesicles with heterologous, membrane-bound machinery. As a model, we focus on bacterial glycoengineering. We first use multiple, orthogonal techniques to characterize vesicles and show how extract processing methods can be used to increase concentrations of membrane vesicles in CFE systems. Then, we show that extracts enriched in vesicle number also display enhanced concentrations of heterologous membrane protein cargo. Finally, we apply our methods to enrich membrane-bound oligosaccharyltransferases and lipid-linked oligosaccharides for improving cell-free N-linked and O-linked glycoprotein synthesis. We anticipate that these methods will facilitate on-demand glycoprotein production and enable new CFE systems with membrane-associated activities.

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Cell-free gene expression (CFE) systems activate transcription and translation using crude cellular extracts instead of living, intact cells. In recent years, these systems have matured from widely used tools in molecular biology to platforms for biomanufacturing and synthetic biology. Among CFE systems, *Escherichia coli*-based methods have been used the most. A body of work dedicated to optimization of extract preparation and reaction conditions has simplified, expedited, and improved the cost and performance of *E. coli* CFE systems. Optimized *E. coli*-based CFE reactions: (i) quickly synthesize grams of protein per liter in batch reactions; (ii) are scalable from the nL to 100 L scale; and (iii) can be freeze-dried for months of shelf stability. The ability to readily store, distribute, and activate freeze-dried cell-free systems by simply adding water has opened new opportunities for point-of-use biomanufacturing and therapeutic production. Additionally, the existing CFGpS system based on *E. coli* S30 extracts has been used to produce model glycoproteins, human glycoproteins, and protective conjugate vaccines. Unfortunately, the existing CFGpS system based on S30 extracts (i.e., cell extracts that result from a 30,000 g clarification spin) is limited by glycosylation efficiency, only producing ~10–20 μg/mL of glycoprotein in batch. Characterizing and enriching cell-derived vesicles comprising membrane-bound glycosylation components offers one strategy to address this limitation, and perhaps make possible a variety of applications involving membrane-bound biology.

Here, we set out to develop methods that enhance membrane-dependent functionality of cell-derived vesicles in bacterial CFE systems, with a focus on bacterial glycoengineering. First, we characterize size distributions and concentrations of native membrane vesicles in extracts, providing a benchmark for analysis and engineering of CFE systems. To do so, we apply canonical strategies (e.g., TEM), and also apply simple and expedited characterization workflows that rely on techniques such as light scattering to directly analyze vesicles in extracts without the need for lengthy protocols. Second, we investigate the impacts of upstream extract processing steps on vesicle profiles, revealing simple handles to modulate vesicle concentration in extracts. Third, we use cell-derived membrane vesicles to enrich a variety of heterologous, membrane-bound proteins and substrates in extracts without the use of synthetically derived membranes. Finally, we apply our findings to improve glycoprotein yields in our existing asparagine-linked (N-linked) CFGpS system and a new membrane-dependent CFGpS system based on serine/threonine-linked (O-linked) glycosylation. By applying our optimized methods to increase concentrations of vesicle-bound glycosylation machinery, we shorten the time associated with extract preparation, increase glycosylation efficiencies, and enhance glycoprotein titers by up to ~170%. Importantly, we go on to show that improvements in glycoprotein titers are generalizable to multiple glycoproteins without the need to re-optimize conditions.

**Results**

**Overview of results.** In this study, we aimed to characterize and engineer membrane vesicles (which form upon fragmentation of cell membranes during cell lysis) in *E. coli* CFE extracts (Fig. 1). Then, we used this knowledge to control enrichment of membrane-bound components for enhancing defined function, including improving glycoprotein synthesis (Fig. 1). To achieve these goals, we: (i) used nanocharacterization techniques to determine the sizes and quantities of membrane vesicles in *E. coli* extracts; (ii) determined how extract processing can control the enrichment of vesicles in extracts; (iii) enriched several heterologous, membrane-bound components in extracts via vesicles; and (iv) demonstrated that increasing enrichment of membrane-bound components significantly improves CFGpS systems for N- and O-linked glycosylation. This work sets the stage for portable biomanufacturing platforms that can broaden access to medicines by making them when and where they are needed.

**Characterization of membrane vesicles in CFE extracts.** Initially, we used several nanocharacterization techniques to analyze the size of vesicles and to visualize these particles in CFE extracts prepared using homogenization and 30,000 g clarification (i.e., S30
**Fig. 1** A platform for engineering cell-free gene expression (CFE) systems with cell-derived membrane-dependent functions. Membrane-bound cargo expressed in living E. coli is carried through into CFE extracts via membrane vesicles. The extract preparation method used to prepare CFE extracts impacts sizes and concentrations of vesicles, and their associated cargo. Here, we develop a facile nanocharacterization pipeline to better understand and characterize the impacts of extract preparation methods on vesicle profiles and their associated cargo. We then apply our findings to improve cell-free glycoprotein synthesis (CFgpS), which is a promising platform for on-demand vaccine development. By increasing concentrations of vesicles and membrane-bound glycosylation machinery, oligosaccharyltransferases (OSTs) and lipid-linked oligosaccharides (LLOs), we overcome limitations in CFgpS and increase glycoprotein titers.

**Fig. 2** Characterization of membrane vesicles in crude CFE extracts. a DLS analysis of crude extracts (blue) and SEC purified vesicles (green). Crude extract data are presented as mean intensity values for a given size ± standard deviation (SD) of n = 9 measurements (3 biologically independent extracts, each measured 3 times). Purified vesicle data are presented as mean intensity values for a given size ± SD of n = 3 measurements (one purified fraction measured 3 times). b NTA of purified vesicles collected from SEC. Mean and mode diameters observed in the particle size distribution are listed in the inset. Data are presented as mean ± standard error of the mean (SEM) of n = 5, 1 min NTA measurements of purified vesicles. c Illustration of particles detected in crude CFE extracts. d Cryo-EM micrographs of crude extracts. Black arrows indicate vesicles with apparent unilamellar morphology. White arrows indicate nested or multilamellar morphologies. Cropped images indicate representative vesicles. Scale bars are 100 nm. Uncropped images are available in Supplementary Fig. 3 and numbered with the corresponding cropped vesicles. e Cryo-EM micrographs of SEC purified vesicles. Cropped images indicate representative purified vesicle particles. Scale bars are 100 nm. Uncropped images are available in Supplementary Fig. 3 and numbered with the corresponding cropped vesicles. Micrographs are representative of three independent experiments. Source data for all panels are provided as a Source Data file.
extracts)\(^{34}\). Dynamic light scattering (DLS) analysis of crude extract revealed two major peaks: one narrower peak with an intensity maximum at ~20 nm, and a broader peak at ~100–200 nm (Fig. 2a). The 20 nm peak likely represents small cell-derived particles. E. coli ribosomes, which are present at ~1 μM in typical CFE reactions and enable the production of protein in our CFE reactions (Supplementary Fig. 1), are ~20 nm in size and likely contribute considerably to the signal measured\(^{33,34}\). We hypothesized that particles measured in the ~100–200 nm peak were vesicles. To directly analyze membrane vesicles without ribosomes and other cellular particles, we identified and purified membranous particles via size-exclusion chromatography (SEC)\(^{35–37}\) (Supplementary Fig. 2A). DLS analysis of purified membrane vesicles revealed an intensity particle size distribution that directly overlapped with the proposed vesicle peak from our DLS traces of crude extracts (Fig. 2a). Nanoparticle tracking analysis (NTA), an orthogonal method for sizing and quantitating nanoparticles in solution, showed an average purified vesicle diameter of 118.5 ± 0.7 nm, corroborating the approximate size range of vesicles measured with DLS (Fig. 2b). The zeta potential of purified vesicles was −14.5 ± 1.0 mV, indicating a negative particle surface charge consistent with phospholipid vesicles (Supplementary Fig. 2B). An illustration of particles detected in extract is shown in Fig. 2c.

Cryo-electron microscopy (cryo-EM) of extracts showed small (≤20 nm) particles and other larger, circular particles consistent with vesicle morphology (Fig. 2d). Cryo-EM micrographs of extracts revealed vesicles between ~40 nm and ~150 nm in size, and we observed intact vesicle morphologies both pre- and post-SEC purification (Fig. 2d, e). Uncropped and annotated cryo-EM micrographs are provided (Supplementary Fig. 3). Comparisons between measurements reveal that DLS, a bulk, in-solution measurement, overestimates vesicle diameter. DLS, however, is a useful tool for quickly characterizing crude extract particle profiles because it can detect particles <50 nm (including ribosomes) that are smaller than vesicles and are below the size limit of detection of NTA. Together, these results show particle profiles of crude extracts and indicate that vesicles are polydispersive, are on the order of tens to hundreds of nm across, and are relatively low in concentration compared with ribosomes and other small complexes.

Extract processing impacts vesicle size distributions and concentrations. To understand how to control membrane vesicles in extracts, we next sought to study how protocols to process extracts impacted vesicle properties. Specifically, we studied cell lysis and extract centrifugation because cell membranes are ruptured during lysis, and centrifugation dictates particle separation. We lysed cells using standard sonication (constant input energy per volume of cell suspension) or homogenization protocols (~20,000 psig)\(^{11,34}\), then subjected lysates to a traditional 30,000g centrifugation protocol (termed ‘S30’ extracts), or a lower g-force protocol where the maximum centrifugation speed was 12,000g (termed ‘S12’ extracts) (Fig. 3a)\(^{11,12}\). These combinations of lysis and centrifugation protocols were selected because they have previously been used to obtain high-yielding E. coli CFE extracts\(^7\). Indeed, all the conditions tested yielded extracts that were active for protein synthesis in standard CFE reaction conditions (Supplementary Fig. 4A). The combination of a standard homogenization and S30 prep represents our base case because extracts used in our previously described one-pot CFGPS platform were prepared with these conditions, as well as the extracts used in our initial characterization here (Fig. 2). Before this work, S12 extracts had not previously been used for making glyco-competent CFE extracts.

Of the conditions tested, the centrifugation protocol had the most impact on vesicle concentrations. We observed higher numbers of vesicles in S12 extracts for both lysis methods, with the reduced centrifugation speed likely being the reason for increased particle concentrations. Specifically, we observed 1.2- and 2.0-fold enrichments of vesicles in sonicated and homogenized S12 extracts, respectively (Fig. 3b). Homogenized S12 extracts contained the highest concentration of vesicles with 6.5 ± 0.3 × 10\(^{12}\) particles/mL (as compared to 3.4 ± 0.1 × 10\(^{12}\) particles in the base case), making it the most promising condition for enriching vesicles.

While centrifugation impacted vesicle concentration, lysis method impacted vesicle size. Sonicated extracts contained smaller vesicles with narrower size distributions than homogenized extracts, regardless of centrifugation protocol. Our observations that lysis method impacts vesicle size is consistent with studies showing that varying experimental parameters to disperse phospholipids (or amphiphiles in general) impacts vesicle sizes\(^7\). Particle size distributions of sonicated extracts reached a single maxima at ~110 nm, with average particle diameters of ~130 nm; homogenized extracts had higher average particle diameters of ~160 nm, displaying distinct peaks at ~120 nm, and considerable shoulder peaks at ~150 nm (Fig. 3c, d and Supplementary Fig. 5A). The particle size distributions observed in homogenized extracts may indicate the presence of multiple, discrete, vesicle populations (Fig. 3c, d). DLS measurements confirmed the observation that sonicated extracts contained relatively smaller, less polydisperse vesicles than homogenized extracts (Supplementary Fig. 5B, C). Notably, direct vesicle analysis in extracts enabled us to gauge the impacts of extract processing in ways that have not been previously accessible and provides benchmarks for intact vesicle concentrations in extracts.

Heterologous membrane-bound cargo can be controllably enriched via membrane vesicles. With a better understanding of the characteristics and concentrations of native vesicles, we sought to enrich extracts with vesicles containing heterologous cargo derived from the periplasmic membrane of E. coli. Since S12 extracts contain higher concentrations of vesicles than S30 extracts, we hypothesized that S12 extracts would also contain higher concentrations of associated heterologous cargo. The highest dynamic range of vesicle concentration between S12 and S30 preparations was observed with homogenization, so we proceeded with homogenization for enrichment experiments (Fig. 3b). We overexpressed six membrane-bound proteins of various sizes, transmembrane topologies, and taxonomical origins to test for enrichment (Supplementary Table 1). The proteins selected for enrichment encompass classes of proteins that could enable expanded functionalities in CFE, including glycosylation enzymes (PgIβ, PgIO, STT3) and signal transduction/sensing proteins (NarX, PR, CB1). We expressed each membrane protein in vivo with a C-terminal FLAG tag, prepared S30 and S12 extracts, then analyzed concentrations of the overexpressed membrane protein using quantitative western blotting. We observed approximately 2-fold membrane protein enrichment in S12 over S30 (S12/S30) extracts for all proteins other than PR, for which we observed ~4-fold enrichment (Fig. 4a, b). As a control, when sfGFP with no transmembrane helices was expressed in vivo, we did not observe significant S12/S30 enrichment (Fig. 4c). Full blots for Fig. 4a–c are shown in Supplementary Fig. 6. Notably, enrichment values obtained via blotting correspond closely with the 2-fold vesicle enrichment observed via NTA in homogenized S12 and S30 extracts with no overexpression (Fig. 3b). All extracts with pre-enriched
membrane proteins displayed protein synthesis activity (Supplementary Fig. 4B).

With an eye towards bacterial glycoengineering applications, we next confirmed that PglB and PglO, key enzymes for glycosylation, were associated with membrane vesicles, as opposed to free in solution (Fig. 4d). Extracts with pre-enriched PglB or PglO were probed with a green fluorescent α-FLAG antibody, then analyzed via SEC. Fluorescence chromatograms are shown in Fig. 4d, with the characteristic vesicle elution fraction highlighted in gray (Supplementary Fig. 2A). The characteristic vesicle elution peak corresponded with green fluorescence for extracts containing PglB or PglO and no corresponding peak was observed in an extract with no overexpressed membrane protein (Fig. 4d). Our results show that heterologous cargo that is embedded in the periplasmic membrane of E. coli cells can be pre-enriched in extract and tuned via vesicles.

Increasing vesicle concentrations improves cell-free glycoprotein synthesis (CFGpS) for N- and O-linked glycosylation systems. We next set out to exploit our ability to enrich vesicles harboring heterologous cargo in an application. We focused on protein glycosylation, because glycosylation plays critical roles in cellular function, human health, and biotechnology. As a model, we sought to increase glycoprotein yields for a defined time, termed ‘CFPS time’. At the CFPS time, reactions were spiked with MnCl₂, quenching CFPS and initiating glycosylation by providing the OST with its Mn²⁺ cofactor. CFGpS reactions charged with S30 or S12 extracts were run for CFPS times of 2, 10, 20, 30, and 60 min using a His-tagged sfGFPDQNAT acceptor protein, where DQNAT is a permissible sequence of all acceptor proteins used are presented in Supplementary Table 2. Endpoint glycoprotein yields were quantified using total acceptor protein yield, determined by sFGFP fluorescence and ¹⁴C incorporation, and % glycosylation, determined by western blotting (Fig. 5a and Supplementary Fig. 7). Fluorescence staining and SEC analysis confirmed the presence and association of LLO and PglB with the vesicles (Supplementary Fig. 8A).

To assess the impact of enriched vesicles on CFGpS, we carried out reactions in two phases (Fig. 5a, inset). First, cell-free protein synthesis (CFPS) of the acceptor protein was run for a defined time, termed ‘CFPS time’. At the CFPS time, reactions were spiked with MnCl₂, quenching CFPS and initiating glycosylation by providing the OST with its Mn²⁺ cofactor. CFGpS reactions charged with S30 or S12 extracts were run for CFPS times of 2, 10, 20, 30, and 60 min using a His-tagged sfGFPDQNAT acceptor protein, where DQNAT is a permissible sequence of all acceptor proteins used are presented in Supplementary Table 2. Endpoint glycoprotein yields were quantified using total acceptor protein yield, determined by sFGFP fluorescence and ¹⁴C incorporation, and % glycosylation, determined by western blotting (Fig. 5a and Supplementary Fig. 7). Fluorescence staining and SEC analysis confirmed the presence and association of LLO and PglB with the vesicles (Supplementary Fig. 8A).

Campylobacter jejuni, which consists of the membrane-bound oligosaccharyltransferase (OST) PglB that catalyzes glycosylation, and a lipid-linked oligosaccharide (LLO) donor of the form: GalNAc-a1,4-GalNAc-a1,4-(Glcb1,3)-GalNAc-a1,4-GalNAc-a1,3-Bac (where Bac is 2,4-diacetamido-2,4,6-trideoxyglucopyranose) from an undecaprenyl-pyrophosphate-linked donor⁸⁰. NTA and western blot analysis of CFGpS extracts revealed 2.5-fold S12/S30 enrichment of vesicles and a corresponding 2-fold S12/S30 enrichment of PglB (Supplementary Fig. 7). Fluorescence staining and SEC analysis confirmed the presence and association of LLO and PglB with the vesicles (Supplementary Fig. 8A).

Fig. 3 Extract processing impacts vesicle size distributions and concentrations. a Illustration of extract processing conditions. Extracts were prepared in triplicate for each condition shown. b Nanoparticle Tracking Analysis (NTA) concentration analysis of vesicles in sonicated (blue) and homogenized (green) extracts. Asterisk indicates base case conditions for extract preparation. Data for (b) are presented as mean ± SD of n = 15 replicates (3 biologically independent extracts; each examined over 5, 1 min NTA measurements). c NTA particle size distribution of vesicles in sonicated (blue) and homogenized (green) S30 extracts. d NTA particle size distribution of sonicated (blue) and homogenized (green) S12 extracts. Data for (c, d) are presented as mean ± SEM of n = 15 replicates (3 biologically independent extracts; each examined over 5, 1 min NTA measurements). Source data for all panels are provided as a Source Data file.
In cell-free systems, we next ported an α-linked glycosylation system to the CFGpS platform\textsuperscript{72,81,82}. We selected the O-OST PglO from \textit{Neisseria gonorrhoeae} that accepts the \textit{C. jejuni} heptasaccharide LLO as a donor, but differs from PglB in acceptor sequence preferences\textsuperscript{83}. For PglO, we used an sfGFP-fusion acceptor protein containing a recently determined 8 amino acid (WPAAASAP, with S being the glycosylated residue) minimum optimal \textit{O}-linked recognition site (termed ‘MOOR’)\textsuperscript{83}. We confirmed residue-specific \textit{O}-linked glycosylation and enrichment of PglO and LLO in vesicles (Supplementary Figs. 10 and 8B). As additional proof of site-specific glycosylation, we performed liquid chromatography mass spectrometry (LC-MS/MS) analysis of the glycoproteins obtained via CFGpS with PglO and PglB and observed the presence of the 1406 Da \textit{C. jejuni} heptasaccharide LLO on the expected tryptic peptides (Supplementary Fig. 11A, B)\textsuperscript{80}. As in PglB-mediated CFGpS, we observed increased endpoint glycoprotein yield and \% glycosylation in reactions charged with S12 extracts. Specifically, reactions with CFGpS times of 20 min resulted in a 69\% increase in glycoprotein yield and an increase from 27\% to 40\% glycosylation in reactions with S12 extracts compared to those containing S30 extracts (Fig. 5d and Supplementary Fig. 12). Corresponding blots are shown in Fig. 5e and Supplementary Fig. 12A, B. Collectively, these results indicate that improvements to glycosylation in S12 extracts translate from the \textit{N}-linked glycosylation system to the O-linked glycosylation system.

To determine whether enhanced glycoprotein production in S12 extract-based CFGpS reactions was transferrable to non-model acceptor proteins, we tested three additional proteins. This included the \textit{C. jejuni} AcrA, a native bacterial glycoprotein with two internal glycosylation sites\textsuperscript{50,84}, as well as two possible carrier proteins, and a cytosolic sfGFP control with no transmembrane helices. On each western blot, left lanes are S12 extracts and right lanes are S30 extracts. Black arrows indicate the membrane protein of interest. Molecular weight (kDa) from protein ladder standards are indicated to the left of each blot. Protein names and enrichment ratios of bands (S12/S30) are shown directly below each blot. All blots are representative of \(n=3\) biologically independent extracts. Cartoons depict the transmembrane topology for each protein. See Supplementary Table 1 for taxonomical origin, transmembrane topology, functions(s), theoretical size, and UniProt ID. Full western blot images for panels (a-c) are available in Supplementary Fig. 6 and Source Data file. d) Fluorescence chromatograms of SEC analysis of extracts probed with a fluorescent α-FLAG antibody. Strains used to prepare extracts were enriched with no membrane protein (gray trace), PglB (dark purple trace), or PglO (light purple trace). Characteristic vesicle elution fraction from 3 independent experiments is highlighted in gray. Source data for all panels are provided as a Source Data file.
MBP, and PD, respectively, when comparing S12- to S30-based reactions. Expression improvements were determined by 14C-leucine incorporation (Fig. 5f and Supplementary Fig. 13A). Our results highlight that the improvements in glycoprotein yield observed in extracts with higher concentrations of vesicles hold for diverse proteins without the need for re-optimization.

**Discussion**

In this work, we set out to benchmark, understand, and quality-control protein-enriched vesicles in bacterial CFE extracts for expanding and enhancing functionality. We showed that upstream extract processing can be used to tune concentrations of vesicles and associated cargo from the periplasm. Then, we applied this knowledge to improve CFGpS, with a specific application focus of glycoprotein synthesis. Our results have several key features.

First, the light scattering tools used here allowed us to quickly quantify intact vesicle numbers and sizes in CFE extracts. This is important because this knowledge informed design rules for enhancing vesicle concentrations and functionality from their associated protein cargo in cell-free systems. Notably, the effective vesicle surface area calculated from NTA measurements (~0.3 m² membrane/mL extract) is consistent with values calculated from phospholipid concentrations in similar extracts. Routine vesicle characterization could provide key information necessary for cost-effective energy metabolism from oxidative phosphorylation and ATP regeneration. Since vesicles are key for activating cost-effective energy metabolism from oxidative phosphorylation in CFE, routine vesicle characterization could...
become a vital quality-control check, leading to improved reproducibility in and between labs. Our results also offer insight into why, despite the presence of vesicles in the E. coli CFE system, CFE-derived membrane proteins cannot be synthesized via insertion into native vesicles without additional vesicle supplementation. With ~6 nM of intact vesicles in CFE reactions (where intact vesicle concentration was calculated from NTA measurements), the concentration of vesicles is orders of magnitude lower than typical protein titers produced in our CFE extracts (~30 µM of reporter protein or higher).

Third, our work opens the door to engineering cell-free systems that rely on enriched membrane-bound components. We show that membrane-bound proteins and LLOs expressed in vivo in the periplasm can be enriched in vesicles, indicating that a population of vesicles is derived from the inner periplasmic membrane. Importantly, our workflow easily interfaces with numerous methods that could be used to alter vesicles and their membrane-bound cargo. For example, using other centrifugation speeds besides 12,000 g could result in changes to vesicle concentration. In addition, additives could be supplemented to cell-free systems to tune biophysical features of membrane properties (e.g., composition, size, fluidity, curvature). Furthermore, unlike the previously used S30 extract procedure, the optimized S12 extract strategy developed here does not require a high-speed centrifuge and is less time-intensive. This simplifies the CFEgP-S platform, enabling the process from inoculation of cell culture to testing CFEgP-S reactions to be completed in a single workday.

And, while we focus entirely on E. coli-based systems here, the reported characterization methods could, in principle, be extended to further optimize insect and CHO-based CFE systems that rely on ER-derived microsomes to perform glycosylation, embed nascent membrane proteins, and perform other membrane-dependent functions.

Towards applications in biomanufacturing, a key feature of the E. coli-based CFEgP-S system is expressing synthetic glycosylation pathways encoding diverse O-antigens from pathogenic bacteria. This feature points toward immediate utility of our CFEgP-S system in the on-demand bioproduction of conjugate vaccines. Here, we show that S12 extracts enable higher glycoprotein titers of two glycoconjugate vaccine carrier proteins modified with a model C. jejuni LLO, indicating that vaccine production may be simplified and more efficient using the optimized methods reported here. Additionally, we have recently shown that our optimized S12 conditions can be used to recapitate efficient, humanized O-linked glycosylation in glycoengineered E. coli extracts. While applications in O-linked glycosylation and conjugate vaccines are imminent, the recapitulation of efficient eukaryotic-type N-linked glycosylation (i.e., glycoproteins with a Man3GlcNAc2 core glycan) for production of therapeutics still remains on the horizon in E. coli-based systems.

Future studies to elucidate translocation and co-translational glycosylation in vesicles will be important. These studies could be especially useful for producing complex, native glycoproteins for which protein glycosylation and folding are co-translational. While it has been shown that glycosylation with PglB can proceed on pre-folded proteins in vitro (using purified, reconstituted components and without the need for translocation or intact membranes), obtaining a more robust understanding of the topology of glycosylation in membrane vesicles is an important future effort for therapeutics production.

Looking forward, we anticipate that our work will accelerate efforts to manufacture proteins that require membrane-dependent modifications, such as glycoproteins. For example, the approach described enables N-linked glycoprotein synthesis yields of >100 µg/mL, which increases accessibility for on-demand vaccine production in resource-limited settings. In sum, our results pave the way for efficient, accessible CFE systems that require membrane-bound activities for expanding system functionality and enabling a variety of synthetic biology applications.

**Methods**

**Extraction preparation.** The chassis strain used for all extracts was CLM2434. Source strains were grown in 1 L of 2xYTPG media at 37 °C with agitation. Cells were grown overnight in OD 3, then harvested by centrifugation (5000g, 4 °C, 15 min). For overexpression of proteins in vivo, CLM24 source strains were grown at 37 °C in 2xYTPG with the appropriate antibiotic(s), listed in Supplementary Table 3. Cells were induced with 0.02% (wt/vol%) L-arabinose at OD 0.6–0.8, shifted to 30 °C, and harvested at OD 3. All subsequent steps were carried out at 4 °C and on ice unless otherwise stated. Pelleted cells were washed 3 times in S30 buffer (10 mM Tris acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate). After the last wash, cells were pelleted at 7000 g for 10 min, flash-frozen and stored at −80 °C. After growth and harvest, cells were thawed and resuspended to homogeneity in 1 mL of S30 buffer per gram of wet cell mass. For homogenization, cells were disrupted using an Avestin Emulsiflex-B15 high-pressure homogenizer at 20,000–25,000 psi with a single pass (Avestin, Inc. Ottawa, ON, Canada). For sonication, input energy was calculated using an empirical correlation.

Cells were sonicated on ice using a Q125 Sonicator (Quontron, New York, CT) with a 3.175 mm diameter probe at a frequency of 20 kHz and 50% of amplitude. Energy was delivered by the Q125 in pulses of 59 s of on energy was delivered. Cells were lysed and clarified in triplicate. For S30 preparation, lysed cells were centrifuged twice at 30,000g for 30 min; supernatants were transferred to a fresh tube for each spin. Supernatants were incubated with 250 rpm shaking at 37 °C for 60 min for runoff reactions. Following runoff, lysates were centrifuged at 30,000g for 15 min. Supernatants were collected, aliquoted, flash-frozen, and stored at −80 °C for further use. For S12 preparation, lysed cells were centrifuged once at 12,000g for 10 min; supernatants were collected and subjected to runoff reactions as described above. Following runoff, lysates were centrifuged at 10,000g for 10 min at 4 °C. Supernatants were collected, aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C.

**Dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) measurements.** DLS measurements were performed on a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) with a measurement angle of 173° in disposable cuvettes (Malvern Instruments Ltd., UK ZEN0040). All measurements were collected in triplicate for 13 scans per measurement. Refractive index and viscosity were obtained from the instrument’s parameter library. The instrument’s ‘General Purpose’ setting was used to calculate intensity and number particle size distributions. For DLS of crude extracts, extracts were diluted 1:10 with 0.1 µm filtered PBS before analysis. For purified vesicle samples, elutions were analyzed directly without dilution. NTA measurements were performed on a NanoSight NS300 using a 642 nm red laser (Malvern Instruments Ltd., UK). Samples were diluted to manufacturer-recommended particle concentrations in sterile PBS until a linear trend between dilution factor and concentration measured was found. Samples were flowed into the cell, and the instrument was focused according to manufacturer recommendations. Measurements were collected at room temperature, using a 1 mL syringe and a syringe pump infusion rate of 30 (arbitrary units). Data for each sample was collected in 5 separate 1 min videos, under continuous flow conditions. Mean particle diameters and particle concentrations were obtained from aggregate NanoSight experiment reports of each run, then averaged across triplicates and corrected for dilution factor.

**Transmission electron microscopy (TEM).** For cryo-TEM measurement, 200 mesh Cu grids with a lacey carbon membrane (EMS Cat. # LC200-CU) were placed in a Pelco easiGlowlow discharger (Ted Pella Inc., Redding, CA, USA) and an atmosphere plasma was introduced on the surface of the grids for 30 s with a current of 15 mA at a pressure of 0.24 mbar. This treatment creates a negative charge on the carbon membrane, allowing for aqueous liquid samples to spread evenly over the grid. Then, 4 µL of sample was pipetted onto the grid and blotted for 5 s with a blot of offset of ~0.5 mm, followed by immediate plunging into liquid ethane within a FEI Vitrobot Mark IV plunge freezing instrument (Thermo Fisher Scientific, Waltham, MA, USA). Grids were then transferred to liquid nitrogen for storage. The plunge-frozen grids were kept vortexted at ~172 °C in a Gatan Cryo Transfer Holder model 626.6 (Gatan Inc., Pleasanton, CA, USA) while viewing in a JEOL JEM1230 LaB6 emission TEM (JEOL USA, Inc., Peabody, MA) at 120 keV. Image data were collected by a Gatan Orius SC1000 CCD camera Model 831 (Gatan Inc., Pleasanton, CA, USA). Image analysis was done using ImageJ.

**Plasmid construction.** All plasmids used in this study are listed in Supplementary Table 3. Supplementary Table 4 includes primers used to clone plasmids containing ORF 1 and ORF 3. For this study, pSP-NPG0 was amplified from an existing plasmid using primers 1 and 2 (Supplementary Table 4). gBlocks for PR, HsCB1, LmSTT3D, and sGFP were ordered with homology to the pSP backbone. pSP plasmids were then
Western blotting and densitometry analyses. SDS-PAGE was run using NuPAGE 4–12% Bis-Tris protein gels with MOPS-SDS buffer (Thermo Fisher Scientific, Waltham, MA, USA). After electrophoresis, proteins were transferred from gels to Immobilon-P polyvinylidene difluoride 0.45 µm membranes (Millipore, USA) according to manufacturer’s protocol. Membranes were blocked in either Odyssey or Intercept blocking buffer (LI-COR, USA) and washed with 1x PBST. A-FLAG blots of membrane proteins were probed using a-FLAG antibody (Abcam 2493) at a 1:5000 dilution in blocking buffer with 0.2% Tween20 (Sigma-Aldrich) as the primary. A-His blots were probed with e-His-antibody (Abcam, ab1187) at a 1:7500 dilution in blocking buffer with 0.2% Tween20 (Sigma-Aldrich) as the primary. Autoradiogram gel images were acquired using Typhoon FLA 7000 Control Software Version 1.2 Build 1.2.1.93. Autoradiogram analysis was performed using Image Studio Lite software to measure band intensity. Fluorescence background was subtracted from blots before determining band intensities. For determining membrane protein enrichment (S2/S30), band intensities of membrane proteins for three independent S12 extract replicates and three independent S20 extract replicates were calculated for each protein. The rounded averages of triplicate ratios (S12/S30) and associated error are reported as enrichment in Fig. 4. For determining glycoprotein yields from CFGpS reactions, band intensities for glycosylated and aglycosylated bands were obtained from independent, triplicate reactions. The fraction of glycosylated protein for each replicate was calculated via band intensities. To determine glycoprotein yields, the fraction glycosylated was multiplied by total protein yield for each replicate as calculated from sGFP fluorescence converted to protein concentration (described below). Yields were plotted using Prism v.9.0.0 (GraphPad).

Lipid dye staining and fluorescence immunoassay of vesicles. All reagents used for immunostaining and SEC were sterile filtered with a 0.1 µm filter (Millipore, USA). To determine vesicle elution fractions, extract was probed with FM 4-64 lipid dye (Life Technologies), a lipophilic styrene dye that has low fluorescence in aqueous solution and becomes brightly fluorescent upon incorporation into membranes. FM-464 dye preferentially stains the inner membrane of E. coli, but has been used to dye the outer membrane as well.29,34 FM-4-64 lipid dye was prepared in stock solutions at 10 mg/mL in 100% DMSO, then diluted 1000-fold in nuclease-free water before use. Then, 80 µL of extract, 10 µL 10x PBS, and 10 µL of FM-4-64 lipid dye were mixed in solution for 10 min at 37 °C prior to SEC. To verify the presence of glycosylation components in vesicles, we probed for the LLO with a red fluorescent soybean agglutinin (SBA) lectin, a protein complex which specifically binds to the C. jejuni LLO,30 and for the OST with an orthogonal green fluorescent a-FLAG antibody as described above. For a-FLAG immunostaining and SBA staining, 90 µL extract and 10 µL of 10xPBS were mixed with 2 µL of a-FLAG-DyLight 488 (MA191878D88, Invitrogen, USA) and 4 µL of SBA-AlexaFluor 594 (S2462, Invitrogen, USA). Antibody and SBA were incubated with extract in the dark with agitation overnight at 4 °C prior to SEC.

Size-exclusion chromatography (SEC) of vesicles. Here, 100 µL of extract mixture (stained with lipid dye or antibody) was flowed over a SEC column with PBS. Elution fractions were collected into a clear polystyrene 96-well plate (Costar 3370, Corning Inc., USA) at a rate of 0.4 mL/min using a Gilson FC 204 Fraction Collector (Gilson, Inc., USA). Poly-Prep chromatography columns (Bio-Rad, USA) were pre-washed with 10 volume bed diameter of 10x PBS (10x PBS, Invitrogen, USA) and washed with sterile PBS 3 times before use. Elution fluorescence was measured using a Synergy H1 microplate reader (BioTek, USA). Excitation and emission wavelengths for SBA-AlexaFluor 594 were 590 and 617 nm, respectively. Excitation and emission wavelengths for a-FLAG-DyLight 488 were 493 and 528 nm, respectively. Vesicles stained with FM 4-64 lipid dye were used to determine the characteristic vesicle elution fraction. Reference samples probed with FM 4-64 were used to determine the characteristic vesicle elution fraction in each experiment. For plots, SBA curves were background subtracted.

CFE reactions. Protein synthesis was carried out with a modified PANOs-SP system in triplicate reactions, with each reaction containing a uniquely prepared extract23. Specifically, 8 µL of each extract tubes (1% w/v) supplemented with 50 ng template DNA. RNA transcripts were supplemented to PglO reactions, respectively. Glycosylation proceeded at 30 °C for 16 h. After glycosylation, GFP fluorescence was used to quantitate the

Quantification of CFE and CFGpS protein yields. The concentration of cell-free-derived sGFP was determined by measuring in-extract fluorescence and then converting to protein concentration using a standard curve relating sGFP fluorescence to protein concentration as determined by a 14C-leucine incorporation assay.24,25,26 Two µL of cell-free extract were spotted on a PANOx-SP system and reacted with 0.2 μM FRET 4-64 lipid dye and 0.1 μM Cy3 fluorescent antibody (Abcam, ab1187) at a 1:7500 dilution in blocking buffer with 0.2% Tween20 (Sigma-Aldrich) as the primary, 0.2% Tween20 (Sigma-Aldrich) as the secondary for all blots. Blots were imaged using a LI-COR Odyssey Fc (LI-COR Biosciences, USA). Densitometry was performed using Image Studio Lite software to measure band intensity. Fluorescence background was subtracted from blots before determining band intensities. For determining membrane protein enrichment (S2/S30), band intensities of membrane proteins for three independent S12 extract replicates and three independent S20 extract replicates were calculated for each protein. The rounded averages of triplicate ratios (S12/S30) and associated error are reported as enrichment in Fig. 4. For determining glycoprotein yields from CFGpS reactions, band intensities for glycosylated and aglycosylated bands were obtained from independent, triplicate reactions. The fraction of glycosylated protein for each replicate was calculated via band intensities. To determine glycoprotein yields, the fraction glycosylated was multiplied by total protein yield for each replicate as calculated from sGFP fluorescence converted to protein concentration (described below). Yields were plotted using Prism v.9.0.0 (GraphPad).

Yields of all acceptor proteins (other than sGFP) were assessed directly via the addition of 10 µM 14C-leucine (PerkinElmer) to the CFEpS reaction to yield trichloroacetic acid precipitable radioactivity that was measured using scintillation counting. Solid-state fluoride fractions were carried out after centrifugation at 15 min at 4 °C. Briefly, 6 µL of the solid fraction of CFEpS reactions run with 20 min CFPs times were mixed with 6 µL 0.5 M KOH and incubated for 20 min at 37 °C. 5 µL of treated sample was then soaked into two separate filters (Millipore, USA, 0.22 µm) in filter printer I. One filter was washed 3 times using 5% trichloroacetic acid (TCA) with 15 min incubations at 4 °C, and then once with ethanol with a 10 min incubation at room temperature. Following melting of scintillation wax (PerkinElmer MelTexit A 1450-441) on top of both TCA-precipitated and non-TCA-precipitated filters, incorporated radioactivity was measured by a Microbeta2 (PerkinElmer). In each experiment, 80 µL of the soluble fraction of CFGpS reactions containing no plasmid DNA template and subtracted before calculation of protein yields. The fraction of incorporated leucine (washed/uncounted washes) was multiplied by the overall leucine concentration in the reaction and the molecular weight of the protein, then divided by the number of leucine residues per protein to determine the amount of protein produced in each reaction. Yields were plotted using Prism v.9.0.0 (GraphPad).

Autoradiograms of CFGpS reaction products. For sGFP-based glycosylation experiments, western blotting of the acceptor proteins followed by densitometry analysis was used to quantify the fraction of acceptor protein glycosylated (see above for detailed description). For other acceptor proteins, autoradiograms were used to quantify glycoprotein from CFEpS reaction products using densitometry. Autoradiograms were run by using SDS-PAGE reactions of CFE reactions (from the same reactions used to calculate yields) using NuPAGE 4–12% Bis-Tris protein gels with MOPS-SDS buffer (Thermo Fisher Scientific, Waltham, MA, USA). The gels were then dried overnight between cellulose films and then exposed for 48–72 h to a Storage Phosphor Screen (GE Healthcare). The Phosphor Screen was imaged using a Typhoon FLA7000 imager (GE Healthcare). Autoradiogram gel images were acquired using Typhoon FLA 7000 Control Software Version 1.2 Build 1.2.1.93. Autoradiogram analysis was performed using ImageJ (Version 2.10.1.33c, Build 5232404693) gel analyzer to determine ratios of glycosylated and aglycosylated full-length acceptor protein. Glycoprotein yields were determined by multiplying fraction glycosylated as determined by ImageJ analysis, by the yields determined from scintillation counting for each replicate. Yields were plotted using Prism v.9.0.0 (GraphPad).

Cell-free glycoprotein synthesis (CFGpS) reactions. For crude extract-based expression of glycoproteins, a two-phase scheme was implemented as previously described33. In this work, protein synthesis was carried out as described above at 15 µL in PCR strip tubes (Thermo Scientific AB-2000 with 50 ng template DNA. RNA transcripts were supplemented to PglO reactions, respectively. Glycosylation proceeded at 30 °C for 16 h. After glycosylation, GFP fluorescence was used to quantitate the
total amount of acceptor protein synthesized, and western blots were used to calculate the fraction of glycosylated and aglycosylated proteins. For additional acceptor proteins shown in Fig. 5e, all CFPgS reaction conditions were held constant other than the DNA template and addition of 10 μM 14C-leucine to enable quantification (described above).

Estimation of vesicle membrane area. Equation 1 below was used to calculate S, the vesicle surface area (m²/mL), where K_empty is average vesicle radius (m), C is concentration of particles measured by NTA (particles/mL).

\[
S = 4 \pi n (\text{K}_\text{empty})^2 C
\]

(1)

Liquid chromatography mass spectrometry (LC-MS/MS). Acceptor proteins were purified using a His purification protocol prior to LC-MS. CFPgS reactions producing glycosylated sGFP-DQNAT and sGFP-MOOR were scaled up to a total volume of 1.2 mL each and run in 50 mL reaction tubes (Falcon, Corning) with 20 min CFPgS times. Following 16 h glycosylation reactions, CFPgS reactions were transferred to 1.5 mL microtubes (Axygen, Corning) and centrifuged at 16,000g for 3 min. Soluble fractions were split in half and loaded onto two equilibrated Ni-NTA Spin Columns (Qiagen 31014) per CFPgS reaction following column equilibration with equilibration buffer (50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole). CFPgS reactions were incubated on columns for 5 min at room temperature followed by centrifugation at 250g for 12 min. Columns were then washed 3 times with 600 μL low-imidazole buffer (50 mM NaH₂PO₄ and 300 mM NaCl and 20 mM imidazole) and centrifuged at 900g for 2 min before elution in 100 μL of high-imidazole buffer (50 mM NaH₂PO₄ and 300 mM NaCl and 500 mM imidazole). Four elution fractions were collected, and the most concentrated fraction collected from each column was dialyzed against 50 mM Ammonium Bicarbonate. Dialysis buffer was changed after 2 h and then allowed to proceed overnight. Glycopeptides for LC-MS/MS analysis were prepared by reducing His-tag purified, dialyzed glycopeptides by incubation with 5 mM DTT at 60°C for 1 h and then digesting with 0.044 μg/μL MS Grade Trypsin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C overnight. LC-MS/MS was performed by injection of 20 μL (or about 35 pmol) for sGFP-DQNAT and 25 pmol for sGFP-MOOR) of digested glycopeptides into a Bruker Elute UPLC equipped with an ACQUITY UPLC Peptide BEH C18 Column, 300 Å, 1.7 μm, 2.1 mm × 100 mm (186003686 Waters Corp.) with a 10 mm guard column of identical packing (186004629 Waters Corp.) coupled to an Impact-II UHPLC TOF Mass Spectrometer. Liquid chromatography was performed using 100% H₂O and 0.1% formic acid as Solvent A and 100% acetonitrile and 0.1% formic acid as Solvent B at a flow rate of 0.5 mL/min and a 40°C column temperature. An initial condition of 0% B was held for 1 min before elution of the peptides of interest during a 4 min gradient to 50% B. The column was washed and equilibrated by a 0.1 min gradient to 100% B, a 2 min wash at 100% B, a 0.1 min gradient to 0% B and then a 1.8 min hold at 0% B, giving a total 9 min run time. Pseudo multiple reaction monitoring (MRM) MS/MS fragmentation was targeted to theoretical glycopeptide masses corresponding to detected peptide MS peaks. Glycopeptides were fragmented with a collisional energy of 30 eV and an isolation window that included the entire glycopeptide isotopic envelope. For LC-MS/MS of glycopeptides, a scan range of 100–3000 m/z with a spectral rate of 8 Hz was used. External calibration was performed prior to data collection.

LC-MS/MS data were collected using Bruker Compass Data Analysis v4.4 (Bruker Daltonics, Inc.). Representative LC-MS/MS spectra from MRM fragmentation were selected and annotated manually. Observed glycan and glycopeptide m/z values are annotated in figures. LC-MS/MS data were exported from Bruker Compass Data Analysis and plotted in Microsoft Excel.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Uniprot accession codes used in this study are: Q5HTX9; Q5FA54; P0AFA2; Q9F7P4; P21554; E9AET9.

All data generated or analyzed during this study are included in the manuscript or Supplementary information or are available from the corresponding author upon request. Accession codes are listed for applicable genes in the Supplementary information. We report no restrictions on data availability. Source data are provided with this paper.

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References
1. Silverman, A. D., Karim, A. S. & Jeytt, M. C. Cell-free gene expression: an expanded repertoire of applications. Nat. Rev. Genet. 21, 151–170 (2020).
2. Hershew, J., Kightlinger, W. & Jewett, M. C. Cell-free systems for accelerating glycoprotein expression and biomanufacturing. J. Ind. Microbiol. Biotechnol. 47, 977–991 (2020).
3. Rasor, B. et al. Toward sustainable, cell-free biomanufacturing. Curr. Opin. Biotechnol. 69, 136–144 (2021).
4. Bogart, J. W. et al. Cell-free exploration of the natural product chemical space. ChemBioChem 22, 84–91 (2020).
5. Zennella, A., Thoring, L., Hoffmeister, C. & Kubick, S. Cell-free protein synthesis: pros and cons of prokaryotic and eukaryotic systems. ChemBioChem 16, 2420–2431 (2015).
6. Katzen, F., Chang, G. & Kudicki, W. The past, present and future of cell-free protein synthesis. Trends Biotechnol. 23, 150–156 (2005).
7. Kightlinger, W. et al. A cell-free biosynthesis platform for modular construction of glycoprotein pathways. Nat. Commun. 10, 1–13 (2019).
8. Kightlinger, W. et al. Design of glycosylation sites by rapid synthesis and analysis of glycosyltransferases article. Nat. Chem. Biol. 14, 627–635 (2018).
9. Karim, A. S. et al. In vitro prototyping and rapid optimization of biosynthetic enzymes for cell design. Nat. Chem. Biol. 16, 912–919 (2020).
10. Carlson, E. D. et al. Highly productive, one-pot cell-free protein synthesis platform based on genomically encoded Escherichia coli. Cell Chem. Biol. 26, 1743–1754 (2019).
11. Contreras-Llano, L. E. et al. Holistic engineering of cell-free systems through proteome-reprogramming synthetic circuits. Nat. Commun. 11, 1–10 (2020).
12. Cai, Q. et al. A simplified and robust protocol for immunoglobulin expression in Escherichia coli cell-free protein synthesis systems. Biotechnol. Prog. 31, 823–831 (2015).
13. Bernath, K., Magdassi, S. & Tawfik, D. S. Directed evolution of protein inhibitors of DNA-nucleases by in vitro compartmentalization (IVC) and nano-droplet delivery. J. Mol. Biol. 345, 1015–1026 (2005).
14. Zawada, J. F. et al. Microscale to manufacturing scale-up of cell-free cytokine production—a new approach for shortening protein production development timelines. Biotechnol. Bioeng. 108, 1570–1578 (2011).
15. Callhoun, K. A. & Swartz, J. R. An economical method for cell-free protein synthesis using glucose and nucleoside monophosphates. Biotechnol. Prog. 21, 1146–1153 (2005).
16. Pardee, K. et al. Paper-based synthetic gene networks. Cell 159, 940–954 (2014).
17. Pardee, K. et al. Portable, on-demand biomolecular manufacturing. Cell 167, 248–259.e12 (2016).
18. Stark, J. C. et al. On-demand, cell-free biomanufacturing of conjugate vaccines at the point-of-care. Sci. Adv. 7, eabe9444 (2021).
19. Hunt, J. P., Yang, S. O., Wilding, K. M. & Bundy, B. C. The growing impact of lyophilized cell-free protein expression systems. Biotechnology 8, 325–330 (2017).
20. Gregorio, N. E. et al. Unlocking applications of cell-free biotechnology through enhanced shelf life and productivity of E. coli extracts. ACS Synth. Biol. 9, 766–778 (2020).
21. Adiga, R. et al. Point-of-care production of therapeutic proteins of good-manufacturing-practice quality. Nat. Biomed. Eng. 2, 675–686 (2018).
22. Thavarajah, W. et al. Point-of-use detection of environmental fluoride via a cell-free riboswitch-based biosensor. ACS Synth. Biol. 9, 10–18 (2020).
23. Salehi, A. S. M. et al. Cell-free protein synthesis approach to biosensing hTRβ-specific endocrine disruptors. Anal. Chem. 89, 3395–3401 (2017).
24. Pardee, K. et al. Rapid, low-cost detection of Zika virus using programmable biomolecular components. Cell 165, 1255–1266 (2016).
25. Liu, X. et al. Design of a transcriptional biosensor for the portable, on-demand detection of cyanic acid. ACS Synth. Biol. 9, 84–94 (2020).
26. Meyer, A. J. et al. Organism engineering for the bioproduction of the triaminotrimethobenzene (TATB) precursor phosphoroglucinol (PG). ACS Synth. Biol. 8, 2746–2755 (2019).
27. Jung, J. K. et al. Cell-free biosensors for rapid detection of water contaminants. Nat. Biotechnol. 38, 1451–1459 (2020).
28. Amalfitano, E. et al. A glucose meter interface for point-of-care gene circuit-based diagnostics. Nat. Commun. 12, 1–10 (2021).
34. Jaroentomeechai, T. et al. Single-pot glycoprotein biosynthesis using a cell-free transcription-translation system enriched with glycosylation machinery. Nat. Commun. 6, 1–11 (2015).
35. Huang, A. et al. Biobots™ explorer: a modular synthetic biology education kit. Sci. Adv. 4, eaat5105 (2018).
36. & Stark, J. C. et al. BioBits™ Bright: a fluorescent synthetic biology education kit. Sci. Adv. 4, eaat5107 (2018).
37. Scrutton, S. C. et al. Robust health: classroom activities exploring engineering, biology, and human health with fluorescent readouts. ACS Synth. Biol. 9, 1001–1009 (2020).
38. Martin, R. W. et al. Cell-free protein synthesis from genomically recoded Escherichia coli. Nat. Commun. 9, 220 (2018).
39. Martin, R. W. et al. A cell-free framework for rapid biosynthetic reactions in glycoprotein folding and degradation. Biochem. J. 313, 1–13 (2000).
40. Zheng, K., Baptog, C. & Bayer, R. The impact of glycosylation on monoclonal antibody conformation and stability. MAbs 3, 568–576 (2011).
41. Kightlinger, W., Warfel, K. F., Delisa, M. P. & Jewett, M. C. Synthetic Glycobiology: Parts, Systems, and Applications. ACS Synth. Biol. 4, 7 (2020).
42. Dudley, Q.M., Karim, A.S., Nash, C.J. & Jewett, M.C. Cell-free prototyping of cell-free protein synthesis in bacterial cell-free system. Biotechnol. Bioeng. 115, 1253–1264 (2018).
43. Hellenius, A. & Aebl, M. Roles of N-linked glycans in the endoplasmic reticulum. Annu. Rev. Biochem. 73, 1019–1049 (2004).
44. Kurkowski, A., Chirmule, C. & Nair, P. Immunogenicity of biotechnologies: causes and association with posttranslational modifications. J. Immunol. Res. 2016, 128473 (2016).
45. Parodi, A. J. Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. Biochem. J. 1049 (2004).
46. Faridmoayer, A., Fentabil, M. A., Mills, D. C., Klassen, J. S. & Feldman, M. F. Synthetic biology: designing and engineering glycocompounds outside of living cells. Front. Chem. 8, 645 (2020).
47. Goerke, A. R. & Swartz, J. R. Development of cell-free protein synthesis. Biotechnol. Bioeng. 91, 425–435 (2005).
48. Ramakrishnan, V. Ribosome structure and the mechanism of translation. Cell 108, 557–572 (2002).
49. Martin, R. W. et al. Single-step isolation of extracellular vesicles by size-exclusion chromatography. J. Extracell. Vesicles 3, https://doi.org/10.3402/jv.v3i3.23340 (2014).
50. Cole, S. D. et al. Methodologies for preparation of prokaryotic extracts for cell-free expression systems. Synth. Biotechnol. 5, 252–267 (2020).
51. Schob, J.A. et al. A cell-free platform for rapid synthesis and testing of active oligosaccharyltransferases. Biotechnol. Bioeng. 115, 739–750 (2017).
52. Matthias, D. et al. Cell-free expression and assembly of ATP synthase. J. Mol. Biol. 413, 5903–5921 (2011).
53. Sachse, R., Dondapati, S.K., Fenz, S.F., Schmidt, T. & Kubick, S. Membrane protein synthesis in cell-free systems: from bio-mimetic systems to bio-membranes. FEBS Lett. 588, 2774–2781 (2014).
54. Schwarz, F. et al. Relaxed acceptor site specificity of bacterial oligosaccharyltransferase in vivo. Glycobiology 21, 45–51 (2011).
55. Schob, J.A. et al. A cell-free platform for rapid synthesis and testing of active oligosaccharyltransferases. Biotechnol. Bioeng. 115, 739–750 (2017).
56. Aebischer, R. et al. How many human proteoforms are there? Proteom. 13, 89–98 (2013).
57. Pan, C. et al. Biosynthesis of conjugate vaccines using an O-linked glycoprotein biosynthesis system. J. Biol. Chem. 283, 34596–34604 (2008).
58. Faridmoayer, A. et al. Engine cell-free protein synthesis in bacterial cell-free system. Biotechnol. Bioeng. 91, 351–367 (2008).
59. Goerke, A. R. & Swartz, J. R. High-level cell-free synthesis of proteins containing site-specific natural amino acids. Biotechnol. Bioeng. 102, 400–416 (2009).
60. Attendorf, K. H. & Stachelin, L. A. Orientation of membrane vesicles from Escherichia coli as detected by freeze-fracle electron microscopy. J. Bacteriol. 117, 888–899 (1974).
61. Herteg, E.L. & Hinkle, P.C. Oxidative phosphorylation and proton translocation in membrane vesicles prepared from Escherichia coli. Biochem. Biophys. Res. Commun. 58, 178–184 (1974).
62. Jewett, M.C., Calhoun, K.A., Voloshin, A., Wuu, J.J. & Swartz, J.R. An integrated cell-free metabolic platform for protein production and synthetic biology. Mol. Syst. Biol. 4, 220 (2008).
63. Brantner, M., Vogel, S., Stoff, K., Spiegel, H. & Schillberg, S. A versatile coupled cell-free transcription-translation system based on tobacco BY-2 cell lysates. Biotechnol. Bioeng. 112, 867–878 (2015).
64. Aebischer, R. et al. IRES-mediated translation of membrane proteins and glycopolymers in eukaryotic cell-free systems. PLoS ONE 8, e82234 (2013).
65. Sachse, R. et al. Synthesis of membrane proteins in eukaryotic cell-free systems. Eng. Life Sci. 13, 39–48 (2013).
66. Katzen, F. & Kudlwicki, W. Efficient generation of insect-cell based cell-free translation extracts active in glycosylation and signal sequence processing. J. Biotechnol. 125, 194–197 (2006).
67. Zemella, A. et al. Cell-free protein synthesis as a novel tool for directed glycoengineering of active erythropoietin. Sci. Rep. 8, 1–12 (2018).
68. Gurramkonda, C. et al. Improving the recombinant human erythropoietin glycosylation using microscale supplementation in CHO cell-free system. Biotechnol. Bioeng. 115, 1253–1264 (2018).
69. Goerke, A. R. & Swartz, J. R. Mimicking the cytoplasmic environment actsives long-lived and efficient cell-free protein synthesis. Biotechnol. Bioeng. 86, 19–26 (2004).
70. Cole, S. D. et al. Quantification of Interlaboratory Cell-Free Protein Synthesis Variability. ACS Synth. Biol. 8, 2080–2091 (2019).
71. Valderrama-Rancón, J. D. et al. An engineered eukaryotic protein glycosylation pathway in E. coli. Nat. Chem. Biol. 8, 434–436 (2012).
72. Alaimo, C. et al. Two distinct but interchangeable mechanisms for lipid-linked oligosaccharides. EMBO J. 25, 967–976 (2006).
73. Guarno, C. & Delisa, M. P. A prokaryote-based cell-free translation system that efficiently synthesizes glycoproteins. Glycoconjugate J. 22, 596–601 (2012).
74. Fishov, I. & Woldringh, C.L. Visualization of membrane domains in Escherichia coli. Mol. Microbiol. 32, 1166–1172 (1999).
93. McBroom, A. J., Johnson, A. P., Vemulapalli, S. & Kuehn, M. J. Outer membrane vesicle production by *Escherichia coli* is independent of membrane instability. *J. Bacteriol.* **188**, 5385–5392 (2006).

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**Author contributions**

All of the authors designed research; J.M.H., K.F.W., J.A.P., and S.M.I. performed research; C.J.S., J.M.H., J.A.P., and E.W.R. contributed new reagents/analytic tools; J.M.H. and K.F.W. analyzed data; and J.M.H., K.F.W., and M.C.J. wrote the paper. All authors reviewed and edited the paper. M.C.J. provided supervision.

**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. M.P.D. has a financial interest in Glycobia, Inc., Versatope, Inc., Ajuta Therapeutics, Inc., and SwiftScale Biologics. M.P.D.’s interests are reviewed and managed by Cornell University in accordance with their conflict of interest policies. The authors declare no other competing interests.

**Additional information**

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