

GlycoCAP: A Cell-Free, Bacterial Glycosylation Platform for Building Clickable Azido-Sialoglycoproteins

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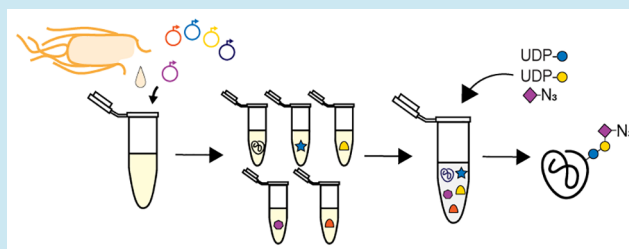
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ABSTRACT: Glycan-binding receptors known as lectins represent a class of potential therapeutic targets. Yet, the therapeutic potential of targeting lectins remains largely untapped due in part to limitations in tools for building glycan-based drugs. One group of desirable structures is proteins with noncanonical glycans. Cell-free protein synthesis systems have matured as a promising approach for making glycoproteins that may overcome current limitations and enable new glycoprotein medicines. Yet, this approach has not been applied to the construction of proteins with noncanonical glycans. To address this limitation, we develop a cell-free glycoprotein synthesis platform for building noncanonical glycans and, specifically, clickable azido-sialoglycoproteins (called GlycoCAP). The GlycoCAP platform uses an *Escherichia coli*-based cell-free protein synthesis system for the site-specific installation of noncanonical glycans onto proteins with a high degree of homogeneity and efficiency. As a model, we construct four noncanonical glycans onto a dust mite allergen (Der p 2): α 2,3 C5-azido-sialyllactose, α 2,3 C9-azido-sialyllactose, α 2,6 C5-azido-sialyllactose, and α 2,6 C9-azido-sialyllactose. Through a series of optimizations, we achieve more than 60% sialylation efficiency with a noncanonical azido-sialic acid. We then show that the azide click handle can be conjugated with a model fluorophore using both strain-promoted and copper-catalyzed click chemistry. We anticipate that GlycoCAP will facilitate the development and discovery of glycan-based drugs by granting access to a wider variety of possible noncanonical glycan structures and also provide an approach for functionalizing glycoproteins by click chemistry conjugation.

KEYWORDS: bacterial glycoengineering, cell-free protein synthesis, click chemistry, lectins, noncanonical glycans



INTRODUCTION

Lectins are glycan-binding receptors that represent a promising class of therapeutic targets. Sialic acid-binding immunoglobulin-like lectins (Siglecs) are one example of a lectin family that has shown particular promise as targets in treating allergic inflammation and preventing anaphylaxis.^{1–14} Siglecs are transmembrane receptors that bind sialic acid-containing glycans and are differentially expressed on immune cells such that each immune cell has its own Siglec expression profile.^{15–19} Most, but not all, Siglecs have immune suppressive activity by virtue of possessing cytoplasmic immunoreceptor tyrosine-based inhibitory motifs that recruit phosphatases.¹⁹ Therefore, Siglecs not only offer an avenue for immune cell inhibition but also provide an approach for cell-specific targeting.

Despite their promise, the full potential of lectin-targeting therapeutics, like Siglecs, remains untapped. This is because lectins specifically bind glycans, and we lack adequate tools to rapidly build and manufacture a multiplicity of structures needed for biochemical, mechanistic, and structural studies to create glycan-based, lectin-binding drugs. Current approaches often utilize mammalian cell culture, which yields heteroge-

neous glycan populations,^{20–23} or chemoenzymatic synthesis, which produces homogeneous glycan populations but can be expensive and difficult to scale up.^{5,7,11,12,24–26}

Cell-free protein synthesis (CFPS) systems from *Escherichia coli*-based extracts have emerged as an approach to rapidly make glycoproteins^{27–29} and could overcome the challenges of current manufacturing methods. Making glycoproteins in CFPS systems involves expressing glycosyltransferases from other organisms and supplementing the reaction with appropriate sugar substrates (e.g., UDP-glucose, lipid-linked oligosaccharides) to install glycans onto proteins. Cell-free glycoprotein synthesis systems have several advantages over conventional cellular manufacturing strategies. First, because *E. coli* lacks endogenous glycosylation machinery, site-specific glycosylation occurs without competition from other host

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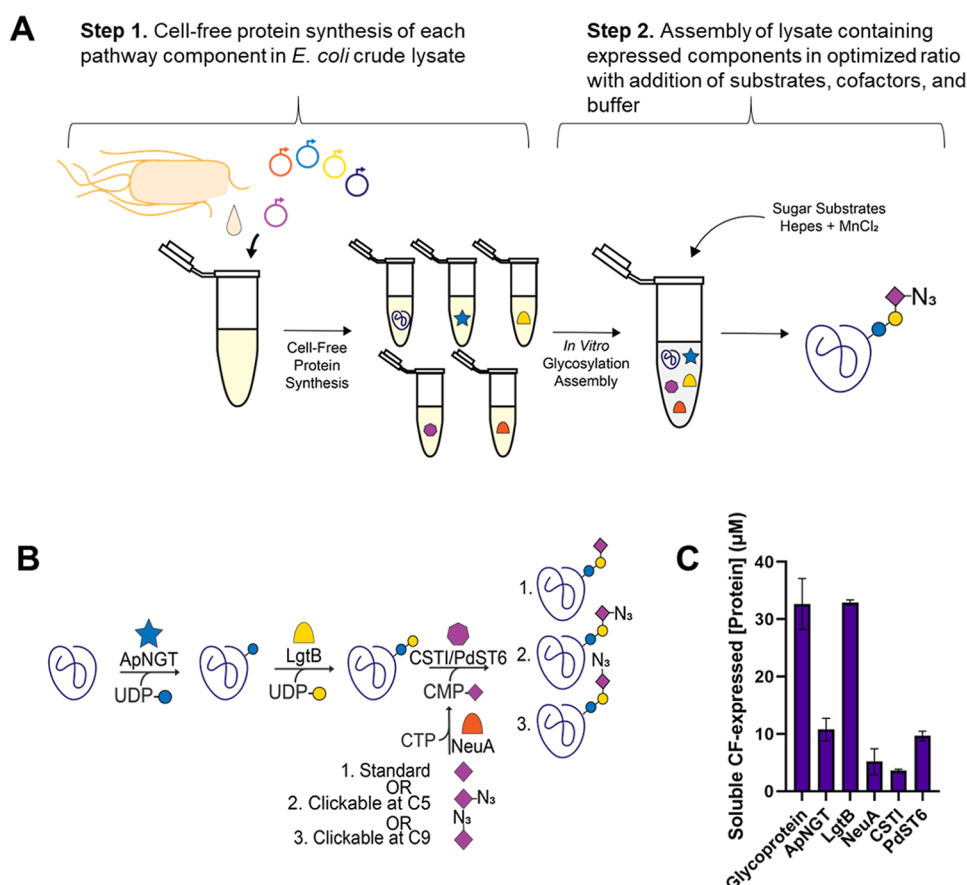


Figure 1. GlycoCAP platform: a cell-free glycosylation platform for building clickable azido-glycoproteins. (A) Plasmids encoding each component of the glycosylation pathway are supplemented in lysate harvested from *E. coli* cells along with necessary cofactors and building blocks for transcription and translation. After overnight expression, cell-free expressed components are mixed in optimized proportions along with sugar substrates and cofactors in an *in vitro* glycosylation (IVG) reaction. (B) Components of the pathway include (i) a target glycoprotein designed with an amino acid sequence recognized by (ii) ApNGT for modification with glucose by N-linked glycosylation; (iii) LgtB elaborates galactose onto glucose in a β 1,4 linkage, and either (iv) CSTI or PdST6 can be added to elaborate a sialic acid on galactose in an α 2,3 or α 2,6 linkage, respectively. Finally, (v) NeuA is required to convert noncanonical sugars, such as C5-azido-sialic acid or C9-azido-sialic acid, into activated sugar substrates for CSTI/PdST6 by conjugating the sugars to a phosphonucleotide. (C) Soluble concentration of each component in the CFPS reaction after expression is detected by radioactive incorporation of [^{14}C]-leucine ($n = 3$; error bars = standard deviation).

glycosyltransferases. Second, cell-free systems are not limited by viability requirements, thus avoiding constraints arising from noncanonical substrates.³⁰ Third, the lack of a cell membrane circumvents the need for membrane transport of provided sugar substrates. Finally, cell-free systems are amenable to scale-up for manufacturing, having demonstrated linear scalability over a six order of magnitude range in volume to reach the 100 L milestone.^{31,32}

To date, cell-free glycoprotein synthesis systems have been developed for making human and bacterial glycoproteins,^{33–42} designing protein glycosylation sites,^{43,44} and modularly constructing glycosylation pathways.⁴⁵ However, while cell-free systems offer promise for making glycoproteins, there is one group of glycan structures that has shown therapeutic promise but has, to our knowledge, never been made in an integrated cell-free system: that group is noncanonical glycans.

Noncanonical glycans and, more specifically, clickable azido-sialoglycans have been used to optimize lectin-binding pocket interactions by chemically modifying sialic acid-containing glycans through click chemistry conjugation of small chemical groups.^{46–48} This approach has shown great promise in targeting Siglecs to treat allergy and autoimmune disease.^{8,11–14,49–52} Clickable azido-sialoglycans also offer oppor-

tunities for creating dual function therapeutics, functionalizing glycoprotein with favorable properties, and facilitating scientific research by providing methods for labeling and localizing proteins of interest.^{53–55}

Here, we set out to leverage the advantages of a cell-free system to harness the promise of noncanonical glycans. Toward this aim, we developed a bacterial-based, cell-free glycosylation platform that enables site-specific installation of clickable azido-sialoglycans onto proteins (called GlycoCAP) (Figure 1A). The key idea was to create cell-free biosynthesis “units” made from crude cell lysates that are selectively enriched with pathway enzymes expressed directly in lysates by CFPS. Then, these units are assembled modularly, in a mix-and-match fashion, to build defined glycosylation pathways harboring noncanonical glycans (Figure 1B). As proof-of-concept, we apply GlycoCAP for the synthesis of four Siglec ligands, which are amenable to strain-promoted and copper-catalyzed click chemistry modification. The GlycoCAP platform contributes a set of tools for constructing noncanonical glycans onto proteins, which could open opportunities for therapeutically targeting Siglecs, among other lectins, and offers strategies for protein labeling, development of dual function therapeutics, and protein localization.

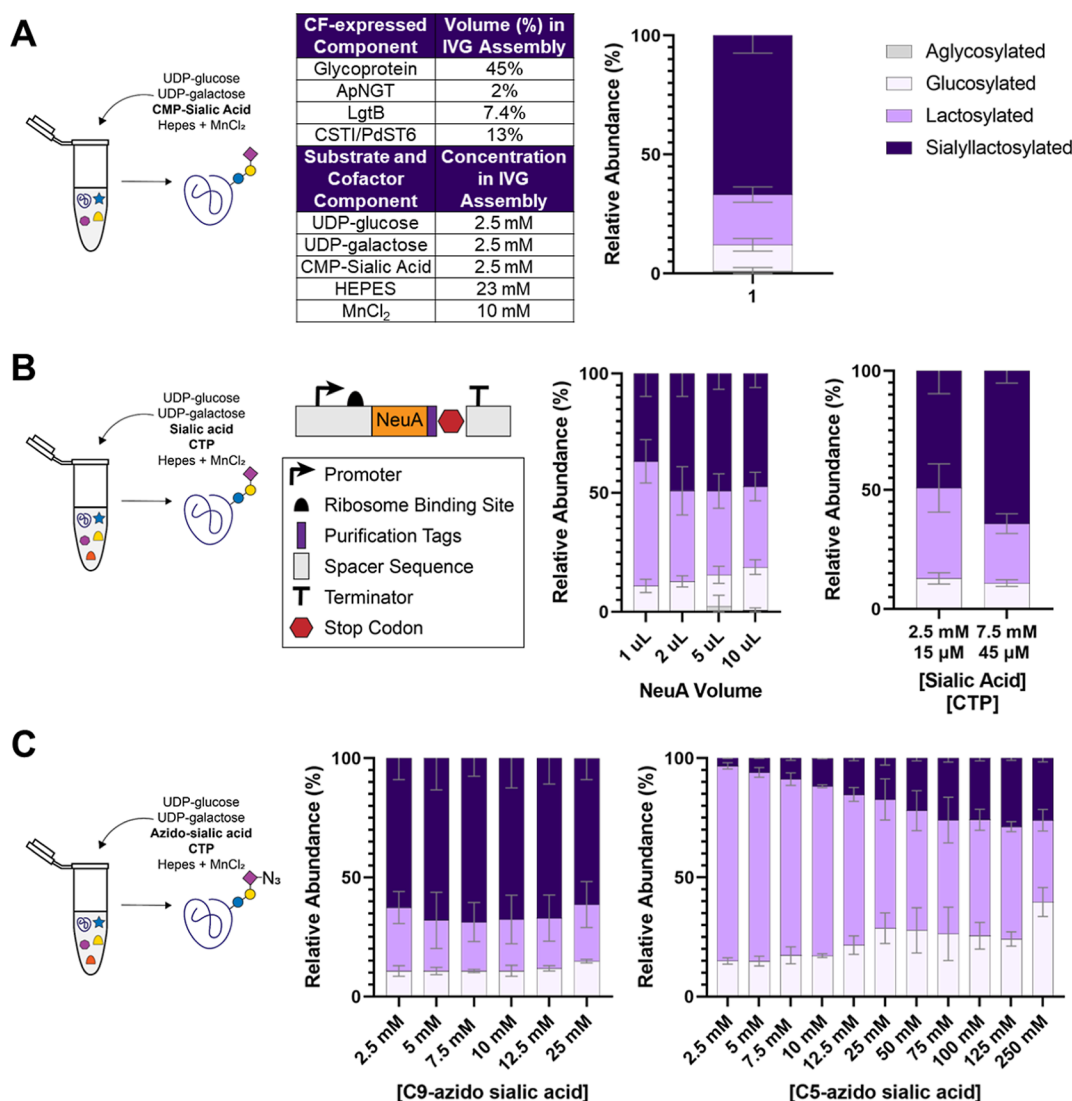


Figure 2. Optimization of GlycoCAP for noncanonical sugar incorporation. (A) IVG reactions were assembled based on optimized ratios (i.e., where 13% of the final reaction volume was made up of either crude lysate expressing PdST6 if an α 2,6 linkage was desired or crude lysate expressing CSTI if an α 2,3 linkage was desired) to achieve glycosylation when activated CMP-sialic acid is supplemented directly and NeuA is not required to install sialyllactose onto the target glycoprotein. Under these conditions, $67 \pm 7\%$ glycosylation efficiency is achieved ($n = 2$; error bars = average error). The color-coded legend applies to all bar graphs shown. (B) Plasmid encoding NeuA was designed for CFPS. Once expressed, NeuA was assembled into the IVG reaction with its needed substrates: nonactivated sialic acid and CTP. Titration of NeuA was performed to determine 2 μ L (which corresponds to 4.5% volume) as the optimal volume to add to the IVG assembly ($n = 4$; error bars = standard deviation). An additional titration of standard sialic acid shows that increasing [sialic acid] to 7.5 mM and [CTP] to 45 μ M enhances the glycosylation efficiency to $64 \pm 5\%$ ($n = 4$; error bars = standard deviation). (C) Titration of noncanonical sialic acids shows that C9-azido-sialic acid is as efficiently incorporated as standard sialic acid, with 7.5 mM sialic acid and 45 μ M CTP being the optimal concentration to achieve $69 \pm 8\%$ efficiency. A concentration of 125 mM C5-azido-sialic acid is required to achieve the optimal glycosylation efficiency of $29 \pm 1\%$ ($n = 2$; error bars = average error). All glycosylation efficiencies are determined by MS of trypsinized samples and estimated by the area under the curve of each pathway intermediate's extracted ion chromatogram based on the theoretical mass of the resulting glycopeptide.

RESULTS

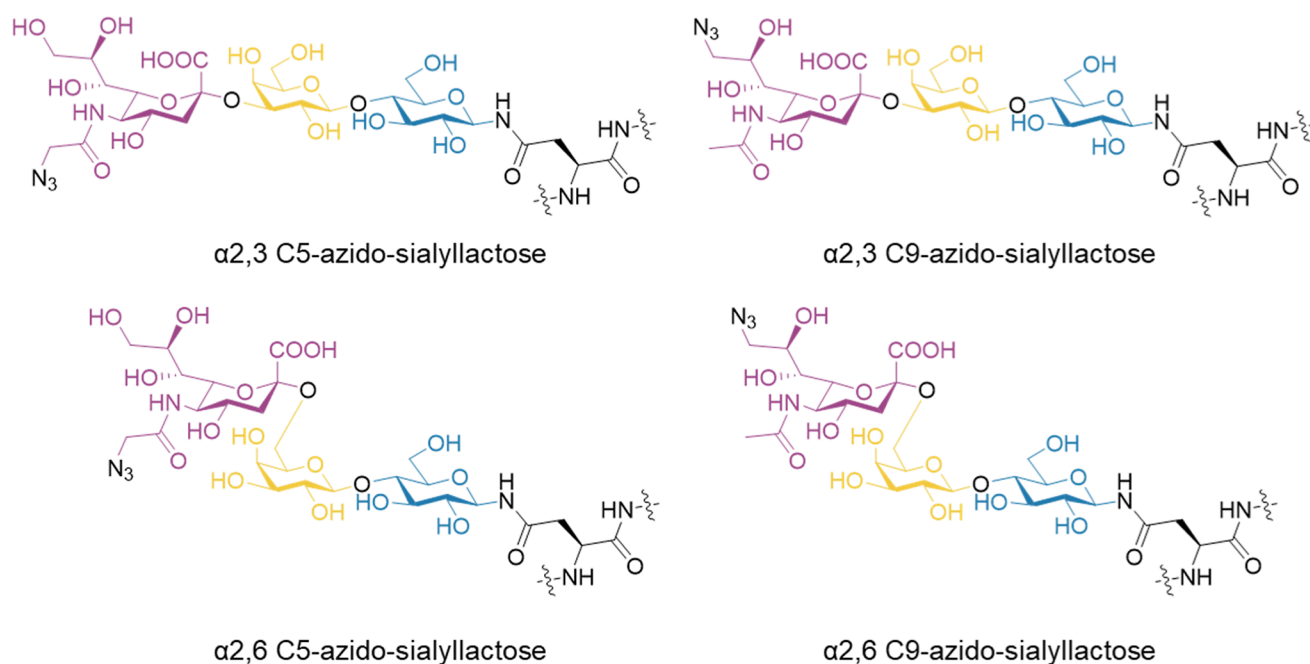
GlycoCAP Platform. The goal of this work was to create a bacterial-based, cell-free platform for installing noncanonical glycans onto desired target glycoproteins. Specifically, we focused on site-specific installation of azido-sialyllactose onto proteins as the azide handle on the glycan grants an avenue for conjugation by click chemistry for Siglec therapeutics. To achieve our goal, we (i) established a base pathway for installing sialyllactose onto proteins, (ii) implemented and optimized a pathway required for activating and incorporating azido-glycan substrates, and (iii) applied our pathway to

produce azide-containing glycoproteins that could be modified through bioorthogonal chemistry.

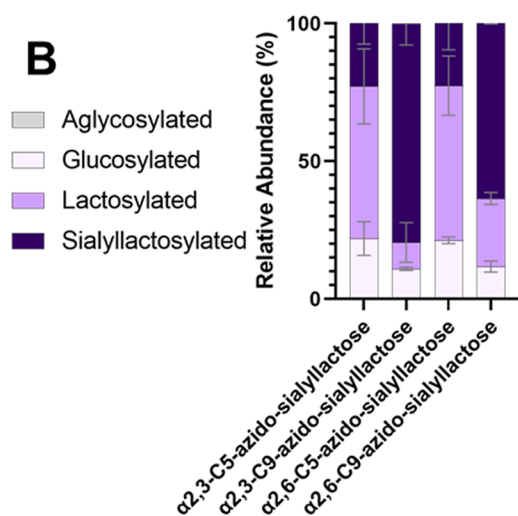
Establishing Conditions for Installing Sialyllactose.

Initially, we set out to construct a minimal trisaccharide structure terminating in sialic acid on a model protein target. This involved two steps. First, we individually expressed multiple components of a bacterial glycosylation pathway we previously established⁴⁵ by CFPS in the lysate of an engineered *E. coli* strain.⁵⁶ Pathway components include (i) the target glycoprotein, which has been designed with an amino acid sequence⁴³ recognized for N-linked glycosylation (Table S1), (ii) ApNGT, which is a glycosyltransferase from *Actinobacillus*

A



B



C

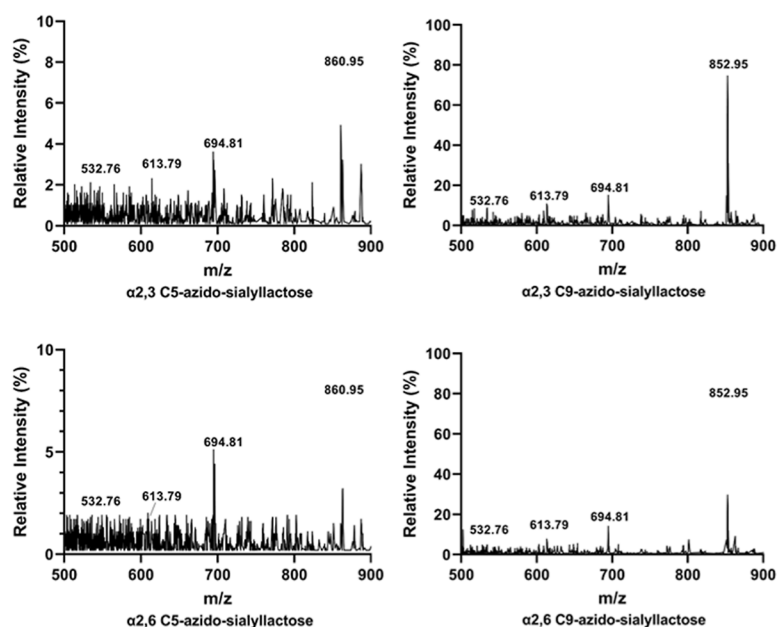


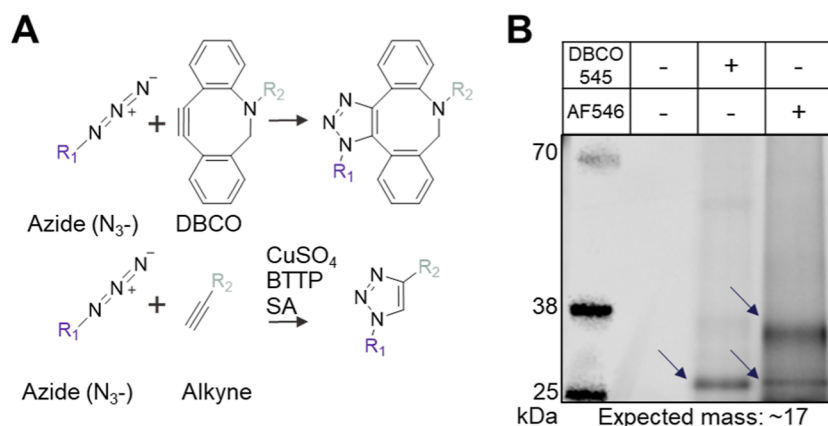
Figure 3. Structures made with optimized conditions of GlycoCAP. (A) Chemical structures installed onto proteins in GlycoCAP. (B) Efficiencies for each species are represented as a bar graph ($n = 2$; error bars = average error). (C) MS confirmation of each species is shown in relation to the peaks of its pathway intermediates in the +2 charge state. MS data show a representative spectra selected from $n = 2$ experiments.

pleuropneumoniae and installs glucose onto an asparagine residue,^{57,58} (iii) LgtB, which is from *Neisseria gonorrhoeae* and elaborates a galactose onto the glucose in a β 1,4 linkage, and (iv) CSTI, from *Campylobacter jejuni*, which elaborates a sialic acid onto the lactose in an α 2,3 linkage, or PdST6, from *Photobacterium damsela*, which elaborates a sialic acid onto the lactose in an α 2,6 linkage. Soluble expression and yield of pathway components were quantified by [14 C]-leucine incorporation (Figures 1C and S1).

In step 2, following the expression of pathway components, we assembled *in vitro* glycosylation (IVG) reactions such that the reactions were composed of an optimized percent volume of each CF-expressed component and optimized molar concentrations of substrates and cofactors based on parameters previously published.⁴⁵ IVG reactions included CF-expressed (i) target glycoprotein (45% by volume), (ii) ApNGT (2%), (iii) LgtB (7.4%), and (iv) CSTI (13%) or PdST6 (13%) along with (v) activated sugar donors UDP-glucose (2.5 mM),

Table 1. Data from Preparative Scale Production of Azido-Sialoglycoproteins Where IVGs Were Performed at a 1 mL Scale

Glycan identity	scale up of GlycoCAP azido-sialoglycoprotein production			
	C5-azido α 2,3-sialyllactose	C9-azido α 2,3-sialyllactose	C5-azido α 2,6-sialyllactose	C9-azido α 2,6-sialyllactose
Total elution volume (mL)	1.2	1.2	1.2	1.2
Protein yield (μ g/mL)	347	196	265	194
Total protein (μ g)	416	235	318	233
Azido-sialylation (%)	18	85.0	16	64
Final product yield (μ g)	73	200	50	148

**Figure 4.** Click chemistry conjugation of azido-sialoglycoproteins. (A) Conjugation by click chemistry with either a strained alkyne (top) or standard alkyne (bottom) is possible with the azido-sialoglycan structures built by GlycoCAP. SA = sodium ascorbate. (B) Fluorescent gel demonstrates successful click conjugation onto a C9-azido-sialoglycoprotein with either a fluorescent strained alkyne (DBCO 545) by strain-promoted click chemistry (lane 3) or a fluorescent standard alkyne (AF546) by copper-catalyzed click chemistry (lane 4). Fluorescent bands are visible at the expected masses for Der p 2 in its monomer form ($\sim$ 17 kDa) and dimer form ($\sim$ 34 kDa) except in the lane where azido-sialoglycoprotein was loaded without any fluorophore conjugation as a negative control (lane 2). Gel representative of $n = 3$ experiments.

UDP-galactose (2.5 mM), and CMP-sialic acid (2.5 mM) and MnCl₂ (10 mM) and HEPES (23 mM) buffer. As a model protein, we used dust mite allergen (Der p 2) for glycan installation.^{59,60} IVG reactions were incubated overnight, resupplemented with sugar substrate, and incubated for an additional 24 h. Glycosylated allergen was isolated by affinity-tag purification, and liquid chromatography–mass spectrometry (LC–MS) was used to calculate the glycosylation efficiency. We determined a base case standard for glycosylation efficiency, which we determined to be $67 \pm 7\%$ (Figure 2A).

Extending the Pathway for Incorporation of Non-activated Sialic Acids. With a base case glycoprotein synthesis system in place, we turned our efforts toward incorporating noncanonical glycans. Since noncanonical sialic acids are only available in their nonactivated form (not conjugated to a phosphonucleotide), incorporation of a noncanonical sialic acid requires the addition of *N*-acetylneuraminase cytidyltransferase (NeuA) (Figure S2 and Table S2), which catalyzes the formation of CMP-sialic acid from canonical or noncanonical sialic acid and cytidine triphosphate (CTP) to produce the appropriate, activated sugar donor for CSTI/PdST6 enzymes. We performed a titration of CFPS-expressed NeuA into a series of IVG reactions where 2.5 mM standard sialic acid and 15 μ M CTP were supplemented. NeuA was added at a volume of 1, 2, 5, or 10 μ L. Adding 2 μ L (corresponding to 4.5% of the reaction volume) was enough to achieve the optimal glycosylation efficiency of $49 \pm 10\%$, meaning the relative abundance of the final desired sialylated product is $\sim$ 49% and the remaining species are earlier pathway intermediates (Figure 2B).

To determine whether the lower efficiency compared to the base case standard is a result of the IVG assembly being substrate limited, we titrated additional sialic acid and CTP. Increasing [sialic acid] to 7.5 mM and [CTP] to 45 μ M achieved a glycosylation efficiency of $64 \pm 5\%$, which is on par with the base case standard using the activated sialic CMP-acid (Figure 2B).

Incorporation of C9- and C5-Azido-Sialic Acids. We next performed a titration of azido-sialic acids where the azide group is either at the 9th carbon of sialic acid (C9-azido-sialic acid) or where an azidoacetyl group is on the 5th carbon of sialic acid (C5-azido-sialic acid) (Figure S3). The goal was to test whether the enzymes of our pathway are permissive to an additional chemical group on their sialic acid substrate. C9-azido-sialic acid was incorporated at an equal or better efficiency than canonical sialic acid, where 7.5 mM C9-azido-sialic acid and 15 μ M CTP led to $69 \pm 8\%$ glycosylation efficiency (Figure 2C). C5-azido-sialic acid could also be incorporated. However, higher concentrations of C5-azido-sialic acid were required and still did not match the base case efficiency with the canonical sialic acid (Figure 2A), indicating that the pathway enzymes are less permissive to C5-azido-sialic acid than to C9-azido-sialic acid, possibly due to disrupted binding pocket interactions caused by the azide group at the 5th carbon position. By adding 125 mM C5-azido-sialic acid and 750 μ M CTP, we were able to achieve a glycosylation efficiency of $29 \pm 1\%$ (Figure 2C).

GlycoCAP Production of α 2,3-C5-Azido-sialyllactose, α 2,3-C9-Azido-sialyllactose, α 2,6-C5-Azido-sialyllactose, and α 2,6-C9-Azido-sialyllactose. With optimal conditions established for noncanonical azido-sialic acid

incorporation, we installed four different clickable azido-sialoglycans onto proteins (Figure 3A). Under optimized GlycoCAP conditions, α 2,3-C5-azido-sialyllactose was produced at $23 \pm 7\%$ efficiency, α 2,3-C9-azido-sialyllactose was produced at $80 \pm 8\%$ efficiency, α 2,6-C5-azido-sialyllactose was produced at $23 \pm 10\%$ efficiency, and α 2,6-C9-azido-sialyllactose was produced at $64 \pm 1\%$ efficiency (Figure 3B). MS confirmed the production of these species and demonstrated the relative proportion of reaction intermediates and the final product present in the final reaction (Figure 3C).

Preparative Scale Production of Azido-Sialoglycoproteins. As a representative synthesis to demonstrate the scalability of the GlycoCAP platform, we performed 1 mL IVGs for each of the four clickable azido-sialoglycoproteins. After purification, we characterized the protein yield by UV absorbance at 280 nm. The abundance of azido-sialylated protein relative to other pathway intermediates was determined by MS and applied as a percentage to the total protein yield to estimate the final product yield. Preparation at this scale yielded approximately 70 μ g of C5-azido- α 2,3-sialyllactose, 200 μ g of C9-azido- α 2,3-sialyllactose, 50 μ g of C5-azido- α 2,6-sialyllactose, and 150 μ g of C5-azido- α 2,3-sialyllactose (Table 1 and Figure S4).

Click Chemistry Conjugation of Clickable Azido-Sialoglycoproteins. To show that installed azido-sialoglycans are amenable to click chemistry conjugation, we next performed both strain-promoted click chemistry with a fluorophore containing a dibenzocyclooctyne (DBCO) group (Figure 4A, top) and copper-catalyzed click chemistry with a fluorophore containing a standard alkyne (Figure 4A, bottom). In the absence of copper, steric strain around the alkyne is required for successful conjugation with DBCO. Copper-catalyzed reactions yield a smaller intervening triazole ring. Samples were run on a NuPAGE Bis-Tris gel and imaged under fluorescent excitation such that only fluorescent bands are visible. Click conjugation was successful with both methods as indicated by the presence of fluorescent bands at the expected masses for the monomer form of the Der p 2 glycoprotein (~ 17 kDa) and the dimer form (~ 34 kDa) (Figure 4B). Additional weight from glycosylation and click modification in addition to incomplete denaturation of Der p 2 due to heat restrictions imposed by the fluorophore's heat sensitivity and the allergen's intrinsic stability likely influence its appearance on the gel slightly above its expected mass. The high intensity band at ~ 34 kDa for the copper-catalyzed click reaction indicates that these conditions favor dimer formation of the target glycoprotein.

DISCUSSION

Here, we developed the GlycoCAP platform. GlycoCAP enables installation of noncanonical glycans onto proteins in a cell-free glycoprotein synthesis system. We demonstrated the utility of GlycoCAP by producing four noncanonical glycans: α 2,3 C5-azido-sialyllactose, α 2,3 C9-azido-sialyllactose, α 2,6 C5-azido-sialyllactose, and α 2,6 C9-azido-sialyllactose, all of which can be functionalized by click chemistry.

GlycoCAP offers several potential advantages for incorporating noncanonical glycans onto proteins. For example, freedom from constraints imposed by living cells may grant access to a wider repertoire of noncanonical glycans than is possible in living cells. As an example, we observed that culturing living *E. coli* with increasing [C9-azido-sialic acid] led to decreasing optical densities under otherwise equivalent

culture conditions (Figure S5). The observation that C9-azido-sialic acid incorporated as efficiently as standard sialic acid in our cell-free system provides proof of concept that our approach can overcome cell toxicity challenges and may be able to build structures otherwise inaccessible.

While GlycoCAP offers some benefits, there remains room for improvement. For example, yields of the desirable products were not 100%. To address this limitation and make the approach suitable for longer, more complex glycans, future efforts can seek to identify glycosyltransferases with higher efficiencies. Reduced permissivity toward C5-azido-sialic acid—possibly due to disrupted binding pocket interactions in NeuA caused by the position of the azide group—could be overcome by future efforts to engineer enzymes to alter promiscuity and efficiency. The GlycoCAP platform could be used to quickly screen a wide range of enzymes toward these efforts.

The GlycoCAP platform offers a range of potential applications. As an example, higher affinity glycan ligands for Siglec binding to generate novel allergy treatments could be pursued. Studies have shown that coengagement of a Siglec receptor with the high affinity IgE receptor (Fc ϵ RI) on the surface of mast cells results in robust inhibition of mast cell activation and anaphylaxis.^{5,12} Siglec-3 (CD33) is expressed on the surface of mast cells and basophils and its endogenous ligand is α 2,3 sialyllactose and α 2,6 sialyllactose—both of which form the basis of the structures made here. GlycoCAP could be used to install clickable azido-sialoglycans onto allergen, which would engage allergen-specific IgE attached to Fc ϵ RI. The azido-sialoglycan could then be chemically modified by clicking on small chemical groups to improve CD33 binding pocket interactions and increase ligand affinity.^{49,51,52} This would generate a dual-specific therapeutic that coengages IgE/Fc ϵ RI and CD33 to inhibit the allergic response. Siglec-8 has been targeted by liposomes decorated with click chemistry modified, sulfated sialoglycans to treat eosinophilic and mast cell diseases,⁷ and Siglecs have been shown to play a role in neurodegenerative disease⁶¹ and cancer.^{62,63} The tools produced here could aid in the therapeutic development for a range of pathologies.

Looking forward, GlycoCAP provides a potentially powerful approach to discover, study, and optimize glycosylation pathways for noncanonical sugars. This could facilitate basic research and open the door for functionalizing glycoproteins, including conjugating a small-molecule drug to an antibody for localized delivery, modulating drug properties by conjugating property-endowing moieties, or barcoding or labeling proteins of interest with a nucleotide sequence or fluorophore. In addition, GlycoCAP could broaden capabilities for targeting lectins and Siglecs, paving the way for a novel class of medicines that are glycan-based therapeutics.

MATERIALS AND METHODS

Plasmid and DNA Design. The protein sequence of Der p 2 allergen was retrieved from Uniprot (accession ID: ALL2_DERPT), signaling sequence removed, and then codon optimized for *E. coli* K12 strains into a DNA sequence using the IDT Codon Optimization Tool. Additional synthetic sequences were added to optimize expression and to enable site-specific glycosylation and affinity purification (Table S1). This insert as well as the NeuA inserts (Table S2) were synthesized into a pJL1 backbone at NdeI and SalI restriction sites by Twist Biosciences.

Plasmid DNA used in CFPS reactions was purified from glycerol stocks provided by Twist Biosciences using the ZymoPURE Midi Kit (Zymo Research D4200). Plasmids have been made available on Addgene (Table S3).

Synthesis of C5- and C9-Azido-Sialic Acids. To make C5-azido-sialic acid, *N*-Azidoacetylmannosamine (317 mg, 1.21 mmol) was dissolved in H₂O (12.1 mL), followed by addition of sodium pyruvate (665 mg, 5 equiv). Neu5Ac aldolase (8 U mg⁻¹) was added, and the mixture was heated to 37 °C and stirred overnight. The solvents were evaporated, and the crude product was purified by column chromatography (0–20% 0.04 M HCl in MeCN, v/v), yielding the product (344.6 mg, 81.4%) as a white solid. ¹H NMR (500 MHz, D₂O): δ 4.16–4.06 (m, 4H), 4.01 (t, *J* = 10.3 Hz, 1H), 3.84 (dd, *J* = 11.8, 2.7 Hz, 1H), 3.75 (ddd, *J* = 9.1, 6.3, 2.7 Hz, 1H), 3.62 (dd, *J* = 11.9, 6.3 Hz, 1H), 3.56 (dd, *J* = 9.3, 1.2 Hz, 1H), 2.33 (dd, *J* = 13.1, 5.0 Hz, 1H), 1.89 (dd, *J* = 13.1, 11.5 Hz, 1H). ¹³C NMR (126 MHz, D₂O): δ 173.5, 171.0, 95.4, 70.2, 70.1, 68.2, 66.5, 63.1, 52.1, 51.9, 38.9 (Figure S3).

C9-azido-sialic acid was synthesized as described previously.⁶⁴

Harvest and Processing of *E. coli* Lysate for CFPS. *E. coli* lysate was prepared using previously published methods.³⁰ A highly productive engineered strain of MG1655-derived *E. coli* strain C321.ΔA.759³⁶ was grown in a Sartorius Biostat C+ 10 L fermentor in 10 liters of 2xYTGP media (yeast extract 10 g/L, tryptone 16 g/L, NaCl 5 g/L, K₂HPO₄ 7 g/L, KH₂PO₄ 3 g/L, and glucose 18 g/L, pH = 7.2) at 34 °C with rotation at 250 rpm. T7 RNA polymerase expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) at OD = 0.6. At OD = 3.0, cells were put on ice and centrifuged at 8000g for 5 min at 4 °C in a chilled JLA 8.1 rotor in a Beckman Avanti J-25I refrigerated centrifuge. The supernatant was discarded, and the cell pellets were washed three times with S30 buffer (10 mM trisacetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate) and pelleted by centrifugation at 10,000g for 2 min at 4 °C in a Thermo Heraeus Multifuge X3R centrifuge. Cell pellets were flash frozen and stored at –80 °C. After thawing, cells were resuspended in 0.8 mL of S30 buffer per gram of wet cell mass, distributed in 1.4 mL aliquots, and sonicated (Qsonica) at 50% amplitude for a cycle of 45 s on, 59 s off until ~950 Joules was reached. Lysate was kept on ice and spun at 12,000g for 10 min at 4 °C. The supernatant (820 μL) was collected in fresh tubes and incubated at 37° with shaking at 250 rpm for 1 h. Tubes were then spun at 12,000g for 10 min at 4 °C, and the supernatant collected from this final spin (500 μL) comprises the cell-free extract used to assemble CFPS reactions. The extract was flash frozen and stored at –80 °C until ready for use.

Assembly of CFPS Reactions. CFPS reactions were assembled based on previously published methods with the following building blocks and reagents:³⁰ 6 mM magnesium glutamate; 10 mM ammonium glutamate; 130 mM potassium glutamate; 2.646 mM ATP; 1.874 mM each of GTP, UTP, and CTP; 0.075 mg/mL folinic acid, 0.376 mg/mL *E. coli* tRNA mixture from strain MRE600 (Roche Applied Science); 0.33 mM nicotinamide adenine dinucleotide; 0.27 mM coenzyme-A (CoA); 4 mM oxalic acid; 1 mM putrescine; 1.5 mM spermidine; 57 mM HEPES at pH = 7.2; 2 mM of each of the 20 standard amino acids; 30 mM phosphoenolpyruvate (Roche Applied Science); 13.3 μg/mL designed gene construct in the pJL1 vector; and 27% v/v of *E. coli* crude

lysate (prepared above). Reagents were purchased from Sigma-Aldrich unless otherwise stated. CSTI was expressed under oxidizing conditions, which was assembled as described above with an additional 30 min preincubation of the extract in 14.3 μM iodoacetamide before supplementation into the CFPS reaction and with the additional following reagents: 4 mM oxidized L-glutathione GSSG, 1 mM reduced L-glutathione, and 3 μM purified DsbC from *E. coli*. CFPS reactions were incubated for 20 h at room temperature, with the exception of LgtB, which was flash frozen after 6 h to prevent protease degradation.

Radioactive Quantification of Proteins Expressed by CFPS. Radioactive quantification was performed based on previously published methods.^{30,65} In brief, CFPS reactions were assembled as described above with the addition of 10.67 μM radioactive amino acid, ¹⁴C-leucine. A negative control CFPS reaction where no DNA was added was included as a reference control. After 20 h of incubation, the samples were mixed with 0.5 N KOH—to precipitate the protein—in a 1:1 volume ratio (i.e., 5 μL of 0.5 N KOH and 5 μL of CFPS reaction) to quantify the total protein expression. Samples were then spun at 12,000g for 10 min, and the supernatant was mixed with 0.5 N KOH in a 1:1 volume ratio to quantify the soluble protein expression. All CFPS + KOH samples were incubated for 20 min at 37 °C. From each of these mixtures, 4 μL was dispensed onto a filtermat (PerkinElmer, 1450-421) and duplicated on a second filtermat and allowed to dry for 20 min. One filtermat for each set of samples was washed 3× in 5% trichloroacetic acid for 15 min at 4 °C, while the other filtermat was left alone. A sheet of scintillation wax was melted on a hot plate over the filtermat, and the radioactive signal was read from each sample by the Microbeta2 scintillation counter.

Protein yields (μg/mL) are calculated by the following formula:

$$\left(\frac{\text{Signal of sample on washed filtermat} - \text{signal of negative control sample}}{\text{signal of sample on unwashed filtermat}} \right) * [\text{¹⁴C-leucine (μM)}] * \text{molecular weight of protein (g/mol)} / (\# \text{ leucine residues} \times 1000)$$

Protein yields (μg/mL) can be converted to (μM) by the following formula:

$$(1000 * \text{protein yield μg/mL}) / \text{molecular weight (g/mol)}$$

Assembly of IVG Reactions. CFPS reactions were centrifuged at 12,000g for 10 min, and the supernatant was transferred to the final IVG reaction. Lysates were mixed in optimized proportions, where the target glycoprotein comprised 45% of the final reaction volume, ApNGT comprised 2%, LgtB 7.4%, CSTI/PdST6 13%, and NeuA (when included) 4.5%. Activated sugar substrates (UDP-glucose, UDP-galactose, and CMP-sialic acid) were supplemented at a concentration of 2.5 mM. Nonactivated sialic acid and CTP were supplemented at 7.5 mM and 45 μM, respectively. For incorporation of azido-sialic acids, C9-azido-sialic acid should be supplemented at 7.5 mM with CTP at 45 μM and C5-azido-sialic acid should be supplemented at 125 mM with CTP at 750 μM. IVG assemblies were incubated at 30 °C for 24 h, at which time sugar substrates were resupplemented at their optimized concentration and the reactions were incubated for another 24 h at 30 °C.

Glycoprotein Purification. IVG reactions were diluted in 50 mM NaH₂PO₄, 300 mM NaCl, pH = 8.0 (Buffer 1) and centrifuged at 12,000g for 10 min. His-tagged, glycosylated Der p 2 allergen was isolated by affinity-tag purification with Ni-NTA magnetic beads (Invitrogen Dynabeads His-tag Isolation

and Pulldown, 10104D). Beads were equilibrated with Buffer 1, then resuspended in the IVG reaction supernatant, and allowed to bind for >10 min at 4 °C with end-over-end rotation. Samples were then washed twice with Buffer 1 and eluted with 500 mM imidazole in Buffer 1 with 10 min of end-over-end mixing at room temperature.

The elution was desalted using Zeba spin columns (Thermo Scientific 89883) according to manufacturer's instructions and collected in nuclease free water (Ambion, AM9937).

LC–MS Analysis of Glycoprotein. After the samples were trypsinized with 0.0044 $\mu\text{g}/\mu\text{L}$ MS grade trypsin (Thermo Fisher Scientific, PI90057) with an incubation of at least 4 h at 37 °C, the samples were run on the Bruker Impact II ESI QTOF. Samples were first passed through an ACQUITY UPLC Peptide BEH C18 column (300 Å, 1.7 μM , 2.1 mm $\times$ 100 mm, 186003686 Waters Corp.) equipped with a 10 mm guard column of identical packing (186004629 Waters Corp.). Then, the samples were eluted at a flow rate of 0.5 mL/min and a 40 °C column temperature starting at a 1 min hold of 95% solvent A (100% water and 0.1% formic acid) and 5% solvent B (100% acetonitrile and 0.1% formic acid) with a linear gradient up to 0% solvent A and 100% solvent B for a total run time of 9 min.

MS Data Analysis. MS data were analyzed using the Bruker software. Theoretical masses in the +2 charge state (Table S4), which was determined to be the predominant form, were used to make the extracted ion chromatogram (EIC) for each pathway intermediate and final product. Glycosylation efficiency was determined by taking the area under the curve (AUC) of each intermediate's EIC and calculating the relative percentage represented by the AUC of a given intermediate relative to summed AUCs of all possible intermediates. Spectra were plotted using m/z values from 500 to 900 at the elution times encompassing EICs and deconvoluted to emphasize species of interest (Table S5).

GlycoCAP at Preparative Scale. CFPS reactions were assembled using conditions described above at sufficient volumes for addition into IVG reactions. IVG reactions were assembled at a total volume of 1 mL and were comprised of CF-expressed components in optimized relative proportions and the necessary substrates and cofactors at optimum molar concentration as described above (Figure 2). After incubation and supplementation as described for IVG assembly, His-tagged glycoprotein was purified with Ni-NTA magnetic beads (Invitrogen Dynabeads His-tag Isolation and Pulldown, 10104D). Purified samples were desalted as described above and analyzed by MS for glycosylation efficiency. Protein yield was determined by UV absorbance at 280 nm using a Thermo Nanodrop 2000 spectrophotometer (extinction coefficient: 15470, molecular weight: 17376 Da).

Click Chemistry Conjugation. Click chemistry reactions were assembled using purified glycoprotein at a total volume of 100 μL . For strain-promoted click chemistry, a stock of 3.4 mM DBCO-PEG4-Fluor 545 (Sigma-Aldrich, 760773) suspended in dimethyl sulfoxide was added to the purified and desalted glycoprotein in water (~ 250 $\mu\text{g}/\text{mL}$) at a concentration of 400 μM and allowed to incubate at 30 °C for 4 h.

For copper-catalyzed click chemistry, ~ 10 μg of purified, desalted glycoprotein product suspended in 100 μL of 1% RapiGest (Waters, 186008090) was combined with 300 μM CuSO_4 , 600 μM BTTP (Click Chemistry Tools, 1414-100), and 200 μM Alexa Fluor 546-Alkyne (Jena Bioscience, CLK-1285-1) and allowed to incubate for at least 1 h at room

temperature to allow the ligand complex to form. Sodium ascorbate (2.5 mM) was then added the reaction, and the reaction underwent an incubation period of 3.5 h at room temperature.

Fluorescent Gel. Licor protein sample loading buffer (LI-COR Biosciences 928-40004) and 4 mM DTT were added directly to click chemistry conjugation reactions containing the click-modified glycoprotein. Samples were heat denatured at 70 °C for 3 min and then run on a NuPAGE 4-12% Bis-Tris gel (Invitrogen, NP0321BOX) at 150 V with MOPS buffer (Thermo Fisher Scientific, NP0001). Gel was imaged on a LI-COR-Odyssey Fc imaging system on the 600 and 800 channels and formatted in ImageStudio.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.3c00017>.

Figure S1: Total and soluble cell-free expression of pathway enzymes, Figure S2: NeuA DNA expression and glycosylation with and without a CAT linker, Figure S3: NMR spectra of synthesized C5-azido-sialic acid, Figure S4: Coomassie-stained protein gel and EIC of scaled up azido-sialoglycoprotein production, Figure S5: C9-azido-sialic acid demonstrating *E. coli* cell toxicity, Table S1: DNA design of target glycoprotein, Table S2: NeuA DNA design with and without a CAT linker, Table S3: Addgene IDs for plasmid accession, Table S4: Theoretical m/z values, and Table S5: Peak lists used to generate mass spectra (PDF)

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Notes

The authors declare the following competing financial interest(s): B.S.B. receives remuneration for serving on the scientific advisory board of Allakos, Inc. and owns stock in Allakos. He receives consulting fees from Third Harmonic Bio, Lupagen, Sanofi, and Acelyrin. He receives publication-related royalty payments from Elsevier and UpToDate. He is a co-inventor on existing Siglec-8-related patents and thus may be entitled to a share of royalties received by Johns Hopkins University during development and potential sales of such products. B.S.B. is also a co-founder of Allakos, Inc. which makes him subject to certain restrictions under university policy. The terms of this arrangement are being managed by Johns Hopkins University and Northwestern University in accordance with their conflict-of-interest policies. M.C.J. has a financial interest in SwiftScale Biologics, Gauntlet Bio, Pearl Bio, Inc., Design Pharmaceuticals, and Stemloop Inc. M.C.J.'s interests are reviewed and managed by Northwestern University in accordance with their competing interest policies. All other authors declare no competing interests.

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