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# Decoration of proteins with sugar chains: recent advances in glycoprotein synthesis

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Chemical or chemoenzymatic synthesis is an emerging approach to produce homogeneous glycoproteins, which are hard to obtain by conventional biotechnology methods. Recent advances in the synthetic methodologies for the decoration of protein molecules with oligosaccharides provide several remarkable syntheses of homogeneous glycoproteins. This short review highlights several of the latest syntheses of glycoproteins including therapeutically important glycoproteins, a highly glycosylated protein, and unnatural glycoproteins in order to illustrate the power of the modern glycoprotein synthesis. Structurally defined glycoproteins are a novel material for understanding the molecular basis of glycoprotein functions and for the development of the next generation of biopharmaceuticals.

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## Introduction

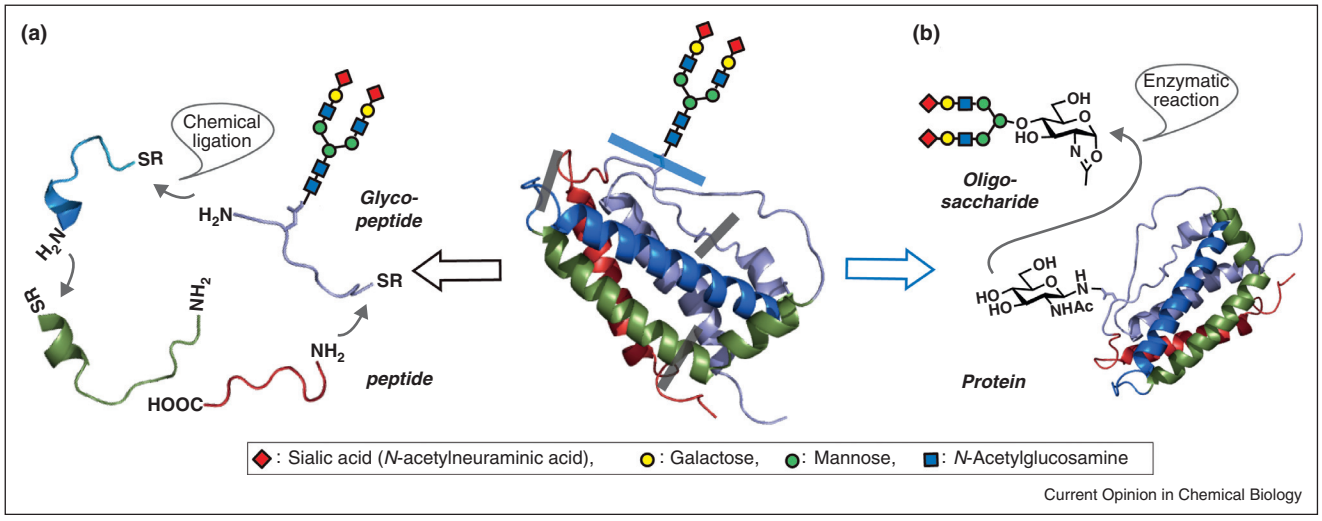
Protein glycosylation is one of the most frequently observed posttranslational modifications (PTM) in eukaryotes. Glycosylated proteins (glycoproteins) play important roles in many biological events such as cell–cell adhesion, immune response, fertilization, or inflammation [1]. This ubiquitous PTM is structurally classified into *N* and *O*-glycosylation. In *N*-glycosylation, an oligosaccharide is attached to the side chain of an Asn residue in the consensus sequence: Asn-Xaa-Ser/Thr (Xaa = any amino acids except for Pro) through the side chain nitrogen atom, whereas in *O*-glycosylation, the oligosaccharide is attached through the oxygen atom to the side chain of Ser or Thr residues without a consensus sequence. The biosynthetic pathways of *N* and *O*-glycosylated proteins involve an array of enzymes such as glycosyltransferases or glycosidases and, consequently, cause heterogeneity in the structure of the attached oligosaccharides [1]. Therefore, the resultant glycoproteins are found as a mixture of diverse glycoforms that have the same protein scaffold but different glycosylation patterns. Recombinant DNA expression technology can produce therapeutic quality glycoproteins, however, it is still not straightforward to suppress the generation of heterogeneity despite the considerable efforts that have been made to this end [2]. This inherent difficulty of producing homogeneous glycoproteins has been a severe hindrance for investigations at a molecular level of the precise correlation between oligosaccharide structures and whole glycoprotein function.

In this context, synthetic methodologies relying on chemical/chemoenzymatic synthesis have been developed and have successfully provided a variety of syntheses of glycoproteins in the last a few years [3,4••,5••,6••,7,8••,9,10•,11–13,14••,15–18]. This short review focuses on several recent syntheses of glycoproteins including therapeutically important glycoproteins, a highly glycosylated protein, and unnatural glycoproteins, in order to illustrate the power of modern glycoprotein synthesis. These remarkable advances in synthetic methodologies allow us to obtain homogeneous glycoproteins that are novel materials for functional studies and for the development of the next generation of biopharmaceuticals. For early achievements in this field, readers are recommended to follow these excellent comprehensive reviews [19–21].

## Modern synthetic strategies for the synthesis of glycoproteins

Before discussing recent synthetic achievements in glycoprotein synthesis, we briefly depict two modern strategies for the preparation of homogeneous glycoforms [20,21]. Two distinct sets of precursors can be considered for glycoprotein synthesis: peptide/glycopeptide or protein/oligosaccharide (Figure 1). In the first case, sophisticated chemoselective peptide coupling reactions such as native chemical ligation (NCL) or the thioester method are employed to connect these peptide/glycopeptide segments leading to the desired glycoprotein structure (Figure 1(a)) [22,23]. To utilize the peptide coupling reactions, preparation of peptide/glycopeptide thioesters, which is an activated form at C-terminus, is essential. Of these components, glycopeptide derivatives are key components that can be prepared by either solid phase peptide synthesis (SPPS) using glycosyl-amino acids for both *N*-linked and *O*-linked glycopeptide derivatives, or the

**Figure 1**




Schematic representation of the modern synthetic strategy of glycoproteins. **(a)** Synthesis from peptides and glycopeptides employing chemical ligation reactions. Both segments are synthesized by SPPS. **(b)** Convergent synthesis from oligosaccharides and proteins relying on enzymatic coupling reaction. In the modern fashion, the protein component is prepared by enzymatic trimming of oligosaccharides of recombinant heterogeneous glycoproteins. Attached oligosaccharide structures can be diverse and the typical example is described here as cartoon representation.

condensation reaction of glycosylamines with protected peptides having a side-chain free Asp residue as originally reported by Lansbury for *N*-linked glycopeptide derivatives which method is recently extended to SPPS [24–27].

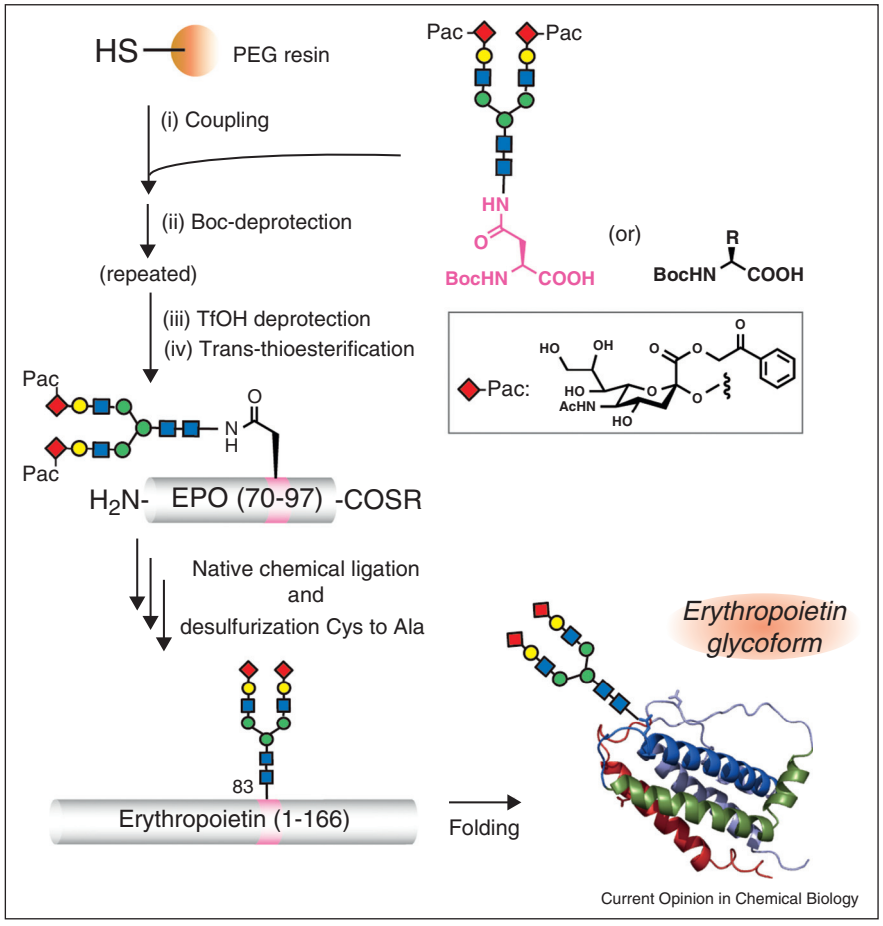
In the second case, an enzymatic reaction is utilized to covalently incorporate the homogeneous oligosaccharide into the protein (Figure 1(b)). This synthetic strategy is broadly applicable to the synthesis of relatively large *N*-linked glycoproteins. For both synthetic strategies, the oligosaccharide components are independently prepared as key building blocks by chemical, enzymatic, chemoenzymatic synthesis, or by isolation from natural sources [19–21]. The most recent reported glycoprotein syntheses have been achieved by either the synthesis from peptide/glycopeptide building blocks or from oligosaccharide/protein as discussed in the following sections.

## Synthesis of an erythropoietin glycoform: a synthetic milestone of therapeutic glycoproteins

Erythropoietin (EPO) is an erythropoiesis hormone glycoprotein that has been developed as a biopharmaceutical for the treatment of anemia associated with chronic kidney disease. This therapeutically important glycoprotein consists of 166 amino acids deduced from the cDNA sequence, which can be processed by removal of the C-terminal Arg residue into 165 amino acids during the secretion pathway, with three *N*-linked oligosaccharides incorporated at Asn 24, 38 and 83, and one *O*-linked oligosaccharide at Ser 126. It is well known that the *N*-linked sialyloligosaccharides, which have sialic acid residues at the non-reducing end, are essential for the exertion of EPO bioactivity *in vivo*. Therefore, preparation of EPO as a homogeneous glycoprotein is essential for the study of the relationship between the precise structures of the attached oligosaccharide and the bioactivity of EPO. Considerable efforts have been devoted to the total chemical synthesis of the glycosylated EPO, however, it has been quite challenging to obtain the intact glycoprotein homogeneously because of the intrinsically complex sialyloligosaccharide structures [12,28•].

In this context, Murakami et al. have successfully synthesized a single glycoform of EPO analogue having one disialyl-undecasaccharide at Asn 83 from the peptides/sialylglycopeptide strategy (Figure 2) [6••]. They prepared a sialylglycopeptide-$^{\alpha}$thioester, which was an essential component for the EPO synthesis, using a phenacyl (Pac) esterified sialyloligosaccharide-Asn as a key building block. This component was derived from a sialyloligosaccharide-Asn, which in turn was isolated from hen egg yolk [29]. The phenacyl ester confers stability on the sialyl-linkage which is usually quite unstable in acidic media. This modification enabled the authors to synthesize a sialylglycopeptide-$^{\alpha}$thioester efficiently by Boc SPPS employing a trans-thioesterification reaction for the detachment of the assembled peptide-$^{\alpha}$thioesters from the solid support without HF treatment. NCL of the glycopeptide-$^{\alpha}$thioester with peptide-$^{\alpha}$thioesters prepared by conventional SPPS followed by *in vitro* folding reaction gave an EPO derivative as a single glycoform.

**Figure 2**




Chemical synthesis of a sialylglycopeptide-$^{\alpha}$thioester and its use in the total synthesis of a homogeneous EPO glycoform reported by Murakami et al. [6••].

This synthetic EPO analogue having an intact *N*-linked sialyloligosaccharide exhibited full bioactivity *in vitro* in good agreement with previously reported bioactivity of a recombinantly prepared EPO having single *N*-linked oligosaccharide [30]. Their concise synthetic strategy comprising a new efficient synthetic method for the synthesis of sialylglycopeptide-$^{\alpha}$thioesters allows us to expect the synthesis of more complex EPO bearing three *N*-linked sialyloligosaccharide at the native glycosylation positions.

## Chemical synthesis of a highly glycosylated antifreeze protein

Total chemical synthesis is also applicable for the preparation of *O*-linked glycoproteins. Antifreeze glycoprotein (AFGP) is an *O*-glycosylated protein which has a repetitive sequence composed of Ala-Thr-Ala unit where Thr residue is glycosylated by Galβ(1-3)GalNAc through an α-linkage (repeat number = 4–50) to thus form a highly glycosylated protein molecule. AFGP can prevent the growth of ice crystals to protect against cryoinjury in subzero degree environment such as the deep sea in the Arctic or the Antarctic [31]. To obtain in-depth structure–activity relationships of such an unusual glycoprotein, Wilkinson et al. carried out the total synthesis of homogeneous AFGPs [8••]. They designed an *O*-glycosylated dodecapeptide and an *O*-glycosylated dodecapeptide-$^{\alpha}$thioester having four *O*-glycosylations as unit peptides to assemble the repetitive sequence efficiently by NCL in conjunction with metal-free Cys to Ala desulfurization reaction. The *O*-glycopeptide was synthesized by Fmoc SPPS in a straightforward fashion, while the *O*-glycopeptide-$^{\alpha}$thioester was prepared by coupling of a side chain protected *O*-linked glycopeptide-$^{\alpha}$carboxylic acid and an alkylthiol in solution phase followed by side chain deprotection [32]. After coupling of these segments, a part of the product was converted into the thioester form by coupling with the alkythiol using PyBOP and used for the next ligation step. By repeating these coupling-thioesterification steps, Wilkinson et al. successfully prepared a set of homogeneous AFGP derivatives having a range of molecular weights and used them to investigate

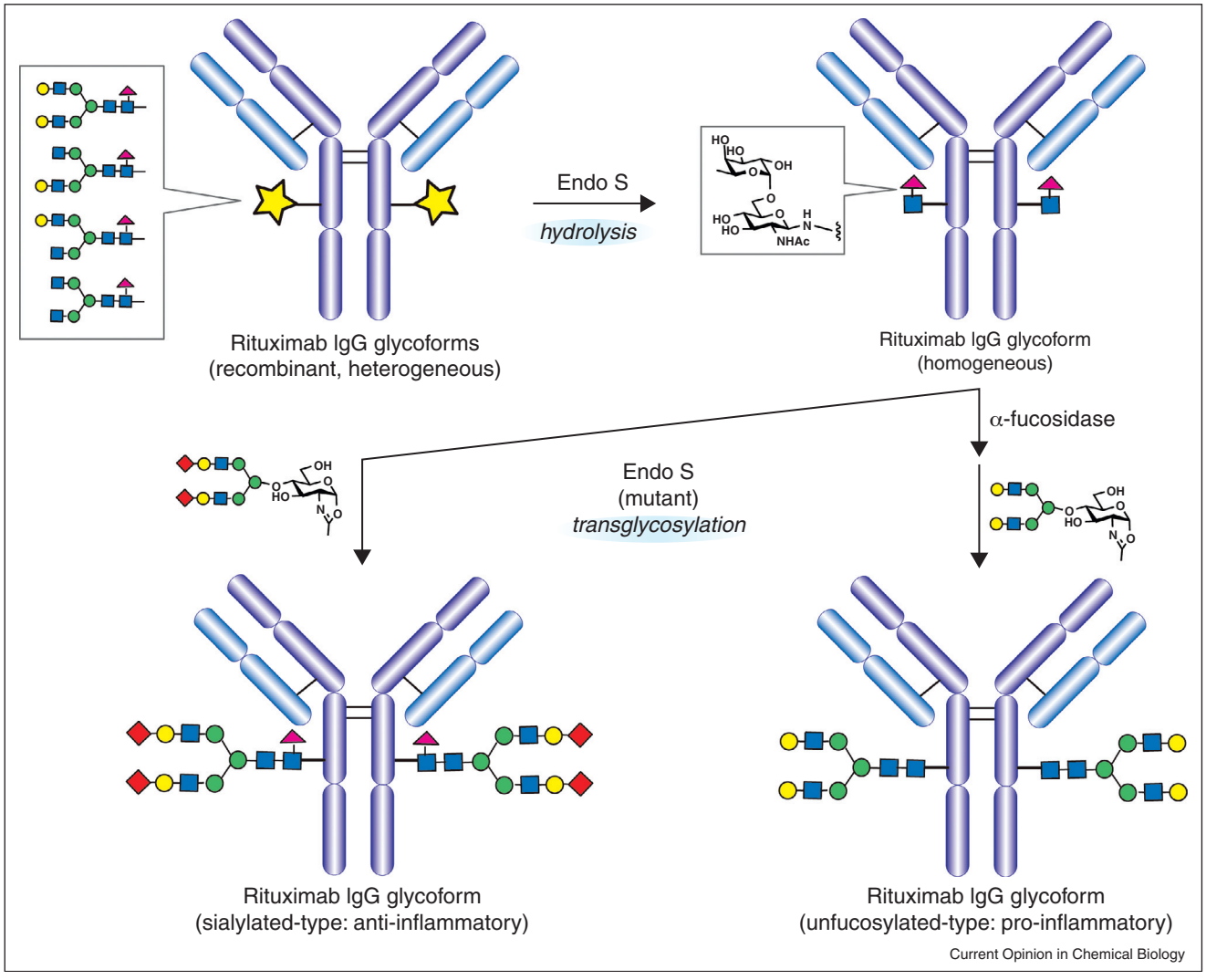
the correlation between the repeat number of AFGP and its anti-freeze activity.

## Chemoenzymatic synthesis of IgG: synthesis of a gigantic glycoprotein

While chemoselective coupling strategy using peptides and glycopeptides as building blocks is a suitable strategy for the synthesis of glycoprotein of moderate size having several glycosylation sites, chemoenzymatic synthesis using an oligosaccharide and a protein can provide relatively large glycoproteins efficiently. Human IgG antibody is a 150 kDa homodimer glycoprotein bearing an *N*-linked oligosaccharide at Asn 297 of the Fc domain. The binding event of IgG to Fcγ receptor, which is responsible for the antibody-dependent cellular cytotoxicity activity, is correlated with the structure of the *N*-linked oligosaccharide attached [33,34]. Therefore, the preparation of a homogeneous IgG glycoform is essential to provide a fine-tuned antibody with maximum efficacy.

To achieve the synthesis of such a gigantic glycoprotein, Huang et al. has demonstrated a chemoenzymatic synthesis of an intact rituximab, which is a therapeutic monoclonal IgG antibody [4••]. Their synthetic strategy was based on two-step glycoengineering: enzymatic trimming of the heterogeneous oligosaccharides of a recombinantly prepared IgG by endo-β-*N*-acetylglucosaminidase from *Streptococcus pyogenes* (EndoS) and subsequent glycan remodeling by transglycosylation with the same EndoS, which activity was identified recently by the Davis group [35••] (Figure 3). EndoS can cleave the β1,4-glycosidic bond of the chitobiose at the reducing end of the *N*-linked oligosaccharide with or without the core α1,6-fucose

**Figure 3**




Chemical synthesis of an IgG glycoform by a two-step glycoengineering strategy using EndoS as reported by Huang et al. [4••]. They demonstrated the syntheses of six kinds of IgG glycoforms and some examples are shown here.

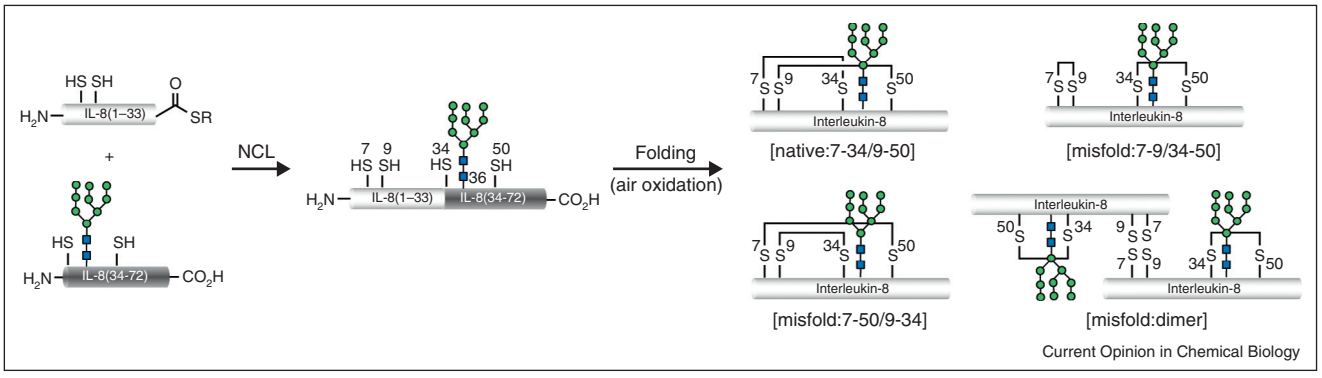
residue [35••]. Utilizing this unique ability of EndoS, Huang et al. prepared homogeneous glycosylated IgG having Fucα(1-6)GlcNAc residue from commercially available rituximab IgG (Figure 3). On the resultant glycosylated-IgG they performed transglycosylation by using an EndoS mutant (D233A or D233Q) and chemically synthesized *N*-linked oligosaccharide-oxazoline, which is a mimic of transition state for the glycosidase. In this glycosidase mutant, hydrolysis activity is eliminated by site-directed mutagenesis but transglycosylation activity is maintained to attach the oligosaccharide to the remaining GlcNAc residue on the IgG [36–38]. As a consequence, Huang et al. demonstrated the syntheses of six kinds of IgG glycoforms having a homogeneous oligosaccharide respectively such as disialyl-undecasaccharide related to anti-inflammatory activity or unsialylated and unfucosylated nonasaccharide related to pro-inflammatory activity. Intriguingly, although several glycoprotein syntheses have employed a similar synthetic strategy using the other types of endo-glycosidase such as EndoA, EndoM or EndoH, only EndoS showed significant activities of hydrolysis and transglycosylation on the IgG glycoprotein [3,9,39].

## The chemical synthesis of a 'misfolded' glycoprotein: a unique unnatural glycoprotein

In the above sections, we introduced typical examples of the syntheses of naturally occurring glycoproteins. A remarkable ability of chemical synthesis is that it can also provide *unnatural* glycoprotein derivatives. Izumi et al. reported a unique chemical synthesis of an intentionally misfolded glycoprotein as a new probe for the investigation of the glycoprotein quality control system in the endoplasmic reticulum (ER) [5••,40]. In the early stage of the glycoprotein biosynthetic pathway in eukaryotic cells, a specific *N*-linked oligosaccharide $Glc_3Man_9GlcNAc_2$ is attached to a nascent polypeptide and processed into $Man_9GlcNAc_2$ (M9) by trimming of the Glc residues. This specific oligosaccharide is used as a tag by UDP-glucose:glycoprotein glucosyltransferase (UGGT) to monitor the completion of the protein folding process [41,42]. UGGT is a key enzyme in this system; it can transfer one glucose residue to the M9 oligosaccharide and convert it to $Glc_1Man_9GlcNAc_2$ (G1M9) only when the protein folding is incomplete (misfolded). Immature glycoprotein with the G1M9 oligosaccharide is passed back to the lectin-like chaperones calnexin/calreticulin for refolding. Despite the curious function of UGGT as a 'folding sensor', the molecular basis for the discrimination of correctly or incorrectly folded structures in the ER has not been elucidated.

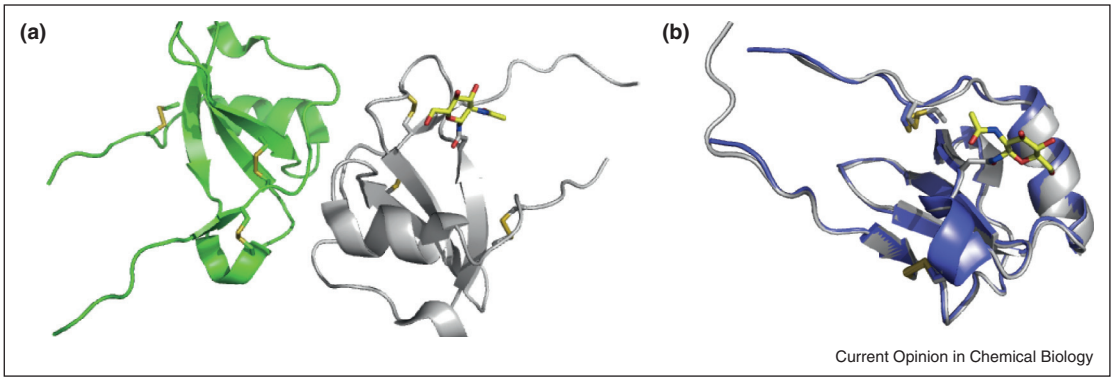
To address this issue, Izumi et al. designed and synthesized a disulfide bond-swapped glycoprotein as a structurally defined misfolded glycoprotein which can be a substrate of UGGT (Figure 4). They chose interleukin-8 (IL-8) as a protein scaffold, which has two disulfide bonds in the native form. Using M9 oligosaccharide isolated from egg yolk [43], a full-length IL-8 polypeptide bearing M9 oligosaccharide was prepared from a chemically synthesized peptide and a glycopeptide segment and was subjected to folding under air oxidation conditions. This kinetically controlled disulfide bond forming reaction gave a native form of the glycosylated IL-8 protein, along with three misfolded forms of glycosylated IL-8 including two kinds of disulfide bond-swapped structures and one unnatural dimer structure assembled by an intermolecular disulfide bond (Figure 4). All structures were characterized by circular dichroism (CD) analysis and by disulfide-bond mapping after enzymatic digestion. It turned out that those three misfolded glycoproteins are well recognized by UGGT and monoglucosylated, whereas the native form was not glucosylated. This means the uniquely designed misfolded glycoprotein can serve as a misfolded glycoprotein and thus can be a powerful probe to investigate the molecular basis of the function of UGGT or other enzymes and proteins involved in the ER glycoprotein quality control system [44•].

**Figure 4**




Unnatural glycoprotein syntheses; schematic representation of the synthesis of intentionally misfolded glycoprotein analogues [5••].

**Figure 5**




X-ray crystal structures of the structurally defined homogeneous glycoproteins: **(a)** quasi-racemate (green: D-protein, gray: glycosylated L-protein); **(b)** superimposed structure of glycosylated L-protein and nonglycosylated L-protein obtained from racemic protein crystallization (blue: non-glycosylated L-protein) [14••].

## Synthesis of a glycoprotein for the quasi-racemic X-ray crystallography

The last example is the chemical synthesis of a defined glycoform of the chemokine Ser-CCL1 to facilitate quasi-racemic glycoprotein X-ray crystallography. X-ray crystallography is one of the most robust methods for the analysis of complex biomolecules. However, it is well known that glycoproteins are difficult to crystallize due to the inherent flexibility of the oligosaccharide moiety, and this has hampered the progress of glycoprotein X-ray crystallography. In 2008, Kent et al. demonstrated 'racemic protein crystallography' that gives facile crystallization and affords high quality protein crystals by using a racemic protein mixture composed of equimolar amounts of a naturally occurring L-protein and the mirror image D-protein which is made from D-amino acids and achiral amino acid glycine [45,46]. Recently, this method has been extended to facilitate the crystallization of glycoproteins as 'quasi-racemic' protein mixtures where the glycosylated L-protein is crystallized with an equimolar amount of the non-glycosylated D-protein [13,14••]. Each component was prepared by total synthesis using modern chemical ligation methods, and the resultant glycoprotein quasi-racemate facilitated crystallization and gave diffraction quality crystals. The X-ray structure obtained from the diffraction data revealed that *N*-glycosylation did not affect either the local or the overall structure of the protein part of the glycoprotein, by comparison with the crystal structure of non-glycosylated form of L-Ser-CCL1 obtained from true racemic protein crystal (Figure 5). This result demonstrated a unique utility for chemical synthesis of glycoproteins and may open a new avenue for the investigation of the role of glycosylation by structural biology.

## Conclusions

In this short review, we have described the potential utility of precisely defined homogeneous glycoproteins with several representative examples. The development of modern synthetic chemical strategies and methodologies has allowed access to both natural and unnatural glycoproteins. Furthermore, it is also now feasible to associate detailed structural analysis of the glycoproteins prepared by chemical synthesis. These advances suggest that glycoproteins, which is one of the major classes of natural products, can be recognized as a target molecule of chemical/chemoenzymatic synthesis methods, just like classical natural product synthesis of small molecules. Structurally defined synthetic glycoproteins may provide an opportunity to systematically uncover the many intriguing biological functions of glycosylation and also to produce a next generation of biopharmaceuticals based on single glycoform glycoproteins. The most recent successes in glycoprotein synthesis will accelerate further developments of new chemical reactions and technologies to underpin the synthesis of more complex glycoproteins or to develop high-throughput synthetic methodologies.

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