Recent Advances in Glycoprotein Synthesis: A Refined Synthetic Probe towards the Biological World

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Abstract

Protein glycosylation is the most complex and diverse form of post-translational modification in human body. Meanwhile, glycosylation of peptides and proteins emerges as a promising strategy to improve the pharmacokinetic profile of peptide- and protein-based therapeutics. Owing to the importance of protein glycosylation, rigorous evaluation of the relationship between the precise structure and biological function of glycoproteins has to be per-formed. Recently, chemical synthesis, chemoenzymatic synthesis and semisynthesis strategy have attracted extensive attentions towards the prepara-tion of structurally defined glycopeptides and glycoproteins; the obtained synthetic glycoforms thus enable the thorough investigation of specific effects of protein glycosylation. This review highlights the recent progress in the development of novel strategies, preparation of homogeneous glycoproteins and exploration of structure-activity relationships. On this basis, the challenges and prospects are discussed.

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Comprehensive Summary

Protein glycosylation is the most complex and diverse form of post-translational modification in human body. Meanwhile, g

Keywords

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Left to Right: Author 1, Author 2, Author 3

Contents

1. Introduction

Protein glycosylation is a most complicated type of post-translational modifications.[1-3] The variety of glycan structure, glycosidic sites and glycosidic linkage, added up to the complexity of protein glycosylation. Protein glycosylation, unlike pure protein and oligonucleotide synthesis, is not template mediated; hence, the glycosylation outcome depends on the activity and concentration of glycan substrates, the structural and conformational properties of the catalytic domain and the different reactivities of each enzyme involved in the glycosylation process.[3] In this way, the resulting mixture of glycoproteins, termed glycoforms, with likely varied glycan structure as well as site occupancy, are regarded as valuable materials towards better understanding of protein glycosylation in live system. Despite the challenges in accessing well-defined homogeneous glycoproteins, it has been one of the most frequently studied area in the past decades, thus enabling the ongoing structural and functional studies of protein glycosylation. Researchers from synthetic chemistry and chemical biology devoted numerous efforts in this regard, and significant achievement was realized.[4] Apparently, we are getting closer to the code of life: novel synthetic methods were developed, and various decent-sized glycoproteins were synthesized, which had never been achieved before. Of particular significance is the breakthrough of synthetic glycoforms: we can now probe the effect of protein glycosylation systematically through a library of glycoproteins, each in homogeneous form, with distinct glycan chains attached to the protein respectively, and we believe that with additional efforts we will be able to connect such information with the actual outcome in cells and even in individual people. Hereby it is our great honor to give a brief summary of the progress made in this field. Rather than lengthy comprehensive prose, we would like to highlight the breaking news in recent three years.

Novel synthetic methods is fundamental for the successful assembly of glycoproteins.[5-10] Synthesis of each component, being glycans and proteins, as well as the glycosidic linkage in between, remains a demanding task. In order to develop viable and efficient strategies to chemically construct homogeneous complex glycopeptides and glycoproteins, extensive efforts have been made in the field. To date, protein chemical synthesis and semisynthesis, enabled by solid phase peptide synthesis and various chemical ligation strategies,[10-14] have made it possible to access native proteins of decent size. Furthermore, considering the presence of various functionalities within the glycoprotein molecule, synthetic tactics are generally required regarding the efficiency and beauty in the successful assembly of glycoproteins.

Of particular significance is the breakthrough of synthetic glycoforms: we can now probe the effect of protein glycosylation systematically through a library of glycoproteins, each in homogeneous form, with distinct glycan chains attached to the protein respectively. Apparently, we are getting closer to the code of life: novel synthetic methods were developed, and various decent-sized glycoproteins were synthesized, which had never been achieved before. and we believe that with additional efforts we will be able to connect

such information with the actual outcome in cells and even in individual people. Hereby a brief summary of the progress made in this field is presented. Rather than lengthy comprehensive prose, we would like to highlight the breaking news in recent three years.

2. Methods Development towards Efficient Assembly of Glycoproteins

2.1. Development and Application of Synthetic Methods

Better understanding of the essence of protein glycosylation calls for more methodological developments and synthetic efforts. Based on the glycosidic linkage, glycoproteins can be categorized different types, commonly known as N-linked, O-linked, S-linked and C-linked glycoproteins, as well as phosphoglycans and glypiated proteins.[1] To date, chemical synthesis of glycoproteins mostly focused on the N- and O-linked glycoproteins, which represents a majority of glycoproteins in nature. While chemical ligation methods contribute to join peptide fragments toward respective proteins, glycans are mostly introduced at the stage of peptide synthesis (Figure 1). The linear approach is quite straightforward, where the glycosyl amino acids are pre-formed as individual building blocks, and are incorporated for the assembly of glycoproteins (Figure 1, Approach A).[5] Alternatively, a convergent approach denotes that the glycan and the protein are prepared separately, and then conjugated together to give the glycoprotein (Figure 1, Approach B). Generally, N-linked glycoproteins, where the glycosyl amine is conjugated to the protein at asparagine site with the formation of an amide bond, can be performed in a convergent manner.[15-16] In this case, an orthogonally protected Asp residue is introduced initially at the glycosylation site, which is selectively unmasked and subjected to aminolysis by glycosyl amine to give the N-glycosylated Asn. Otherwise, N-linked glycoproteins can be obtained in linear fashion as well, incorporating the structurally defined N- glycosyl-Asn building blocks into solid phase peptide synthesis to generate glycopeptides. These building blocks, obtained either through the chemical coupling of a glycosyl amine precursor to an Asp derivative, or through isolation from natural sources followed by suitable protection,[17-18]have made a number of N-linked glycopeptides and glycoproteins with various glycan structures accessible. For example, the asparagine with a homogeneous complex-type biantennary sialyloligosaccharide can be isolated from egg yolk or egg powder,[17, 19]whereas ones with high mannose type N-glycan can be isolated from soybean flour,[20-21] generally on milligram-to-gram scale. However, O-linked glycoproteins, where the O-glycans are attached to the protein via serine, threonine or tyrosine residues, are more frequently subjected to stereoselectivity issue during the formation of O-glycosidic linkage, and are thus preferably prepared in a linear way (namely, Approach A). The O-linked glycosyl amino acids, generally termed "cassettes", are prepared as building blocks and then incorporated into the peptide fragments.[22] Based on their respective chemical property, novel methods towards the preparation of S-linked and C-linked glycopeptides are also developed, adding to the toolbox of glycopeptide and glycoprotein synthesis.

Figure 1 Approaches to N- and O-linked glycopeptides.

To improve the synthetic efficiency of convergent aspartylation, a modified aminolysis protocol was introduced using aspartyl active esters.[23-24] Compared with the thio- or oxoester counterparts, aspartyl selenoesters demonstrated enhanced reactivity, among which the seleno-phenyl ester afforded an optimal balance between high reactivity and sufficient stability. In particular, the selenoester-mediated glycosylation takes place without additional coupling reagents or catalysts, and is highly compatible with free C-terminal carboxylic acid, as well as multiple functional groups within the complex-type sialylundecasaccharide. Facilitated by these method, a couple of N-linked glycopeptide fragments bearing mono-, di-, and oligosaccharide were synthesized in high yields, including ones derived from human interleukin-5 and erythropoietin (EPO).

Another interesting discovery for the convergent synthesis of N-linked glycopeptides is the thioamide-directed activation of aspartate strategy (Figure 1, Approach C).[25] Basically, the incorporation of a thioamide N-terminal to an Asp residue facilitates Ag(I)-promoted conversion into respective N- glycosylated Asn residue in the presence of a glycosyl amine. An optimal result was obtained when using 1.2 equiv of Ag_2CO_3, and a moderate excess (4 equiv.) of glycosyl amine, with (oxo)amide observed as a minor byproduct. This thioamide-triggered approach is highly chemoselective, and leads to topographically-defined N-glycosylated

asparagine derivatives. Expectedly, further investigations are welcome such as incorporation of more complex glycans, and potential application to on-resin glycosylations.

The frequent occurrence of aspartimide formation represents a formidable challenge in N-linked glycopeptide synthesis, especially during the process of convergent aspartylation (Figure 2A). Continuous efforts were engaged in this regard, with modified protocols developed over years. For example, pseudoproline-assisted aspartylation was devised as a general approach.[26-27] By converting the consensus sequence for N-glycosylation N*XS/T* residues into a pseudoproline moiety, a twist in the protein backbone can be generated,[28] thus preventing the formation of aspartimides, both in peptide elongation and convergent aspartylation (Figure 2B). At the end of glycopeptide synthesis, global acid treatment regenerated the native sequence concomitantly. This finding is crucial to the success of convergent aspartylation, that it has become the method of choice for N-linked glycoprotein synthesis, with the construction of some most challenging synthetic targets such as N-linked RNase,[26] erythropoietin (EPO)[26-27] and E-cadherin[29] glycopeptides. Considering that the protecting group of aspartic acid had an impact on the reaction outcome, a cyanosulfurylide moiety was developed as a novel carboxylic acid protecting group for Asp, to prevent aspartimide formation during peptide synthesis (Figure 2C); yet, the applicability is to be demonstrated in a broader context of glycopeptide synthesis.[30] To avoid the exposure of aspartic acid residues during peptide synthesis, a late-stage oxidation strategy was introduced, employing homoserine as a precursor of aspartic acid (Figure 2D).[31] Upon the full assembly of peptide sequence, oxidation using (2,2,6,6-tetramethyl-piperidin-1-yl)oxyl (TEMPO) and bis(acetoxy)iodobenzene (BAIB) led to the conversion into aspartic acid at respective sites. The method extended to the synthesis of a glycopeptide containing aspartic acids and an O-sulfated glycan.

Figure 2 (A) Aspartimide formation and (B-D) preventative measures

A proteoglycan is a subtype of O-linked glycoprotein found in cell membranes within mucus and connective tissue; it is composed of core proteins covalently-bonded to glycosaminoglycans (GAGs).[32] Owing to the acid lability of sulfates, proteoglycans are recognized as a most difficult synthetic target integrating the sulfated glycosaminoglycans with the peptide backbone.[33-36] Syndecan-1 is a prototypical transmembrane proteoglycan commonly found on cell surface, which carries heparan sulfates (HS) and chondroitin sulfate (CS) glycan chains at its ectodomain.[32] While the sulfation patterns of HS and CS dictate many activities, the core protein can potentially impact HS functions. Hence, the preparation of a collection of site-specifically modified syndecan glycoforms and sulfo-forms is highly desirable. However, the presence of sulfates poses severe challenge to its synthesis: as sulfates are highly acid labile, common solid phase peptide synthesis is not applicable. To this end, suitable protection on sulfates led to successful synthesis of syndecan glycopeptides.[37] Towards better understanding of the interplay between the glycosaminoglycan chain and core protein backbone, construction of larger syndecan molecules up to glycoprotein sized level, was highly desirable. In this regard, a novel "glycopeptide cassette" approach was introduced,[38] where a short-sequenced, orthogonally functionalized glycopeptide was designed as a cassette to allow for site-specific sulfation and subsequent chemical ligation; starting there, microwave-assisted Ag(I) mediated ligation, along with native chemical ligation, Ser/Thr ligation and desulfurization to give ectodomain (23-120) of syndecan-1, representing the longest O-sulfated syndecan-1 molecule synthesized ever (Figure 3).

Figure 3 "Glycopeptide cassette" approach towards O-sulfated glycoprotein synthesis

Well-established chemical ligation strategy is fundamental to the successful preparation of glycoproteins with site-specific glycosylation. Distinct from the conventional linear and convergent approaches, Kajihara et al. developed a novel bifunctional thioacid-mediated strategy, for the (semi)synthesis of N-linked glycoproteins (Figure 4).[39] The key point rests on a well-designed bifunctional glycosyl asparagine thioacid, which can couple two peptide fragments at its N- and C-termini, respectively, thus leading to the target glycoproteins within short steps. The strategy takes place via a two-step transformation: 1) a diacyl disulfide coupling (DDC) between a glycosyl asparagine thioacid and a peptide α-thioacid, which affords a native amide bond via a disulfide bond intermediate without any polymerization reactions; and 2) the resultant glycopeptide has a thioacid form at its C-terminus, which will undergo thioacid capture ligation (TCL) with another peptide bearing a disulfide functional group at its N-terminus to afford the full-length glycoprotein backbone.

Figure 4 (Semi)synthesis of N-glycoproteins via a bifunctional glycosyl asparagine thioacid

Likely due to the steric hindrance of glycans, which could slow the competitive polymerization of the thioacid moiety, the DCC reaction proceeds chemoselectively with the glycosyl asparagine thioacid; subsequently, an intramolecular S-N acyl transfer readily occurs on the diacyl disulfide intermediate, yielding the corresponding amide product predominantly. Yet, all internal peptide cysteines should be protected by the acetamidomethyl (Acm) or phenylacyl (Pac) protecting group because their thiol groups can prevent essential disulfide bond formation in DDC and TCL. The stereochemistry of this chemical transformation was carefully characterized, via extensive NMR and reverse-phase HPLC analysis, which confirmed that each individual DDC and TCL reaction did not give rise to epimerization. The established method was extended to the synthesis of the cytokine glycoproteins CCL1 and interleukin-3 (IL-3), consisting of 73 and 133 amino acid residues, respectively. Together, this thioacid-mediated strategy represents an additional strategy along with existing protein chemical synthesis strategies, such as native chemical ligation (NCL) and expressed protein ligation (EPL).

Considering the high nucleophilicity, cysteine residues have become a popular handle in protein modification. Thus, to directly install a glycosyl unit onto the thiol group of cysteine residues, preferably in aqueous media, is a conceptually straightforward and attractive task. A radical-based glycosylation approach was developed, which chemoselectively afforded axial S-glycosidic bonds under photocatalytic conditions (Figure 5A).[40] The approach utilized bench-stable allyl glycosyl sulfones as the glycosylating agents, to react with an S pyridinyl masked disulfide as a cysteine surrogate. O_2 should be avoided in the reaction system, as it interferes with the radical process. Moreover, a second-generation was developed with the reactive disulfide generated *in situ* . The utility of this approach successfully was exemplified with the glycosylation of various peptides, including a cell penetrating peptide-R8, Tyr-amyloid P component (27-38) and Amyloid β/A4 protein precursor 770 (135-155), and a series of MUC1 peptide 20-mer bearing S-linked Tn-antigen mimetics. Alternatively, glycosyl Bunte salts were utilized for the selective "tagging" of cysteine residues, which created glycomimetics with a disulfide bond at the conjugation site (Figure 5B).[41] The reaction takes place in water under basic condition, under an argon atmosphere. Moreover, a modified two-step protocol was developed, for donors of relatively low reactivities. Via the newly formed disulfide linkage, it has been shown that the GlcNAc-attached analogue, exhibited better blood Glc-lowering activity and higher enzymatic stability *in vivo* compared with the native GLP-1, whereas the conjugation of other sugars, such as Glc and SiaLac, did not always lead to the enhanced pharmacological activity. This information should be useful for the future design of glycosylated GLP-1 analogues. Worth mentioning, the native disulfides within the peptide substrate are well preserved during these two types of cysteine selective transformation.

Figure 5 Strategies for S-modified glycoproteins.

C-Mannosylation is a rare type of protein glycosylation and occurs in the endoplasmic reticulum in a protein co-translational manner.[42] An α-mannose is attached to C2 atom of the indole ring of Trp7 via a carbon-carbon bond. *C* -Mannosyl tryptophan was firstly discovered in human RNase 2,[43] and later in various glycoproteins such as interleukin-12, properdin, thrombospondin, mucins (MUC5AC and MUC5B), and erythropoietin receptor.[44] Owing to the knowledge gap in the biological profile of protein C-mannosylation, it has been a synthetic target of pursuit. Recently, metal-catalyzed C-H glycosylation strategy was extended to the preparation of C-mannosyl tryptophan and C-mannosyl glycopeptides. Using a new isoquinoline acid auxiliary installed on the N terminus of tryptophan, a Pd-catalyzed auxiliary-directed remote C–H glycosylation of tryptophan was developed, employing mannosyl chloride as a glycosyl donor.[45] Thus, a C2-mannosyl tryptophan was obtained with excellent α-stereoselectivity, giving rise to the first total synthesis of insect C-glycopeptide hormone Cam-HrTH-I (Figure 6A). Alternatively, late-stage C-H glycosylations of structurally complex amino acids and peptides was accomplished. A Ni-catalyzed reductive hydroglycosylation reaction was developed, where glycosyl bromides could be coupled with a variety of acetylenic amino acids and peptides with high regioselectivity and stereoselectivity (Figure 6B).[46] No epimerization of the amino acids was observed in the reaction, as determined by careful HPLC analysis for the present C-glycosylation. In particular, a highly favorable 1,2-*trans* diastereoselectivity was obtained for the C2-axially substituted

mannopyranosides, rendering the present protocol a promising method for the preparation of C-glycosyl peptides of biological and therapeutical significance.

Figure 6 (A) Pd-catalyzed C-H glycosylation for the preparation of C-mannosyl Trp and glycopeptides. (B) Ni-catalyzed reductive hydroglycosylation for the preparation of C-linked glycopeptides.

2.2. Development and Application of Enzymatic Methods

Despite the prosperity in the chemical synthesis of glycoproteins, it still encounters challenges. First of all, it requires an excess use of glycosyl building blocks, which are usually obtained through sophisticated preparation steps, thus leading to low overall efficiency. In addition, the added steric hindrance of glycans may account for decreased reactivity for the coupling of glycosyl building blocks, and even chemical ligation as well.[47] In addition to the chemical reactions discussed above, enzymatic and chemoenzymatic synthesis is emerging as an attractive approach that complements and expands the chemical methods for constructing homogeneous glycoproteins.[9] Clearly, to meet the intrinsic diversity of carbohydrates and glycoproteins in nature, the pursuit of novel enzymatic approaches has been an ongoing trend. In particular, since the currently available *in* v*i* v*o* techniques generally provide heterogeneous mixtures of different glycoforms, enzymatic *in* v*itro* methodologies have been pursued.

In addition to their hydrolytic activity, endoglycosidases can also effectively transfer oligosaccharides onto corresponding hydroxyl-containing substrates by transglycosylation.[48] This dual capability makes endoglycosidases, especially the ENGases, valuable tools for the convergent synthesis of oligosaccharides and glycoconjugates: once the initial GlcNAc moiety is installed, either from de novo chemical synthesis or from enzymatic degradation of the naturally occuring heterogeneous structure, the following enzymatic transformation readily takes place to afford various glycan structures (Figure 7). As N-linked glycoproteins adopt a pentasaccharide "core region" common to all N-glycans, ENGases-catalyzed *en bloc* transfer of respectively activated glycosyl donor appears an ideal choice for glycan remodeling, leading to N-linked glycoforms with increased diversity. Approaches to effect improvements in ENGase catalyzed glycosylation processes include the use of N-glycan oxazolines as activated sugar donors,[49-50] in combination with mutant variants of ENGases (known as "glycosynthase') with altered activity towards product hydrolysis.[51-52]

Figure 7 ENGase catalyzed preparation of N-linked glycoproteins

In addition to endoglycosidase-catalyzed *en bloc*transglycosylation, glycosyltransferases mediated glycan chain elaboration appears as another approach.[53] Such glycosyltransferases can be used to add individual glycan units to give various synthetic glycoforms. A prominent example is the successful preparation of a core m1 structured O-mannosyl glycopeptide, reported by Flitsch et al., employing human protein *O* -linked mannose β1,2-*N* -acetylglucosaminyltransferase 1 (POMGnT1) and bovine β1,4-galactosyltransferase (β1,4-GalT) respectively.[54] Similar strategy was reported by Nishimura et al., on the preparation of a synthetic library of core m1 O-mannosyl glycopeptides, which revealed specific binding pattern and conformational change at *O* -mannosylated position.[55] Recently, Li et al. reported a divergent synthesis of core m1, core m2 and core m3 O-mannosyl glycopeptides via a chemoenzymatic approach.[56]Taking advantage of an orthogonally protected mannosyl module, three core structured O-mannosyl amino acid building blocks can be accessible within minimal steps. Subsequent enzymatic galactosylation and sialylation, mediated by bacterial β1,4-galactosyltransferase from*Neisseria meningitidis* (NmLgtB) and a α2,3-sialyltransferase 1 M144D mutant from *Pasteurella multocida* (PmST1 M144D), further expanded the diversity of synthetic O-mannosyl glycopeptides.

Moreover, development of enzymatic tools towards broader application scenario has been pursued. For example, to facilitate the proteoglycan synthesis, the utility of human xylosyltransferase I (XT-I), the enzyme responsible for installation of initial xyloside on protein backbone, was investigated.[57] Furthermore, the utility of human β-1,4-galactosyltransferase 7 (β4GalT7) was extended to the synthesis of Gal-Xyl bearing glycopeptides.[58]Together, XT-I can be coupled with β4GalT7 for one pot synthesis of glycopeptides bearing galactose-xylose disaccharide, thus opening the door for expeditious chemoenzymatic synthesis of proteoglycan type glycopeptides and glycoproteins. Recently, yeast spores were developed as novel natural carriers

of glycosyltransferases.[59] Encapsulation of β1,4-galactosyltransferase from *Homo sapiens* (HsGalT), α2,3-sialyltransferase 1 from *Pasteurella multocida* (Pm2,3ST1) and α2,6-sialyltransferase from *Photobacterium damselae*(Pd2,6ST), on the surface of yeast spores, enabled facile assembly of diverse naturally occurring sialyl-galactosylated glycans via tandem reaction cascade. Furthermore, O-GlcNAcase (OGA) was adopted to aid the chemical synthesis and folding of disulfide-rich proteins, based on the establishment of a removable glycosylation modification (RGM) strategy.[60] In line with the knowledge that protein glycosylation contributes to the maintenance of its correct geometry *in vivo* , the attachment of simple O-GlcNAc moiety at the Ser/Thr sites was found to effectively facilitate protein folding process by stabilization of their folding intermediates. Upon successful folding, the RGM groups can be cleanly removed by O-GlcNAcase (OGA) to afford the correctly folded proteins with multiple disulfide bonds. Using this strategy, the first total synthesis of native interleukin-5 (IL-5) was achieved, which was otherwise unsuccessful due to the formation of misfolded IL-5 isoform (Figure 8).

Figure 8 Preparation of disulfide-rich proteins via removable glycosylation modification (RGM) strategy

2.3. Efforts towards Effort-less Purification

Material loss during the purification of peptide fragments, especially the multiple rounds of HPLC purification, accounts for the accumulative low yield of glycoprotein synthesis. To circumvent the possible purification of ligation intermediate, a one-pot four-segment ligation strategy was employed for the preparation of O-glycosylated histone H2A carrying an *O* -GlcNAc at Ser40 (Figure 9).[61] The synthesis involves a peptide thioester and two orthogonally activatable thioester equivalents, cysteinyl prolyl ester (CPE) and N-alkylcysteine (NAC). These equivalents were demonstrated to have different optimal pH conditions for thioesterification (CPE: pH $>$ 7.8; NAC: pH 4–6), thus allowing for the sequential ligation to take place in one-pot manner. For the attachment of O-GlcNAc, methylbenzyl (MBn) was utilized due to its lability in TFA cocktail during the global deprotection stage. Even though slight hydrolysis of glycosidic linkage was noted during TFA deprotection, the glycosylated segment was obtained in reasonably high yield (21%), which led to the successful assembly of the whole polypeptide sequence via one-pot ligation, with an overall yield of 54%; the following metal-free desulfurization afforded the O-glycosylated histone H2A.

Figure 9 Preparation of O-GlcNAc modified histone H2A via a one-pot four-segment ligation strategy

To save on the labors and the severe handling loss due to multiple rounds of HPLC purifications, an N-terminal bifunctional protection/purification handle (PPH) was developed (Figure 10A).[62] The handle was designed to serve dual roles: a photo-labile linker basically works as a temporary protection of N-terminal Cys, and a His6 tag allows for quick and easy purification of the peptide fragments via immobilized metal affinity chromatography (for example, Ni-NTA beads). A pH switch was installed, which enabled facile loading and elution of the peptide by adjusting the pH under mild conditions (pH 7.0 to 3.0). This protection/purification handle strategy is practically successful for the construction of glycoproteins with tandem repeats, via a couple of sequential cycles of chemical ligation and affinity purification. A quick illustrative example is the preparation of an 80-mer mucin 1 (MUC1) peptide fragment from 20-amino acid tandem repeats. Furthermore, an antifreeze glycoprotein (AFGP) bearing α-linked O-GalNAc at its Thr residue within each ATA tripeptide repeating unit,[63] was assembled up to 240-mer, which represents the largest chemically synthesized protein decorated with glycans reported to date. Driven by similarly practical considerations, a tag-assisted strategy was introduced to facilitate the step-by-step purification during the assembly of glycopeptides (Figure 10B).[64] A hydrophobic tag was initially introduced at the C-terminus, followed by peptide chain assembly. Thus, after each round of (glycosyl) amino acid coupling and Fmoc removal, the tag was there to enable quick purification via centrifugation; upon the completion of peptide chain assembly, the tag could be selectively removed using a cocktail of TFA/TFE/DCM. Thus, the resulting glycopeptides can be subjected to fragment coupling to elongate the peptide backbone, or undergo enzymatic glycan modification to obtain more sophisticated glycan structures. Following this protocol, a collection of O-glycopeptides containing various glycan chains were successfully obtained, including the SARS-CoV-2 glycopeptides, the 20-mer MUC1 glycopeptides, and a 31-mer glycosylated glucagon-like peptide-1 (GLP-1).

Figure 10 To ease the purification, (A) protection/purification handle (PPH) and (B) hydrophobic tag were introduced to glycopeptide synthesis.

3. Comprehensive Study of Protein Glycosylation Using Synthetic Glycoforms

Among the variety of post-translational modifications on protein, glycosylation is one of the most complicated modifications. Since the biosynthesis of glycans is regulated by the substrate specificity of glycosyltransferases and glycosidases, resulting in considerably diverse glycan structures (glycoforms), to understand the biological processes arising from each glycoform is never a simple routine work. Despite that the biological activity of a glycoprotein is typically modified by the glycan part, homogenous glycoproteins (glycoforms) are rarely accessible from natural sources and need to be prepared by synthesis.

Apart from chemical synthesis, semisynthesis approach exhibits advantages to obtain synthetic glycoforms with expanded structural diversity.[10, 13] Protein expression, generally carried out in *E. coli* , is able to offers unglycosylated fragments in large scale. Additionally, expression of certain peptide fragment may overcome the solubility problem encountered in chemical synthesis. Thus, with the encouraging development in synthetic chemistry and biology, allowing scientists to precisely control the subtle differences in glycan structure and amino acid sequence, it is now more possible than ever before to quantitatively interrogate the structure-property relationships of protein glycosylation, and in particular, to uncover the molecular basis of such relationships. The improved understanding promises future medical translation.

3.1 Synthetic Glycoforms with Single Glycosylation Site

The improved understanding promises future medical tr Interleukin 6 (IL-6) is a cytokine that exerts both immunostimulating and regenerating effects through binding to the IL-6 receptor with different localization.[65] Although many of the biological effects of human IL-6 have been studied in detail, little is known about the influence of its glycans. A systematic study was carried out with a representative set of IL-6 glycoforms, including complex-type and oligomannosidic N-glycans.[66] The chemoenzymatic semisynthesis of the library of IL-6 glycoforms involved three segments,[67] where the short glycopeptide segment was synthesized via by pseudoproline-assisted convergent aspartylation; the N-terminal and C-terminal segments were obtained recombinantly from the corresponding fusion protein (Figure 11). Moreover, the sialylated glycopeptides were obtained by enzymatic sialylation using the bacterial sialyltransferases PdST6 or PmST3 and purified by RP-HPLC. In all cases the sialylations required optimization of the reaction conditions. Of note, as the recombinant IL-6 peptide (49–183) is not compatible due to low recovery from preparative RP-HPLC, it was prepared by means of corresponding SUMO fusion protein followed by proteolysis. The obtained synthetic IL-6 glycoforms were then compared with the nonglycosylated reference IL-6 *E. coli.*

It was found that the native helical fold of the set of IL-6 glycoforms was independent of the glycan, where only the overall intensity of the circular dichroism spectra varied to a small extent. In addition, the N-glycan of IL-6 appears no significant effect on the binding to the cellular IL-6 receptor, judged by a proliferation assay using an IL-6-dependent cell line. Strikingly, all the IL-6 glycoforms showed different *in vivo* plasma clearance rates in rats. The unexpected differences in the biological properties of the various IL-6 glycoforms could be further revealed by providing sufficient amounts of a systematically varied library of synthetic IL-6 glycoforms.

Figure 11 Chemoenzymatic semisynthesis of IL-6 glycoforms with N-glycans at Asn44

Interleukin-2 (IL-2) is a cytokine that exerts a wide spectrum of effects on the immune system, and it plays crucial roles in regulating both immune activation and homeostasis.[68] Moreover, it is clinically applied for immunotherapy against various diseases. Proleukin ®, the recombinant human IL-2, is indicated for the treatment of adults with metastatic melanoma and metastatic renal cell carcinoma.[69] Hence, IL-2 remains one of the most extensively studied cytokines to date. Native human IL-2 is a 133 amino acid cytokine that is glycosylated at Thr 3. To expand the understanding of the structure-activity relationship, particularly with regard to glycosylation of IL-2, a concise synthetic route was devised.[70] Briefly, a synthetic glycopeptide fragment (1-5) salicylaldehyde ester was coupled with a recombinant protein fragment (6-133) via Ser/Thr

ligation (Figure 12). Using this semisynthetic protocol, the poor solubility issue, recognized as a significant hurdle in previous reports, was carefully circumvented. Thus, apart from the non-glycosylated IL-2, three different IL-2 glycoforms were prepared on multi-milligram scale, bearing O-linked monosaccharide GalNAcα-, disaccharide Galβ1-3GalNAcα-, or trisaccharide Neu5Acα2-3Galβ1-3GalNAcα- at Thr3. Biological evaluation of the homogenous glycoprotein library reveals that the activity of IL-2 in activating individual T cell subset is glycan dependent, thus highlighting the possibility of further improvement in clinical application.

Figure 12 Semisynthesis of IL-2 glycoforms with O-glycans at Thr3

Interestingly, the same set of O-glycans were found to have an effect on the progression of Alzheimer's disease. The study on O-glycosylation of amyloid-β (Aβ) peptides was carried out using three synthetic glycoforms, bearing O-linked monosaccharide GalNAcα-, disaccharide Galβ1-3GalNAcα-, or trisaccharide Neu5Acα2-3Galβ1-3GalNAcα-, at Tyr10.[71] Each glycosyl amino acid building block was chemically synthesized with defined structure. A modified solid phase peptide synthesis protocol was devised, with a removable polylysine tag to aid the purification and improve the overall yield. Amongst these synthetic glycoforms, a concentration-dependent inhibitory effect was noted, induced by Tyr10 O-glycosylation against the fibril formation of Aβ42. Besides, fewer β-sheets were observed with glycosylated Aβ42 than unmodified Aβ42; especially for trisaccharide modified Aβ42, a random coil secondary structure was clearly indicated. Moreover, increased susceptibility of disaccharide modified Aβ42 fibril toward degradation by extracellular degradation enzymes indicates a possible protective role of O-glycosylation in Aβ pathological aggregation.

Interleukin-17A (IL-17A) is a proinflammatory cytokine that plays essential roles in regulating both innate immunity and host defense in human body.[72] To address the functions of N-glycosylation at Asn45 of IL-17A, synthetic IL-17A glycoforms were prepared, each bearing O-GlcNAc, GlcNAcβ1-4GlcNAc or complex type sialylundecasaccharide, respectively.[73] A four-fragment chemical ligation strategy was adopted, where O-GlcNAc or GlcNAcβ1-4GlcNAc building blocks were introduced at respective site, followed by Endo-M-catalyzed transglycosylation[74] to elaborate the glycan chain to give the corresponding complex type sialylundecasaccharide (Figure 13). Of note, modified transglycosylation protocol was necessary to afford an improved efficiency. Compared with the glycosylated IL-17A, the nonglycosylated isoform exhibited poor stability and a much higher tendency to aggregate during the preparation, indicating protective effect induced by glycosylation against aggregation; in addition, relatively high thermal stabilities were observed for the glycosylated IL-17A. Moreover, N-glycosylation was found to interfere with the interactions between IL-17A and its receptor IL-17RA, where the binding affinity decreased as the N-glycan gets larger. Although speculations remain to be uncovered due to the absence of detailed structural information regarding N-glycans in natural IL-17A, the current research set an example to follow for interrogating the functions of glycosylation.

Figure 13 Synthesis of IL-17A glycoforms with N-glycans at Asn45

3.2 Synthetic Glycoforms with Multiple Glycosylation Sites

Adiponectin is a circulating glycoprotein mainly produced from adipocytes. It is a key regulator of glucose and lipid metabolism, thus increasing the systemic insulin sensitivity and energy homeostasis.[75] The mammalian adiponectin collagenous domain contains four 5-(2S,5R)-hydroxyl lysine residues, which are glycosylated with a glucosyl-galactose disaccharide. To fill in the knowledge gap regarding its glycosylation, and towards the therapeutic application, the effect of glycosylation on adiponectin collagenous domain was explored.[76] An exhaustive collection of homogeneously glycosylated adiponectin collagenous domains with all of the possible 15 glycoforms have been chemically and individually synthesized (Table 1), using stereoselective glycan synthesis and chemical peptide ligation.

Table 1 Synthetic glycoforms of adiponection collagenous domain

Briefly, a facile, large-scale synthesis of the (2S,5R)-hydroxylysine was developed; starting there, stereoselective glycosylation afforded Glcα1-2Galβ1-(2S,5R)-hydroxylysine building block for the glycopeptide fragment preparation. Ser/Thr ligation joined the two peptide fragments (39-60) and (61-107) together. Subsequently,

the evaluation of biological activities and pharmacological properties indicates that the glycan plays an important role of the collagenous domain of adiponectin, in the inhibition of cancer cell growth as well as the regulation of systemic energy metabolism. The current work showcases the power of chemical synthesis in systematically addressing the role of glycosylation on activity and specificity, and opens the door to explore the opportunity of using the synthetic glycopeptide as a potential supplementary towards drug development.

To tackle the challenge in biomass transformation, Tan et al. systematically probed the effects of O-mannosylation of a family 1 carbohydrate binding module (CBM) of *Trichoderma reesei*cellobiohydrolase I (*Tr* Cel7A), using a panel of 23 individual synthetic glycoforms (Table 2).[77] To elucidate the potential site-specific, size-specific and synergistic effects of CBM glycans, regarding its binding affinity for a variety of lignocellulose-derived cellulose and lignin substrates, the synthetic glycoforms were divided into six sets, including the unglycosylated CBM 1, monoglycosylated CBMs (2–4, 5–7, 8–10), multi-glycosylated CBMs (11–20), and CBM glycoforms with varied amino acid sequence or glycan structure. It appears that glycoforms that contain the same mannose structures distributed across all available sites (13, 19, and 20), have the most beneficial glycosylation pattern for improving binding selectivity to cellulose over lignin. Although a single dimannosyl residue at the Ser3 site could also be helpful for increasing the preference for cellulose binding, the binding specificity of CBM 6 is not as high as those of the multi-mannosylated variants. These data led to further investigation using molecular dynamic simulation.[78] Thus, via synthesizing, characterizing, and systematically analyzing a collection of synthetic CBM variants, the beneficial effects of glycosylation on CBM binding preferences were clearly indicated across multiple biomass-derived cellulose and lignin substrates.

Table 2 Synthetic glycoforms of carbohydrate binding module of*Tr* Cel7A

The outbreak of global pandemic COVID-19 spurred the investigation of the glycosylation on spike protein of coronavirus SARS-CoV-2, particularly regarding its receptor binding domain (RBD).[79-82] Driven by this idea, a collection of RBDs containing homogeneous N- and O-glycans were prepared via a semisynthesis approach.[83] Namely, the glycosylated fragments (319-335) and (336-360) were chemically synthesized and the rest part was prepared recombinantly (Figure 15). The N-linked glycans at Asn331 and Asn343 was introduced as GlcNAc, $Man_3GlcNAc_2$ pentasaccharide or the complex-type nonasaccharide; O-glycan at Thr323 was an O-linked GalNAc. The binding of these synthetic glycoforms of RBD to its host receptor, angiotensin converting enzyme 2 (ACE2), as well as to anti-SARS-CoV-2 RBD monoclonal antibodies (mAbs) was studied. The established platform represents a versatile strategy for accessing synthetic glycosylated RBDs, which facilitates the understanding the specific role of glycosylation on these viral RBDs and paves the way for the future development of effective therapeutics and vaccines.

Figure 14 Synthetic RBD glycoforms with various N- and O-glycans

ADP-ribosylation of nuclear proteins is a critical feature of various DNA damage repair pathways.[84-85] Muir et al. reported the development of an efficient and modular semisynthetic route to full-length ADP-ribosylated histones H2B and H3, installed at Ser6 and Ser10, respectively (Figure 15).[86] The synthetic route involved chemically synthesized ADP ribosylated histone tail peptide thioester ($H2B_{(1\text{-}16)}$S6ADPr and $H3_{(1\text{-}14)}$S10ADPr), and recombinant truncated histone ($H2B_{(17\text{-}125)}$A17C and $H3_{(15\text{-}135)}$A15C) expressed in *E. coli* . Native chemical ligation of the peptide thioesters with the corresponding recombinant fragments, followed by free radical-mediated desulfurization smoothly afforded the native full-length ADP-ribosylated histones H2BS6ADPr and H3S10ADPr. Furthermore, these histones were used to generate various ADP-ribosylated chromatin substrates. To this end, three different ADP-ribosylated histone octamer complexes were refolded, which contained either ADP-ribosylated H2B, ADP-ribosylated H3, or both, in addition to a control octamer complex in which all histones are unmodified.

Figure 15 Semisynthesis of ADP ribosylated histones

Analysis by native polyacrylamide gel electrophoresis (PAGE) indicated that the bulky, anionic ADP-ribose moiety did not impede efficient mononucleosome formation; similar thermal stability was observed among the ADP-ribosylated and unmodified mononucleosomes, further indicating that proper nucleosome assembly

was maintained. However, ADP-ribosylation exhibited inhibitory effect on chromatin fiber compaction, which implied its role in chromatin relaxation in the context of DNA repair. Furthermore, the crosstalk between ADP-ribosylation and lysine methylation was studied, where ADP-ribosylation of H3S10 inhibits G9a-mediated H3K9 methylation, indicating the modulation of the H3-tail dynamics by ADP-ribosylation. Collectively, native full-length ADP-ribosylated H2B and H3 were prepared through protein semisynthesis, and were utilized to generate chemically defined ADP-ribosylated mononucleosomes and nucleosome arrays. This work sheds light on a role of histone mono-ADP-ribosylation in DNA repair and also highlights the value of designer ADP-ribosylated chromatin substrates in further investigation.

In the era of antibiotic resistance, the discovery of new antibiotics with new skeletons and mode of action is of high pursuit. As a group of nonribosomal cyclic glycopeptide natural products, mannopeptimycins demonstrate promising activities against clinically important resistant Gram-positive pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci*(VRE).[87] However, the high structural complexity of mannopeptimycins poses a formidable obstacle to their total synthesis.[88] Mannopeptimycins comprise six amino acids with alternating D- and L-configurations, four of which are nonproteinogenic: D- and L-β-hydroxyenduracididines (βhEnd),[89] L-β-methyl phenylalanine (βMePhe), and D-tyrosine (D-Tyr), where D-βhEnd and D-Tyr were glycosy: Novel Antibacterial Glycopeptides lated (Figure 16). Chen et al. finished the first total synthesis of mannopeptimycins α and β, a convergent (3+3) assembly of the cyclic peptide backbone.[90] The N-linked mannose moiety was installed on cyclic guanidine substrates (D-βhEnd) via the gold catalyzed glycosylation of the mannosyl ortho-alkynylbenzoate donor, and Boc-βMePhe-OH was prepared via Pd-catalyzed C-H arylation chemistry. Li et al. reported the total synthesis based on a streamlined peptide assembly strategy, and proceeded macrocyclization via β-hydroxyenduracididine ligation.[91] These impressive achievements shed light on the synthesis of nonribosomal cyclic glycopepitdes with high structural complexity, which will enable exploration of previously inaccessible mannopeptimycin derivatives, and promote the development of new analogues with enhanced antibacterial activity.

Figure 16 by Chemical synthesis of mannopeptimycins: novel antibacterial glycopeptides

4. Conclusions and Perspectives

Owing to the importance of protein glycosylation, rigorous evaluation of the relationship between the precise structure and biological function of glycoproteins has to be performed, which heavily relies on the access to structure-defined glycoproteins with single glycoform. Since these single glycoforms are hard to obtain from natural sources, innovative synthetic methods and technologies are highly valued in this regard.

The size, physical properties, high structural diversity and complexity of glycoproteins poses significant challenge in the preparation of glycoproteins. Recent efforts in this area summarized herein include 1) efficient chemical synthesis, chemoenzymatic synthesis and semisynthesis methods, 2) simplified purification protocols, and 3) glyco-conjugation and glyco-engineering strategies. On this basis, a great number of native glycoproteins are now chemically accessible, as illustrated with the massive successful cases discussed above. The obtained synthetic glycoforms furnish new insights into the role of glycosylation. Furthermore, the repertoires of synthetic tools facilitate the ongoing research with a broader health-promoting application scenario. For example, significant progress is witnessed on the glyco-remodeling/glycoengineering of immunotherapeutics, including therapeutic antibodies,[92-94] cell-based immunotherapies (e.g. chimeric antigen receptor (CAR)-T cell therapy[95]), and synthetic antitumor vaccines (e.g. MUC1 glycopeptide based vaccines[96]), which exhibits generally improved immunological profile, and holds promise for clinical translation. Besides, Glycosylation of peptides and proteins has emerged as a promising strategy to improve the pharmacokinetic profile of peptide- and protein-based therapeutics. Hence, it is expected that with the facile access to structurally defined synthetic glycoforms, future endeavors will help unravel the associations between glycosylation and human diseases, and will open up novel therapeutic avenues.

Apparently, preparation of glycoproteins to mimic nature complexity is a fascinating target, and also an attractive, arduous and problem-driven journey, calling for continuous efforts and wisdom to come. While

the challenge in protein synthesis can be overcome via introduction of glycosylation, it can be anticipated that the ongoing development of protein chemical synthesis and semisynthesis tools, exemplified with various chemical ligation, conjugation and purification technologies,[97-104] will contribute to the progress of glycoprotein synthesis. Meanwhile, a primary concern under development is the construction of diverse forms of glycosidic linkage, between each glycan unit and also between the glycan chain and protein backbone, where stereoselectivity and regioselectivity issues are frequently interrogated. It is foreseeable that the prosperity in carbohydrate chemistry will lead to new progress in glycoprotein synthesis, to an unprecedent future.

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