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Current Opinion in Chemical Biology

Context and complexity: The next big thing in synthetic glycobiology

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Complex glycans participate in many essential life processes. Studies of glycan-mediated biological events have traditionally employed structurally defined fragments of the more elaborate natural molecules. However, it is now clear that this approach may sometimes be insufficient and this realization has prompted a desire to synthesize glycans of similar size and complexity to those found in nature. We highlight here recent work describing the synthesis of such molecules.

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Current Opinion in Chemical Biology 2013, **17**:990–996

This review comes from a themed issue **Synthetic biomolecules**

Edited by **Shang-Cheng Hung** and **Derek N Woolfson**

For a complete overview see the Issue and the Editorial

Available online 26th October 2013

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http://dx.doi.org/10.1016/j.cbpa.2013.10.009

Introduction

Most naturally occurring glycans are large, complex molecules that are difficult to isolate from nature as single glycoforms. Moreover, the quantities available from natural sources are generally too small for detailed biochemical or structural biological investigations. Such studies have therefore relied on synthetic glycans produced by chemical or chemoenzymatic synthesis [1]. Given the complexity of naturally occurring glycans, and historical limitations in synthetic carbohydrate chemistry, the usual approach has been to prepare small fragments of the natural molecules for use in subsequent investigations.

In many cases, the use of small glycan fragments to probe biological function is effective. However, an increasing number of studies call into question the viability of this strategy. For example, Kasper and coworkers recently showed [2•] that optimal protection by glycoconjugate vaccines occurs when multiple repeating units of the targeted glycan (~30 monosaccharide residues) are conjugated to a short peptide. This differs from the standard approach in glycoconjugate vaccine preparation where short glycans are appended to a larger carrier protein [3]. In another study [4•], Takahashi and coworkers demonstrated that the binding of synthetic β-(1→3)-linked-glucans to the immunoreceptor protein Dectin-I increases with chain length, but that an oligomer containing 17 residues still binds more weakly than the native polysaccharide. Finally, bacterial polysaccharide (*e.g.*, lipopolysaccharide) biosynthesis occurs *via* the action of a number of proteins that assemble and transport large, generally lipid-bound, glycans from the cytoplasm, through the cell membrane to the periplasm and ultimately to the cell surface [5]. Taken together, these examples suggest that understanding the biological function of many glycans at a molecular level will require access to molecules that resemble the natural glycoconjugates in size and complexity, not just small fragments of them. We highlight here recent advances in the preparation of large,[1] structurally defined carbohydrate-containing molecules. In most cases, the studies described below were carried out to provide molecules to address specific biological questions. However, given space limitations, the downstream investigations motivating these syntheses are not discussed.

Chemoenzymatic synthesis of N-linked glycans

A large number of carbohydrate-processing enzymes, and recombinant mutants, are now well characterized and many are available for use in enzymatic or chemoenzymatic synthesis [6–10]. In particular, the ready availability of enzymes involved in N-linked glycan biosynthesis [11] has allowed efficient access to these molecules, and different strategies have been developed to achieve this task.

In one investigation [12••], Ito and coworkers devised a strategy in which an unnatural tetradecasaccharide **1** (Figure 1 A) was assembled by chemical methods and then, through the sequential use of glycosidases, 'degraded' to natural N-linked glycans. The attachment of a monosaccharide that could be selectively cleaved at the non-reducing end of each arm of the precursor glycan was key to implementing this approach. As an example, conversion of **1** into decasaccharide **2** was achieved by first treatment with a β-hexsosaminidase to remove the

[1] The term "large" is admittedly subjective. For the purpose of this article, we define a large glycan as a molecule containing 10 or more monosaccharides, or smaller glycans attached to a protein or complex lipid. The coverage does not include molecules in which carbohydrates have been attached either to each other, or to other molecules, by unnatural linkages (*e.g.*, through triazoles generated *via* click chemistry).

GlcNAc residue, followed by an *endo*-mannosidase, which cleaved the Glc–Man disaccharide in Arm C and finally a β-galactosidase. This approach was demonstrated on a glycan attached to a fluorophore-labeled glycine and its application to a protein functionalized with a tetradecasaccharide analogous to **1** could be envisioned.

In another study [13••], Wang and coworkers reported the preparation of 15 glycoprotein derivatives built upon cyclic peptides containing two N-linked glycosylation sites. One or both of these glycosylation sites was modified by the attachment of oligosaccharides ranging in length from 5 to 11 monosaccharide residues. The most complex derivative bore an octasaccharide at one glycosylation site and an undecasaccharide at the other. The assembly of these molecules was achieved by the chemical synthesis of a cyclic peptide containing a β-GlcNAc residue at one or both of the N-linked glycosylation sites (Figure 1B). Attachment of the glycan was achieved using mutants of one of three endo-glycosidases, EndoD, EndoA or EndoM [14,15]. These enzymes recognize (synthetic) oligosaccharides possessing an oxazoline moiety at the reducing terminus as substrates (*e.g.*, **3**), resulting in the formation of a β-GlcNAc-(1→4)-β-GlcNAc linkage. Glycopeptides containing two different glycans were synthesized by the controlled addition of a glycan to one of the two glycosylation sites, separation of the regioisomers by HPLC and addition of a second glycan. Although not reported, a merging of the aforementioned method developed by Ito and coworkers with this approach could be envisioned. Implementation of this strategy would involve addition of a glycan analogous to **1**, bearing an oxazoline at the reducing end, to a peptide bound β-GlcNAc residue, followed by glycosidase trimming. Key to the success of this approach would be identifying an *endo*-glycosidase, either naturally occurring or through mutation, that would transfer this unnatural glycan.

In a third approach Boons, Paulson [16••] and coworkers harnessed the power of glycosyltransferases to assemble a series of asymmetrically substituted N-linked glycans possessing 10–17 monosaccharides. The approach relied upon the chemical synthesis of decasaccharide **4** (Figure 1C), in which one galactose residue was rendered inert to the action of glycosyltransferases by acetylation. Subsequent glycosylation with various transferases, and judicious liberation of the masked galactose residue, allowed the differential extension of the arms of the core decasaccharide to provide a range of products with asymmetric substitution patterns (*e.g.*, **6–8**).

These approaches elegantly couple the power of chemical synthesis with enzymatic transformations to deliver complex glycans that are invaluable functional probes. However, for many other systems, an enzymatic approach is not possible given either a lack of knowledge of the relevant biosynthetic pathways or difficulties in accessing enzymes, or their substrates, in suitable quantities. The reminder of this article thus focuses on purely chemical approaches.

Synthesis of homogenous N-linked glycoproteins

The preparation of pure N-linked glycoproteins of defined structure has been a topic of interest for several years [17]. Recently, investigations by two independent groups have led to the chemical synthesis of glycosylated forms of full-length erythropoietin (EPO) a therapeutic glycoprotein [18] possessing four glycosylation sites. Recent advances [19] in native chemical ligation (NCL) [20] facilitated the successful synthesis of these glycoproteins. Interested readers are directed to recent reviews on the synthesis of glycoproteins [17,21].

Kajihara and coworkers [22••] achieved the first total synthesis of an EPO derivative bearing an intact complex type sialyloligosaccharide at one of the N-linked glycosylation sites, asparagine 83 (Figure 2A). To facilitate the ligation of the peptide fragments, glutamic acid 21 and glutamine 78 in the native protein were mutated to alanine. The approach relied on the isolation of an undecasaccharide linked to asparagine from egg yolks (after proteolytic degradation of the parent glycoprotein and purification [23]) and its elaboration into a fragment suitable for NCL. A series of ligations with peptide thioesters produced by solid phase peptide synthesis afforded the 166 amino acid-residue target. A similar approach was also used to synthesize a homogenously glycosylated derivative of the cytokine interferon-β [24].

Shortly after Kajihara's report on the preparation of an EPO derivative functionalized with a single glycan, Danishefsky and coworkers described the synthesis of the native protein with oligosaccharides at all four glycosylation sites [25••]. The three N-linked glycosylation sites all contained a chitobiose (β-GlcNAc-(1→4)-β-GlcNAc) moiety whereas the O-linked glycosylation site was occupied by a tetrasaccharide containing two sialic acid residues (Figure 2B). The O-linked glycans were installed through the chemical synthesis of a glycosylated amino acid that was used in peptide synthesis and subsequent NCL. In contrast, the N-linked glycosylation was introduced by condensation of a synthetic chitobiosyl amine with aspartic acid residues present in advanced peptide intermediates [26].

Synthesis of oligosaccharide fragments of polysaccharides

There is increasing interest in the total chemical synthesis of structurally defined large oligosaccharides, which are likely more appropriately described as polysaccharides [27–34,35••,36]. The first example dates back two decades to Ogawa's preparation of a 28-residue glycan fragment of a complex glycolipid [27].

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