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Precision Probing of O-GalNAc Glycosylation Using Bump-and-Hole Engineering

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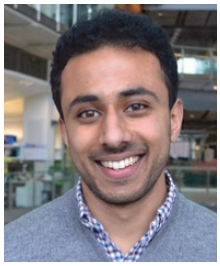
Abstract: Glycosylation is a profound influencer of glycoprotein function. Glycans have a critical impact on health and disease, yet the tools to study them have trailed behind proteins and nucleic acids. O-GalNAc glycosylation involves the addition of *N*-acetylgalactosamine (GalNAc) to protein substrates. Dysregulation of O-GalNAc glycosylation is implicated in many pathologies such as cancer. Studying O-GalNAc glycosylation is complicated by the lack of a consensus sequence for initiation and the complex interdependence between a large family of 20 GalNAc transferases (GalNAc-Ts) in human cells. These issues necessitate precise methods of interrogating enzyme function. Herein, we discuss our own advances into the generation of precision tools to study O-GalNAc glycosylation and other glycosylation types. We discuss the use of bump-and-hole engineering to illuminate the roles of individual GalNAc-Ts. Engineering biosynthetic pathways enables cell line-specific uptake of chemical, editable sugars in co-culture settings. We provide an insight into the state-of-the-art in this field.

Keywords: Bioorthogonal · Chemical tools · Glycans



Abdul Zafar earned his MSci from Imperial College London in 2021. For his Masters project in the Schumann lab, he was awarded the departmental Alfred Bader prize for excellence in organic chemistry. Now a PhD student in the lab, Abdul's research focuses on studying the interplay between glycosyltransferases in yielding mature glycoproteins.

Dr. Benjamin Schumann is a Senior Lecturer at Imperial College London and Group Leader at the Francis Crick Institute, leading the Chemical Glycobiology Laboratory. His group uses chemical tools to precisely probe the biosynthesis of glycoproteins and their fundamental implications in health and disease. Prior to his current position, he trained with Peter H. Seeberger at the Max Planck Institute of Colloids and Interfaces and with Carolyn R. Bertozzi at Stanford University.

1. Introduction

It is becoming increasingly clear how glycan modifications not only decorate but fundamentally influence the functions of underlying biomolecules. The influence of the glycosciences in the clinic is ever-increasing, especially in improving and fine-tuning immunotherapeutics.[1–7] The power of modern genetics is apparent in the diagnosis and definition of congenital disorders of glycosylation, some of which may have been misdiagnosed in the past.[1,2]

Protein O-GalNAc glycosylation is simultaneously one of the most abundant and least understood posttranslational modifications. These glycans are biosynthesized by addition of *N*-acetylgalactosamine (GalNAc) from a uridine diphosphate (UDP)-sugar donor to a Ser/Thr/Tyr side chain by one of 20 GalNAc transferase (GalNAc-T) isoenzymes.[3–7] In the secretory pathway, multiple GalNAc-T isoenzymes act combinatorially to manifest reliable glycosylation in most trafficking proteins. Mutations or altered expression levels of GalNAc-Ts are associated with congenital disorders of O-glycosylation or other pathological conditions including, very frequently, cancer.[8–11] While strategies in molecular cell biology such as knockouts have been extremely valuable to understand the activities of individual GalNAc-Ts in physiology,[12–15] the complex interplay of these transferases is subject to competition and compensation events that require orthogonal approaches. Understanding the cellular activities of transferase enzymes has been traditionally challenging. Unlike hydrolases that can often be traced by covalent activity-based probes, transferases often proceed without covalent intermediates.[16] Advances have been made toward transferase inhibitors, which have only started to be optimized for glycosyltransferases.[17–20] Chemical biology has found a suitable technology in the so-called bump-and-hole (BH) tactic, originally described for the very first molecular glue degraders and later for kinases.[21–23] There, the tactic modified a promiscuous inhibitor to contain a 'bump' that is not bound by the wildtype (WT) enzyme but rather a rationally designed variant enzyme containing a complementary 'hole'. Application to substrates instead of inhibitors has established bump-and-hole systems to dissect the activities of methyltransferases, ADP-ribosyltransferases, and palmitoyltransferses, to name just a few.[24–26] If the 'bump' contains a bioorthogonal tag, the chemically modified substrate is incorporated into substrate glycoproteins (Fig. 1). Treatment with clickable reporter groups such as fluorophores or biotin allows for ensuing enrichment and mass spectrometry analysis to profile substrate proteins. Due to the need for structural input and nucleotide-sugar libraries, the BH approach could not be applied to glycosyltransferases until relatively recently.

It should be emphasized that a plethora of techniques in chemical biology, mass spectrometry, computational modelling, and structural biology have been key to expanding our horizons on O-GalNAc glycosylation.[13–15,27-33] This account is not a compre-

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hensive overview of these techniques – instead, the reader is referred to the corresponding reviews.

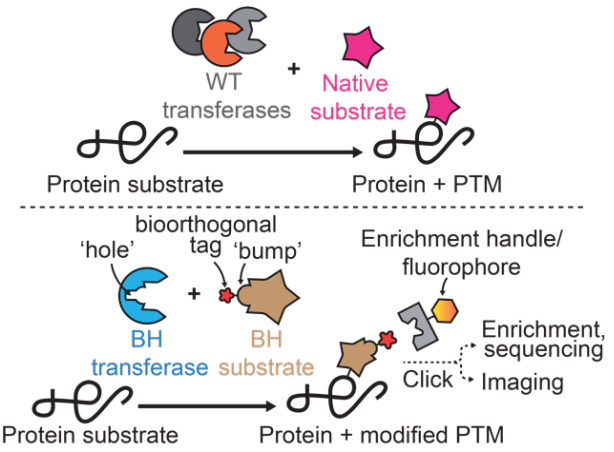

Fig. 1. Design principles for generating a bump-and-hole transferase-substrate system. The introduction of a rationally designed 'hole' through mutagenesis in a target transferase enables acceptance of a complementary 'bumped' substrate equipped with an enrichment handle/fluorophore for downstream tracing.

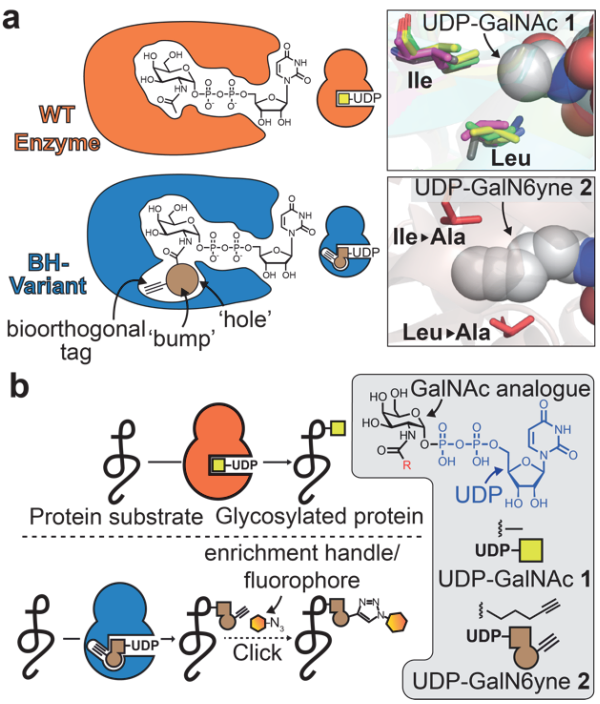

Fig. 2. Application of bump-and-hole engineering to GalNAc-Ts. A) Mutation of highly conserved 'gatekeeper' Ile/Leu residues across GalNAc-T isoenzymes to Ala enables acceptance of UDP-GalNAc analogues featuring elaboration at the acetamide position. WT GalNAc-T crystal structures (top right): GalNAc-T1 (PDB 1XHB), -T2 (PDB 4DOT), -T10 (PDB 2DZI), -T12 (PDB 6PXU). BH GalNAc-T2 crystal structure (bottom right, PDB 6NQT). B) Scheme for isoenzyme-specific substrate tracing using BH GalNAc-Ts.

2. Discussion

The BH tactic for glycosyltransferases was first applied to GalNAc-Ts in the Bertozzi lab.[34,35] The approach is not dissimilar to the successful re-programming of the galactosyltransferase B4GALT1 by Qasba, Hsieh-Wilson and coworkers to accept UDP-GalNAc analogues instead of UDP-galactose.[36,37] Engineered B4GALT1 can be used to trace GlcNAc-terminating glycoproteins.[36,38,39]

GalNAc-T BH engineering was designed to enable screening of substrate profiles for individual isoenzymes. The approach was fuelled by a collection of synthetic, bioorthogonal UDP-GalNAc analogues with 'bumps' that would prevent use by WT transferases.[34,40–42] Existing crystal structures indicated that three hydrophobic amino acids, Ile, Leu and Phe, are in close proximity to the GalNAc acetamide that can be diversified by synthetic chemistry.[43–49] Using a small collection of GalNAc-T2 variants in which these gatekeeper residues were replaced with alanines, suitable enzyme/substrate combinations were identified to glycosylate synthetic peptides using bioorthogonal UDP-GalNAc analogues instead of UDP-GalNAc **1** (Fig. 2A). The double substitution Ile253Ala/Leu310Ala exhibited a particularly productive turnover with UDP-GalN6yne **2** (Fig. 2B). Michaelis-Menten kinetics experiments suggested that kinetic properties were largely conserved, with some variation of K_M and k_{cat} depending on the substrate. This work was expanded from the isoenzyme GalNAc-T2 to T1 and T10, with the corresponding gatekeeper residues conserved in sequence alignments and available crystal structures.[43,44,46] Whether the approach is applicable to the rest of the GalNAc-T family is to be investigated.

Following the first successful examples of BH engineering, we have applied the principle to other GT families. Specifically, we have successfully engineered human xylosyltransferases that are involved in glycosaminoglycan biosynthesis,[50–54] and the glycosyltransferase MGAT5 that modifies *N*-glycan precursors with the sugar *N*-acetylglucosamine (GlcNAc).[20,55,56] Both cases were underpinned by an iterative process of producing multiple enzyme variants and/or nucleotide-sugar substrates. While the newest developments of computational modelling have provided the opportunity to comprehensively map the flexibility of enzyme-substrate interactions,[56,57] it is not currently possible to predict which variant uses which substrate. Collaborations with structural and theoretical biologists are therefore essential to ensure success of the approach.[58]

Following the successful *in vitro* validation of GalNAc-T BH enzyme-substrate pairs, the next logical step was the establishment of the tactic in living cells. While stable expression of genes encoding for (engineered) glycosyltransferases in cell lines is generally trivial, a particular challenge is the delivery of bumped UDP-GalNAc analogues (Fig. 3A). Monosaccharides or monosaccharide-1-phosphate analogues can be delivered to cells through the use of caging groups that are enzymatically removed in the cytosol.[35,59] Biosynthetic transformations are then usually employed to convert free monosaccharides to the corresponding nucleotide-sugars. However, bulky, bioorthogonal modifications are reliably recognized only by the biosynthetic enzymes of some nucleotide-sugars such as cytidine monophosphate sialic acid (CMP-Sia).[60–62] For analogues of GlcNAc and GalNAc, existing salvage enzymes display a more nuanced structure-activity relationship.[63–66] We and others have developed strategies to deliver such analogues, based on foundational enzyme engineering by Kohler and coworkers.[65,67,68] Specifically, the human pyrophosphorylase AGX1 converts GlcNAc-1-phosphate and GalNAc-1-phosphate to the corresponding UDP-sugars. When it was discovered that AGX1 does not accept bulky acylamide side chains, Yu *et al.* engineered the enzyme in another bump-and-hole approach. The Phe383Gly variant accepted a diazirine-containing GlcNAc-1-phosphate analogue to biosynthesize the corresponding UDP-sugar.[68] Based on this strategy, we found that the related variant Phe383Ala exhibits beneficial activity towards GalNAc-1-phosphate analogues. Our initial strategy featured the use of caged GalNAc-1-phosphate analogue **3** in conjunction with a stable cellular expression of $AGX1^{F383A}$. This enzyme-substrate combination allowed robust biosynthesis of UDP-GalN6yne **2** in cells, and similar

biosynthesis strategies have been deployed for related compounds.[35,42,59] In proof-of-principle experiments, we compared substrate proteins of GalNAc-T1 and GalNAc-T2, uncovering differential glycosylation of a peptide of apolipoprotein AI.[35] These studies complemented and refined data of comprehensive glycoproteomics experiments in knockout cell lines,[11,13,14] benchmarking our approach. Supplying UDP-GalN6yne **2** through use of the non-phosphorylated monosaccharide was not possible at this point because the first biosynthetic enzyme, the kinase GALK2, was equally non-permissive towards modifications as AGX1.[35,59,69] Later, we by-passed this first step as well by expressing in human cells the promiscuous bacterial kinase NahK.[64,69] We thus engineered a biosynthetic pathway to generate bumped, bioorthogonal analogues of UDP-GlcNAc and UDP-GalNAc from accessible monosaccharide precursors.

Access to all parts of a cellular bump-and-hole approach allowed for establishing the tactic in human cells. Expression plasmids were generated that combined genes for NahK, AGX1^{F383A} and WT- or BH-GalNAc-Ts. When genes encoding these enzymes were stably expressed, selective incorporation of GalN6yne into cell surface glycoproteins could be followed by cell-surface incorporation of clickable fluorophores. Only cells expressing the enzymes of the engineered biosynthetic pathway and BH-GalNAc-Ts exhibited selective and profound alkyne incorporation into cell surface glycoproteins. Employing clickable biotin as an enrichment handle allowed for profiling of GalNAc-T substrate proteins as well as glycosylation sites by mass spectrometry.[35,69] In a more recent example, the BH-GalNAc-T strategy served to highlight the impact of O-GalNAc glycosylation on the evolutionary trajectory of SARS-CoV-2. The peptide sequence adjacent to the furin cleavage site in the spike protein was subject to extensive variation during the emergence of variants of concern (Alpha, Delta, Omicron), often exchanging neutral with basic amino acids. While it was suggested that an increase in furin activity may account for some of the evolutionary trajectory,[70] foundational data by Ten Hagen and coworkers suggested that glycosylation by GalNAc-T1 plays a major role in this process as well.[71] Using the BH approach, we mapped the GalNAc-T1 glycosylation site to Thr678 on the spike. Key to this finding was the presence of an alkyne on GalN6yne, allowing the incorporation of a clickable, positively charged imidazolium tag that facilitates assignment by mass spectrometry.[72,73] Extension of the initial GalNAc, especially with the negatively-charged sugar *N*-acetylneuraminic acid, appears to prevent furin cleavage from occurring. Variants of concern circumvent this effect by introducing amino acid substitutions that prevent glycosylation by GalNAc-T1.[74] Thus, glycosylation is proposed to be a direct driving factor for viral evolution. Additional GalNAc transferases have since been found to glycosylate nearby sites.[75] Expansion of the bump-and-hole tactic to these and other family members may uncover new substrate specificities, in conjunction with the enhanced detectability of probes by mass spectrometry.

A corollary of the need for an engineered biosynthetic pathway in the BH strategy is the potential for cell specificity: In a co-culture system, one cell line can be specifically equipped by transfection with the ability to use the per-acetylated precursor **4** of GalN6yne by NahK/AGX1^{F383A} (Fig. 3B). Since this procedure only tags the glycoproteome of the engineered cell line, treatment with clickable biotin allows for cell-selective glycoprotein analysis. Bio-Orthogonal Cell-specific TAgging of Glycoproteins (BOCTAG) is particularly useful for proteome analyses without cell sorting, with an early proof-of-principle provided by mass spectrometry analysis of enriched glycoproteins in bulk lysates of cellular co-cultures. In our hands, the bioorthogonal signal could also be boosted by co-expression of an engineered GalNAc-T, endowing further utility to the system.[69] It should be noted that some glycosyltransferases are known to accept large substrate modifications, most notably sialyltransferases and fucosyltransferases.[76,77] Furthermore, endogeneous GlcNAc transferases have exhibited tolerance towards elaboration at the acetamide position on the GlcNAc (an epimer of GalNAc), but as of yet, WT-GalNAc-Ts or other GalNAc transferases do not

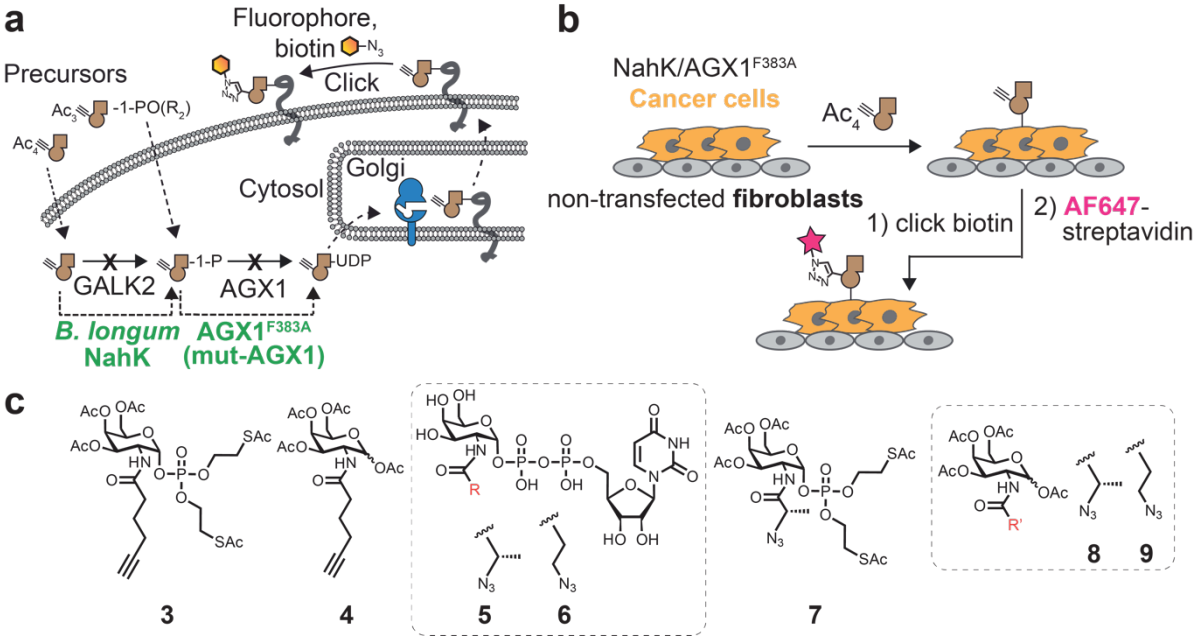

Fig. 3. Next generation chemical tools applied to trace glycans in living cells. A) Engineering a GalNAc salvage pathway in conjunction with BH-GalNAc-Ts to enable endogenous biosynthesis and incorporation of GalNAc analogues into glycoproteins bearing bioorthogonal chemical handles. B) Application of the BOCTAG system for cell-specific labelling of cell lines in co-culture. Cancer cells expressing the genes for the biosynthetic enzymes of the BOCTAG system in co-culture with non-transfected fibroblasts can incorporate GalN6yne bearing an alkynyl handle. Conjugation of biotin *via* click reaction followed by fluorophore labelling with AF647-streptavidin enables cell line-specific visualisation of glycoproteins. C) Structures of caged GalNAc analogues, UDP-GalNAc analogues, and peracetylated GalNAc analogues.

appear to significantly accept UDP-GalN6yne as a substrate.[78,79] More complex model systems are tractable by BOCTAG, for instance in the chemical tagging of the tumour-specific glycoproteome in an orthotopic cancer mouse model. In parallel, Chen and coworkers developed a strategy to deliver bioorthogonal analogues of UDP-GlcNAc in a cell-specific manner, by bump-and-hole engineering the AGX1 isoenzyme AGX2.[80] In this case, the primary application was in chemical tagging of cytosolic Ser/Thr-linked O-GlcNAc glycosylation after biosynthesis of a tagged UDP-GlcNAc analogue by AGX2^{F383G}. Following validation of the approach for cell-specific bioorthogonal glycoprotein tagging, the authors mapped in a transgenic mouse model the cytosolic proteome of cardiomyocytes. A number of enzymes in glycolysis and citric acid cycles were found, as well as mitochondrial proteins involved in oxidative phosphorylation. This work offers an exciting expansion of our toolbox for quantitative glycobiology.

Our initial BOCTAG protocol featured the use of an alkynyl sugar that happens to also be used by engineered GalNAc transferases. We sought to expand the technology, introducing GalNAc analogues with azido-sugars that would offer increased flexibility in biological applications.[38] We further opted to evolve the glycan specificity in such second-generation BOCTAG reagents. In an earlier study, we found that the use of chemically branched acylamide side chains renders UDP-GalNAc analogues resistant towards interconversion to the corresponding UDP-GlcNAc analogues by the cellular epimerase GALE.[59] This finding allows for tailoring of bioorthogonal GalNAc analogues: for instance, delivering UDP-GalNAzMe **5** to cells should result in selective tagging of O-GalNAc glycans, whereas UDP-GalNPrAz **6** should lead to tagging of a larger variety of cell surface glycans. Similar to UDP-GalN6yne **1**, both azide-containing compounds are too sterically encumbered to enter the native GalNAc salvage pathway. Again, delivery was possible through either expression of solely AGX1^{F383A} with a caged GalNAzMe-1-phosphate **7**,[42,59] or through expression of the BOCTAG enzyme combination NahK/AGX1^{F383A} and feeding per-acetylated GalNAc analogues **8** or **9** (Fig. 3C). In both instances, successful and traceable UDP-sugar delivery was key to achieving detectable cell surface incorporation. The GalNPrAz precursor **9** is applicable to BOCTAG, allowing for cell-selective tagging of glycoproteins in a co-culture system as visualized by fluorescence microscopy.[42]

3. Conclusions and Outlook

Originally tackled through robust and comprehensive efforts in biochemistry, the past years have seen profound inroads of the glycosciences into quantitative biology. The therapeutic opportunities in the field are influencing pre-clinical and clinical study design, in a development that is only going to expand from here. Precise detection methods are still in high demand, though – traditionally, glycans could be characterized through an arsenal of lectins that is being constantly expanded. Being able to determine the glycosylation sites introduced by individual, disease-relevant glycosyltransferase enzymes is an orthogonal, equally important step to further our understanding. Providing glycan- and cell specificities in detection reagents is of outstanding relevance for the modern glycosciences.[81,82] We coined the term of Chemical Precision Tools to illustrate a collective move of the field toward these goals, underpinned by advances made by the many great carbohydrate chemists and chemical biologists. The field is fast-growing, with many outstanding challenges. These include better methods to characterise glycopeptides to address the biological impact of glycan microheterogeneity. Advances in mass spectrometry and the application of cutting edge nanopore-sequencing may enable more precise and sensitive glycan profiling.[83-85] Improvements in chemical and chemoenzymatic glycan syntheses as well as the identification of novel carbohydrate-active enzymes are likely to expand the accessibility of reagents, alongside the scope of their application.[85,86] The future is bright for precision tools to shed light on the relevance of glycans in physiology.

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