Photocrosslinking Sugars for Capturing Glycan-dependent Interactions

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Abstract

Photocrosslinking approaches are an important strategy to covalently capture biomolecular interactions. Covalent capture by photocrosslinking is particularly powerful for glycan-dependent interactions because these binding events are typically low-affinity and unable to withstand common purification protocols. Implementation of photocrosslinking methods is based on the incorporation of a photoactivatable crosslinking functional group onto glycan-containing molecules. Upon activation with UV radiation, the photocrosslinking group is converted to a reactive species, which forms a covalent bond with neighboring molecules. Resulting crosslinked complexes can be analyzed by immunological and mass spectrometry techniques to identify glycan-dependent binding partners. In some cases, photocrosslinker incorporation can be achieved in living cells, enabling detection of glycan-dependent interactions in their native context. In this mini-review, we describe new sugar-based photocrosslinking reagents and illustrate how they have been used to photochemically characterize glycan-dependent interactions.

A. Introduction

Glycosylated proteins and lipids engage in specific interactions with other biomolecules. These glycan-dependent binding events regulate many aspects of normal physiology, as well as pathophysiological events such as host–pathogen interactions. While some glycan-dependent interactions are well-characterized, in many cases the identity of one of the binding partners is not known. For example, a specific glycoconjugate may be known to play a physiological role, but the corresponding glycan-interacting protein remains unknown. Alternatively, a glycan-binding protein (also known as a lectin) may be implicated in a biological process, but the glycoconjugate(s) that it recognizes has not been identified. While many factors contribute to the difficulties associated with identifying glycan-dependent interaction partners, one critical aspect is the fact that glycan-dependent interactions are typically transient and low affinity (1). The low affinity complexes have rapid off-rates and can dissociate during affinity purification methods.

Photo-activated crosslinking technology offers a way to overcome the challenge associated with isolating low-affinity complexes (2). Photoactivatable crosslinking functional groups fall into three classes: diazirines, aryl azides, and benzophenones (3). These functional groups are stable in the absence of ultraviolet (UV) radiation. Upon activation with UV radiation, the photocrosslinking functional group is converted to a highly reactive species that can react with a nearby functional group, forming a new covalent bond. In this way, the photocrosslinker can be used to convert a low-affinity non-covalent interaction to a non-dissociable, covalent interaction. While application of photocrosslinking functional groups

in biology has a long history (4), their utility has escalated in recent years due to substantial improvements in mass spectrometry approaches that can be used to analyze the covalently crosslinked complexes (5).

The chemistry of photoactivated crosslinking is controlled by UV illumination, yielding advantages over other forms of crosslinking. First, photoactivatable functional groups are stable in the absence of UV radiation, allowing them to be selectively incorporated at specific locations within living cells. Second, once activated, photocrosslinking groups are highly reactive. As a result, photocrosslinkers will covalently crosslink to any nearby molecule, regardless of functional group composition. If no other biopolymer is nearby, the crosslinker will quickly deactivate by reacting with water or with itself. Because of the short half-life of the reactive intermediate, little non-specific crosslinking occurs. The net result is highly specific crosslinking of functionally relevant interactions. This outcome can be contrasted to those obtained with traditional crosslinkers (*e.g.* formaldehyde) where non-selective incorporation and slow crosslinking kinetics conspire to yield high levels of non-specific crosslinking.

Photocrosslinking capture of biomolecular interactions can be performed either in cell lysates or in intact, living cells. The earliest experiments were performed in lysates. Typically, these photoaffinity labeling experiments involved the addition of a photocrosslinking group to a bioactive small molecule (6). The functionalized small molecule was then incubated with lysate and subjected to UV radiation, resulting in covalent crosslinking between the small molecule and its target. Lysate-based photocross-

linking remains a powerful approach, but is now complemented by cell-based photocrosslinking methods (7–10). In these strategies, photocrosslinking groups are introduced into biomolecules in living cells. UV irradiation of the intact cells results in photochemical capture of biomolecular interactions occurring in their native context. New photocrosslinking building blocks can be used to examine a variety of biomolecules. Indeed, photocrosslinking amino acids (2), nucleic acids (11), lipids (12), and sugars (13) have been reported. In many cases, these photocrosslinking building blocks can be metabolized by cells and incorporated into biopolymers in place of their naturally occurring counterparts. In this review, we describe strategies that have been employed to introduce photocrosslinking groups onto carbohydrate-containing molecules and the applications of these reagents to photochemically capture carbohydrate-dependent biomolecular interactions.

B. Photocrosslinking Reagents and Their Applications

B-1. Sialic Acid Analogs for Photocrosslinking the Sialome

Sialic acids are a family of nine-carbon ketoses with a carboxylate group at the C-1 position (14, 15). Four photocrosslinking sialic analogs have been metabolically incorporated into cellular glycoconjugates. These analogs vary in the identity of the photocrosslinking group, the position of photocrosslinker attachment, and in the inclusion of additional unnatural functionality.

The first reported metabolically incorporated photocrosslinking sialic acid analog contained the aryl azide at the *N*-acyl position (SiaAAz) (Fig. 1a) (16). Bertozzi and co-workers showed that this sialic acid analog could be incorporated into sialylated glycoconjugates by culturing cells with either the photocrosslinking sialic acid or cell-permeable analog. Alternatively, cells could be cultured with an *N*-acetylmannosamine (ManNAc) precursor bearing the *N*-acyl aryl azide functional group (ManNAAz). Cells metabolized the aryl azide ManNAc to the corresponding aryl azide sialic acid and incorporated it into cell surface glycoconjugates. While the analog was efficiently incorporated, no photocrosslinking applications were demonstrated in this initial report.

In 2005, the Paulson group reported a second sialic acid analog modified with an aryl azide, this time at the C-9 position (9-AAz-Neu5Ac) (Fig. 1b) (8). Metabolic incorporation of 9-AAz-Neu5Ac into glycoconjugates was demonstrated in a B cell line, BJAB K20, that is deficient in normal sialic acid production due to a defect in UDP-GlcNAc-2-epimerase activity. Culturing sialic acid-deficient BJAB K20 cells with 9-AAz-Neu5Ac restored cell surface sialylation while also introducing the aryl azide photocrosslinker. Paulson and co-workers used metabolically incorporated 9-AAz-Neu5Ac to identify binding partners for CD22. CD22

is a member of the sialic acid binding, immunoglobulin-like lectin (Siglec) family of proteins that interact with sialylated molecules displayed on the same cell (*cis* interactions) or opposing cells (*trans* interactions) (17). Siglec–ligand interactions mediate B-cell receptor signaling, homing of immune cells, and T-cell signaling. Photocrosslinking experiments performed in BJAB K20 cells revealed that the primary *cis* interaction partners of CD22 are other CD22 molecules. In subsequent photocrosslinking experiments to identify *trans* ligands for CD22, excess soluble CD22 was applied to BJAB K20 cells cultured with 9-AAz-Neu5Ac (18). Candidate *trans* interaction partners were identified by mass spectrometry and evaluated by additional cell-based assays. Overall, use of 9-AAz-Neu5Ac enabled the determination that CD22 is the primary *cis* ligand and the B-cell receptor IgM is the primary *trans* ligand for CD22.

Sialic acid analogs with the diazirine photocrosslinker can also be metabolically introduced into glycoconjugates. The Kohler lab pursued this approach by preparing a diazirine-modified ManNAc analog, ManNDaz (19, 20). To improve cell permeability, ManNDaz was fully acetylated (Ac₄ManNDaz) (Fig. 1a). Once Ac₄ManNDaz enters the cells, the acetyl groups are removed by nonspecific cellular esterases. Released ManNDaz is converted to diazirine-modified sialic acid (SiaDaz) by endogenous enzymes and SiaDaz is metabolically incorporated into cellular glycoconjugates in place of natural sialic acid. Like 9-AAz-Neu5Ac, SiaDaz can be used to covalently capture homomultimerization of CD22 in BJAB K20 cells. Metabolism of Ac₄ManNDaz to SiaDaz-containing glycoconjugates has been demonstrated in a variety of cell lines, and SiaDaz can substitute for natural sialic acid in both glycoproteins and glycolipids. For example, T-lymphocyte Jurkat cells cultured with Ac₄ManNDaz produce a several diazirine-modified glycolipids, including the ganglioside GM1. The ability to selectively introduce a photocrosslinker into glycolipids enables the covalent capture of lipid–protein interactions. The Kohler lab demonstrated this concept by examining the well-characterized interaction between cholera toxin subunit B (CTxB) and the ganglioside GM1 (21, 22). Jurkat cells were cultured with Ac₄ManNDaz and then exposed to CTxB. Subsequent UV irradiation resulted in crosslinking of CTxB to GM1. These results suggest that photocrosslinking sialic acid analogs could be used to discover previously unrecognized protein–glycolipid interactions.

The diazirine photocrosslinker has also been included in a bifunctional unnatural sialic acid. In 2009, the Chen group reported 9AzSiaDaz, which bears the diazirine modification at C-5 and an azide functionality at C-9 (Fig. 1c) (23). Once metabolically incorporated, the diazirine can be activated with UV radiation to covalently crosslink sialic acid binding proteins, while the azide

Fig. 1. Metabolically incorporated photocrosslinking sialic acid analogs capture glycan-dependent interactions. Metabolic engineering approaches enable incorporation into cellular glycoconjugates of sialic acid functionalized with aryl azide at C-5 (SiaAAz) (a) or C-9 (9-AAz-Neu5Ac) (a), with diazirine at C-5 (SiaDAz) (b), or bifunctionalized with diazirine at C-5 and azide at C-9 (c). Glycoconjugates bearing photocrosslinking sialic acid are indicated by grey ovals. UV irradiation of living cells activates the photocrosslinkers resulting in covalent crosslinking to nearby molecules. Neighboring molecules are indicated by white crescents and covalent crosslinks are depicted by red lines. The crosslinked complexes can be purified using antibodies or the azide as a purification handle. Further analysis by immunoblot or LC-MS/MS identifies proteins involved in sialic acid-dependent interactions. The dashed green box on the cartoon immunoblot encloses a band representing a crosslinked complex.

enables enrichment of the target proteins by chemoselective conjugation to an affinity tag functionalized with an alkyne. The Chen group demonstrated the functionality of 9AzSiaDAz by using it to crosslink and purify CD22 from the Daudi human B lymphoma cell line. The cell lysate was reacted with alkyne-biotin following purified using streptavidin and analyzed by Western blot. The photocrosslinked CD22 was observed in 9AzSiaDAz-treated cells. The 9AzSiaDAz reagent should enable a sialome-wide approach to identifying glycan-dependent interactions.

B-2. Photocrosslinking GlcNAc Analog for Identification of O-GlcNAc Interactions

Metabolic engineering to introduce a photocrosslinker onto intracellular glycans has also been reported. The Kohler lab devised a way to introduce the diazirine functional group on the intracellular O-GlcNAc modification (Fig. 2) (24). This approach exploits the hexosamine salvage pathway, which converts GlcNAc to UDP-GlcNAc *via* three enzymatic steps catalyzed by *N*-acetylglucosamine kinase (NAGK), *N*-acetylglucosamine-phosphate mutase (AGM1), and UDP-GlcNAc pyrophosphorylase (UAP1). Unfortunately, the endogenous human enzymes in the hexosamine salvage

Fig. 2. A photocrosslinking GlcNAc analog covalently captures interactions of *O*-GlcNAc-modified proteins. Metabolic incorporation of diazirine-modified GlcNAc begins with the synthesis of a cell-permeable, diazirine-modified analog of GlcNAc-1-P ($\text{Ac}_3\text{GlcNDaz-1-P(AcSATE)}_2$). After deprotection inside of cells, this molecule is transformed to UDP-GlcNDaz by the action of the F383G mutant of UAP1, and then *O*-GlcNAc transferase (OGT) adds GlcNDaz to intracellular proteins. UV irradiation activates the diazirine, resulting in photocrosslinking of *O*-GlcNDaz-modified proteins to neighboring molecules. The crosslinked complexes can be isolated by immunopurification and characterized by immunoblot or LC-MS/MS analysis.

pathway fail to activate diazirine-modified GlcNAc (GlcNDaz) to the corresponding UDP sugar (UDP-GlcNDaz). To bypass this metabolic restriction, Kohler group prepared GlcNDaz-1-P in which the hydroxyl groups are peracetylated and the phosphate is protected with two *S*-acetyl-2-thioethyl (AcSATE) groups. $\text{Ac}_3\text{GlcNDaz-1-P(AcSATE)}_2$ is cell-permeable and treatment with this compound results in intracellular accumulation of GlcNDaz-1-P. However, wild-type UAP1 does not accept diazirine-modified substrate. Examination of the X-ray crystal structure of human UAP1 revealed that that UDP-GlcNAc resides in a compact hydrophobic pocket. To accommodate *N*-acyl diazirine substituent, an UAP1 mutant (F383G) was prepared and introduced in HeLa cells. As predicted, cells expressing UAP1(F383G) and cultured

$\text{Ac}_3\text{GlcNDaz-1-P(AcSATE)}_2$ produce UDP-GlcNDaz.

Once UDP-GlcNDaz is produced, the GlcNDaz must be transferred to substrate proteins by OGT. *In vitro* experiments with purified OGT and UDP-GlcNDaz demonstrated that OGT is capable of transferring GlcNDaz and producing the *O*-GlcNDaz modification (24, 25). Evidence that this also occurs in cells came from an indirect experiment. Lysate from cells producing UDP-GlcNDaz was incubated with an *O*-GlcNAc recognizing antibody and subjected to UV irradiation to activate the diazirine and induce crosslinking. These experiments resulted in crosslinking between the *O*-GlcNAc recognizing antibody and lysate proteins, implying that the *O*-GlcNDaz modification was produced in cells. Next, the Kohler lab performed crosslinking in these UDP-GlcNDaz-producing HeLa cells to identify endogenous proteins that interact directly with the *O*-GlcNAc-modified FG-repeat regions of nucleoporins.

The effects of *O*-GlcNDaz engineering on cellular metabolism were also examined. *O*-GlcNDaz residues are resistant to removal by *O*-GlcNAcase (25), raising the possibility that the unnatural modification might accumulate in cells. Furthermore, the total amount of UDP-GlcNAc/UDP-GlcNDaz in the engineered cells is about two-fold higher than the amount of UDP-GlcNAc in normal cells (24). Despite these facts, no significant alterations in *O*-GlcNAc-ylation were observed, suggesting that *O*-GlcNDaz labeling does not significantly perturb normal *O*-GlcNAcylation patterns. *O*-GlcNAc metabolism is also linked with GalNAc *via* UDP-galactose 4-epimerase (GALE) (26). But UDP-GalNDaz production was not observed, indicating that the diazirine is specifically targeted to GlcNAc-containing glycans. However, it is not yet known whether GlcNDaz is incorporated into other GlcNAc-containing glycoconjugates, such as *N*-linked glycans, glycosaminoglycans, or gangliosides. Thus, the next challenge will be to analyze other glycans produced by these cells.

B-3. Glycopolymers for Identification of Fucose- α (1,2)-galactose Binding Proteins

Photocrosslinking can also be used to identify glycan-binding proteins in cell lysates (13). Here the challenge is that glycan-dependent interactions are typically low affinity. Thus, these interactions do not survive typical affinity purification methods. Hsieh-Wilson and co-workers surmounted this challenge by synthesizing new photoactivatable glycopolymers (Fig. 3) (27). Their goal was to identify proteins that bind fucose- α 1-2galactose ($\text{Fuc}\alpha(1\rightarrow2)\text{-Gal}$). $\text{Fuc}\alpha(1\rightarrow2)\text{Gal}$ is found on the non-reducing termini of many glycans and has been implicated in neuronal development, learning, and memory (28, 29). However, no mammalian proteins that recognize $\text{Fuc}\alpha(1\rightarrow2)\text{Gal}$ were known. The Hsieh-Wilson group synthesized affinity probes incorporating three key features: mul-

tivalent $\text{Fuca}(1\rightarrow2)\text{Gal}$ structures to achieve improved binding avidity, a photoreactive nitrophenylazide to allow crosslinking to bound proteins, and a biotin tag to enable affinity purification. The resulting photoactivatable $\text{Fuca}(1\rightarrow2)\text{Gal}$ glycopolymer was incubated with rat brain lysate and photoirradiated to capture fucose-binding proteins. Subsequent purification and mass spectrometry analysis lead to identification of fucose-binding proteins, including

synaptic vesicle glycoprotein 2a (SV2a), which plays critical roles in trafficking and synaptic vesicle recycling. In sum, use of a novel photocrosslinking glycopolymer provided new insight into the functional role of the $\text{Fuca}(1\rightarrow2)\text{Gal}$ glycan in a complex physiological process.

B-4. PhosDAz Reagent Converts Azides to Diazirine Photocrosslinkers

Future efforts in identifying sugar-dependent interactions may be facilitated by a new strategy to functionalize organic azides. While several of the examples discussed above rely on metabolic incorporation of sugars functionalized with photocrosslinkers, in many cases the metabolic machinery may not tolerate the large photocrosslinking appendages. However, sugars bearing smaller functional group modifications can often be efficiently incorporated into glycoconjugates. Thus, an alternative strategy is to metabolically incorporate a small, bioorthogonal functional group into glycoconjugates and then derivatize the incorporated handle to introduce a larger photocrosslinking functionality. Indeed, a variety of azide-containing sugar analogs (ManNAz (30), GlcNAz (31, 32), GalNAz (33), 6-azidofucose (34, 35)) are metabolized by cells. Azides are particularly popular for bioorthogonal labeling because they are readily incorporated into glycoconjugates and can participate in multiple chemoselective ligation reactions (36). One of these chemoselective reactions is the Staudinger ligation, in which azides are reacted with phosphine-conjugated probes to produce labeled sugars for a variety of applications (30). Recently, the Jewett group reported a diazirine-modified phosphine probe (PhosDAz) that reacts with organic azides (Fig. 4) (37). PhosDAz transforms azides to diazirines through the traceless Staudinger reaction. This group showed that PhosDAz could be used to convert azide-modified sugars to diazirine-modified sugars. While the current PhosDAz reagent does not have the right solubility to be used on cells or in biological buffers, future versions of this probe might have properties appropriate for application in glycoconjugate interaction discovery.

Fig. 3. Photoactivatable glycopolymers identify glycan-binding proteins from lysates. Glycopolymers containing glycan motifs, photocrosslinking functionalities, and a purification handle can be prepared synthetically. These polymers are mixed together with biological samples to allow glycan-binding proteins to bind the glycan structures. Subsequent UV irradiation activates the photocrosslinkers, converting the low affinity, non-covalent glycan-protein interactions into covalently crosslinked complexes. After purification, glycan-binding proteins can be identified by mass spectrometry approaches.

Fig. 4. PhosDAz transforms organic azides into photocrosslinkers. The PhosDAz reagent incorporates both triaryl phosphine and diazirine functionalities. PhosDAz reacts with azide-containing molecules, enabling covalent addition of the diazirine photocrosslinker through a traceless Staudinger ligation. This derivatization reaction has been performed on azide-containing sugars in organic solvent, but not yet in aqueous solution.

C. Conclusions and Outlook

During the past decade, photocrosslinking approaches have undergone a renaissance driven by dramatic improvements in the mass spectrometry methods available for analysis of crosslinked complexes. The availability of new mass spectrometry-enabled proteomics methods has spurred production of new photocrosslinking reagents. Photocrosslinking approaches have the potential to have a significant impact in glycobiology, because glycan-dependent interactions are typically low-affinity and not readily captured by existing biochemical or cell-based methods.

New photocrosslinking approaches to glycan-dependent interaction discovery can be applied in cells or in lysates. So far, the cell-based methods are limited to glycans that contain either sialic acid or GlcNAc. Further, the efficiency of metabolic engineering efforts is highly cell type-dependent (38). Future metabolic engineering efforts may enable photocrosslinkers to be incorporated onto other monosaccharides and in a wider variety of cell lines. Alternative approaches may build upon derivatization reagents, like PhosDAz, that make it possible to convert azide-functionalized sugars to diazirine-functionalized sugars. If PhosDAz-like re-

agents could be deployed in biological settings, they could enable photocrosslinking of a broader range of glycoconjugates. At the same time, lysate-based crosslinking has less severe steric limitations than in-cell methods. The Fuca(1→2)Gal photoactivatable glycopolymer reported by Hsieh-Wilson and co-workers offers a template for future approaches to identify proteins that recognize specific glycan structures. With continued development of these complementary technologies, photocrosslinking approaches applicable to nearly all glycan-dependent interactions may soon be available.

In summary, the introduction of new photocrosslinking reagents and the accessibility of powerful mass spectrometry methods combine to position photocrosslinking as a powerful tool for glycan-dependent interaction discovery. These reagents and approaches promise a new page for functional glycobiology.

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