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A photo-cleavable biotin affinity tag for the facile release of a photo-crosslinked carbohydrate-binding protein

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ABSTRACT

The use of photo-crosslinking glycoprobes represents a powerful strategy for the covalent capture of labile protein complexes and allows detailed characterization of carbohydrate-mediated interactions. The selective release of target proteins from solid support is a key step in functional proteomics. We envisaged that light activation can be exploited for releasing labeled protein in a dual photo-affinity probe-based strategy. To investigate this possibility, we designed a trifunctional, galactose-based, multivalent glycoprobe for affinity labeling of carbohydrate-binding proteins. The resulting covalent protein–probe adduct is attached to a photo-cleavable biotin affinity tag; the biotin moiety enables specific presentation of the conjugate on streptavidin-coated beads, and the photolabile linker allows the release of the labeled proteins. This dual probe promotes both the labeling and the facile cleavage of the target protein complexes from the solid surfaces and the remainder of the cell lysate in a completely unaltered form, thus eliminating many of the common pitfalls associated with traditional affinity-based purification methods.

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1. Introduction

Carbohydrate–protein interactions mediate a number of cellular recognition events that are critical in the immune response, cancer metastasis, fertilization, and the adhesion of viruses, bacterial pathogens, and toxins.[1] Proteins that recognize and translate these glycan-mediated signals are called lectins. Lectins specifically and noncovalently interact with cell-surface carbohydrates and are highly abundant throughout nature.[2] The interaction between a single carbohydrate ligand and its target protein is often transient and characterized by low affinity ($K_d = 10^{-3}$–10^{-6} M), which imposes several difficulties on the quantification of these interactions. However, the affinity is amplified by a clustered presentation of carbohydrate ligands on cell surfaces, which results in the so-called clustered glycoside effect, an enhancement by orders of magnitude above the linear sum of the individual carbohydrate affinities.[3]

Photo-affinity labeling (PAL) is a simple yet powerful biochemical approach for rapid and accurate identification of ligand–receptor interactions and ligand-binding sites. PAL involves the formation of a covalent crosslink at the ligand-binding site of its receptor through a conjugated UV-photo-activating group.[4] In particular, the technique is suitable for covalently capturing low-affinity interactions, membrane-bound proteins, and protein complexes that are difficult to access by traditional affinity-purification methods. Commonly used photo-affinity labels have contained aryl azides, benzophenones, and diazirines and have been employed to covalently modify biomolecules in a range of biological experiments.[5] Diazirines, such as (trifluoromethyl)phenyldiazirine, have been widely recognized as ideal photo-activatable groups for biological applications because of their small size, minimal diffusion and exceptional reactivity upon irradiation with relatively long wavelengths of light (≈365 nm).[6] The latter feature is essential for site-specific labeling at biomolecular interfaces and for achieving low levels of background labeling.

Considering their importance, a variety of glycan-containing photo-affinity probes have been prepared for capturing low-affinity carbohydrate-binding proteins.[7,8] For example, Lee et al. first demonstrated the use of *N*-acetyl-D-galactosamine conjugated to a biotinylated photo-affinity label for the detection of lectins on rabbit hepatocytes,[8a] while Pieters and co-workers showed that a lactose (Lac)-based photo-affinity reagent efficiently and covalently captured the β-D-galactose (Gal)-specific lectin, galectin-1.[8c] Moreover, Sakurai et al. evaluated PAL reaction of

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benzophenone, aryl azide and aliphatic diazirine photo-crosslinking groups connected to Lac for Lac-specific multiple lectins, *Erythrina cristagalli* agglutinin, peanut agglutinin, and *Ricinus communis* agglutinin (RCA).[8e] The presentation of multiple carbohydrate ligands increases the binding of the ligand to the complementary lectin by exploiting the glycocluster–lectin interactions.[9,10] Therefore, various multivalent glycoprobes have been reported for enhancing photo-labeling efficiency.[11,12] In this regard, Shin and co-workers elegantly showed that biotinylated photo-affinity glycoprobes with three mannose (Man) or fucose (Fuc) residues increased the labeling of the Man- and Fuc-specific lectins, Concanavalin A and *Aleuria aurantia*, respectively.[11] We recently described a trivalent Gal-containing trifunctional glycoprobe that enables the selective capture of a Gal-binding lectin from *Ricinus communis*, Agglutinin 120 (RCA_{120}), in a mixture of proteins and in cell lysates.[12]

After photo-labeling, the labeled proteins are typically purified by affinity-based separation methods. Biotin is a widely used affinity tag for avidin (streptavidin)-based purification because of the extraordinarily high binding affinity between biotin and avidin ($K_d \sim 10^{-15}$ M). However, harsh conditions, such as denaturing buffer,[13] acid cleavage,[14] or treatment with excess biotin,[15] are needed to release the captured protein from avidin-coated beads. These methods can lead to the degradation of the labeled proteins and the introduction of protein contaminants due to the release of nonspecifically bound high-abundance proteins. In addition, endogenous biotinylated proteins can be co-eluted with the proteins of interest.[16]

To alleviate these drawbacks, various cleavable linkers have been developed and assembled in the design of affinity probes, such as disulfide[17] or diazobenzene,[18,19] enzymatically cleavable peptides,[20] and acid- and nucleophile-based cleavable[21,22] linkers. Despite many outstanding successes, the release of enriched biomolecules is still governed by the presence of an external cleavage reagent, which introduces an additional component to the mixture. An ideal linker is stable under various conditions, is compatible with post-ligation analytical processes and, most importantly, circumvents the need for exogenous reagents. *o*-Nitrobenzyl derivatives have shown promise as photo-crosslinkers because of their rapid photolysis, yielding a nitroso from the nitro group and an aldehyde or ketone from the oxidation of the benzyl alcohol group.[23] Consequently, various *o*-nitrobenzyl ether-containing photo-cleavable affinity tags have been developed for purification and enrichment of labeled biomolecules.[21,24–26] Such photo-controlled functionalities, which allow the on-demand release (e.g., by UV light) of enriched proteins/peptides, have attracted increasing interest for probe development in quantitative proteomics.[27] For example, an alkyne-containing photo-cleavable biotin was developed to increase the enrichment sensitivity of *O*-GlcNAcylated peptides; the incorporation of an azido-modified UDP-GalNAz (UDP-*N*-azidoacetylgalactosamine) sugar substrate enabled the enzymatic labeling of the resulting *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) through Cu(I)-catalyzed [3+2] azide–alkyne cycloaddition.[28] The photo-reactive group allowed triggered cleavage, which enables the quantitative isolation and easy identification of captured targets. Significant efforts have been devoted to the implementation of cleavable linkers[29] in the fields of biochemistry, proteomics, and cell biology for iterative protein ligation on solid-supported methods,[30] nonetheless, several applications remain unexplored. For example, the use of a photo-affinity glycoprobe combined with a photo-cleavable biotin affinity tag has not been successfully applied, although this design should facilitate the efficient PAL of carbohydrate-interacting proteins and post-ligation cleavage and purification procedures. Herein, we report the development of a PAL-capture probe that contains a light-sensitive photo-cleavable moiety and the application of the probe to the study of carbohydrate-recognizing proteins (lectins).

In the context of our continuous efforts toward the development of small affinity-based probes, we investigated a two-step strategy that involves first photo-labeling the carbohydrate-binding protein with the ligand-based probe, followed by conjugating the labeled proteins to a purification tag that contains a photo-cleavable linker (Fig. 1a). We recently reported a first-generation, trifunctional glycoprobe[12] in which the purification tag (biotin) was directly attached to the ligand and the diazirine labeling agent. Due to the high affinity between biotin and streptavidin, captured proteins were not effectively released from the purification beads, and endogenously biotinylated proteins seriously obstructed the identification of the probe-labeled proteins. In this study, we envisaged a system in which biotin is attached to a photo-cleavable *o*-nitrobenzyl group, which should allow the straightforward release of the labeled proteins from the purification beads because only the probe-labeled proteins would contain the photo-cleavable moiety. We therefore developed the second-generation, multifunctional, Gal-based probe **1**, which contains both a bioorthogonal alkyne handle and a photo-affinity label, and we devised a photo-reactive azide-biotin affinity tag (**2**) for labeling lectins in isolation or in crude cell lysates via PAL (Fig. 1b). The ability of this two-step photo-labeling and cleavage approach for an effective purification of covalently cross-linked target proteins in the presence of mouse brain lysates and subsequent UV light-dependent release of the target proteins from the solid affinity support was evaluated.

2. Results and discussion

2.1. Synthesis of trivalent Gal- and biotin-based photo-affinity probes

Based on the success of the application of PAL to carbohydrate-binding proteins, we attempted to incorporate multiple structural elements into glycoprobe **1** (Fig. 1b) to maximize the specific labeling of interacting proteins. Probe **1** contains three essential elements: (i) a trivalent Gal unit for affinity binding to the target lectin, (ii) a (trifluoromethyl)-phenyldiazirine photo-crosslinking agent, and (iii) a cyclooctyne functionality to allow attachment of a suitable tag for later-stage visualization and enrichment through the well-established strain-promoted [3+2]-azide–alkyne cycloaddition (SPAAC).[31] The appended photo-affinity label in probe **1** generates highly reactive carbene upon irradiation at 365 nm, a wavelength that imparts minimal photochemical damage to proteins.[32] The carbene intermediates trigger rapid and irreversible attachment to C– or heteroatom–H bond of Gal-binding proteins to produce stable photoadducts. While our previously reported PAL strategy included a biotin purification tag as part of the carbohydrate photoprobe,[12] in this case, a second probe, probe **2** (Fig. 1b), contains another photosensitive 1-(2-nitrophenyl)-ethyl carbamate moiety between a purification tag and an azide group. The design of probe **2** offers several advantages: First, the azide group in **2** is available for affixing the purification tag to the 2-fluorocyclooctyne of the cross-linked biomolecules through SPAAC. Second, the terminal biotin group facilitates enrichment of the labeled proteins by streptavidin beads. Third, and most importantly, the biotin-tagged cross-linked proteins can be released upon brief exposure to UV light (365 nm), while nonspecifically bound protein contaminants and endogenously biotinylated species will lack the photo-cleavable linkage and thus remain attached to the beads. After affinity purification, the captured proteins are released by a second exposure to UV light, which cleaves the photo-labile 1-(2-nitrophenyl)-ethyl carbamate moiety. The released products are then analyzed by

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