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High efficiency labeling of glycoproteins on living cells

Ying Zeng^{1,5}, T. N. C. Ramya^{1,5}, Anouk Dirksen^{2,3}, Philip E. Dawson^{2,3}, and James C. Paulson^{2,4}

¹Department of Chemical Physiology, The Scripps Research Institute, 10550 North Torrey Pines Rd, La Jolla, CA 92037, USA.

²Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Rd, La Jolla, CA 92037, USA.

³Department of Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Rd, La Jolla, CA 92037, USA.

Abstract

We describe a simple method for efficiently labeling cell surface glycans on virtually any living animal cell. The method employs mild Periodate oxidation to generate an aldehyde on sialic acids, followed by Aniline-catalyzed oxime Ligation with a suitable tag (PAL). Aniline catalysis dramatically accelerates oxime ligation, allowing use of low concentrations of aminooxy-biotin at neutral pH to label the majority of cell surface glycoproteins while maintaining high cell viability.

Keywords

glycoprotein; sialic acid; oxime ligation; aniline; periodate oxidation; metabolic labeling; live cell labeling

The expanding interest in glycoproteomics and the biological roles of glycoconjugates has increased efforts to develop efficient tools to label cell surface glycoproteins. Several elegant approaches have exploited metabolic labeling of cells, and even whole model organisms, using analogs of glycan precursors that carry bio-orthogonal groups (e.g. azide, alkyne, ketone or aldehyde), allowing the chemical ligation of reporter groups onto cell surface glycoconjugates^{1,2}.

The chemistries used for conjugation with these functional groups each have both advantages and disadvantages for use with living cells. The conjugation of azides with substituted triphenylphosphines using the Staudinger-Bertozzi ligation can be performed on living cells, but suffers from slow reaction kinetics³. Conjugation of azides with substituted-alkynes (or *vice versa*) with the Huisgen cycloaddition, or 'click chemistry', has rapid reaction kinetics, but requires a copper catalyst that is toxic to living cells^{4,5}. The newly described ligation of azides with ring-strained alkynes is compatible with living cells and has rapid reaction kinetics, but requires reagents that are not currently commercially available². Finally, imine (oxime or

4Correspondence should be addressed to: Prof. James C. Paulson, The Scripps Research Institute, 10550 N Torrey Pines Rd, Mail Drop: MEM-L71, La Jolla, CA 92037, USA, Tel: 858 784 9634, Fax: 858 784 9690, jpaulson@scripps.edu.

⁵These authors contributed equally to this work.

Author Contributions

P.D., A.D. and J.P. conceived of the method for PAL on cells. A.D. and Y.Z. performed preliminary experiments. Y.Z., T.N.C.R. and J.P. designed the reported experiments, Y.Z. and T.N.C.R. performed the majority of the experiments. Y.Z. and T.N.C.R. wrote the manuscript. All authors participated in the analysis of the data and edited the manuscript. Y.Z. and T.N.C.R. contributed equally to this work and should be considered co-first authors.

hydrazone) ligations of commercially available aminooxy or hydrazide reagents to aldehydes or ketones are convenient, but require acidic conditions (pH 5–6) and high reagent concentrations (2–5 mM) to compensate for slow reaction rates⁶.

A limitation of all these methods is the need for culturing cells with a glycan precursor containing a bio-orthogonal group prior to labeling. As an alternative, aldehydes can be readily introduced into cell surface glycans by mild periodate oxidation, known for nearly 40 years to selectively oxidize the polyhydroxy side chain of sialic acids^{7,8}. The recent demonstration that oxime ligation on complex biomolecules is dramatically accelerated using aniline as a nucleophilic catalyst^{9–12} inspired us to explore the efficiency of this reaction with aldehydes on living cells introduced by metabolic labeling or periodate oxidation. While aniline can be efficiently used as a nucleophilic catalyst for labeling biomolecules in solution by oxime and hydrazone ligations, we chose to employ the oxime ligation, which gives a more stable product than the hydrazone ligation¹³.

The two approaches used for the introduction of aldehydes onto cell surface sialic acids for subsequent ligation with aminooxy-biotin are illustrated in Fig. 1a. Cells were subjected to mild periodate oxidation (1 mM NaIO₄ at 4 °C for 30 min; **Supplementary Fig. 1**) to selectively introduce an aldehyde at C-7 of sialic acids^{7,8,14}. Alternatively, aldehydes were introduced by metabolic labeling using a novel sialic acid analog, 9-deoxy-9-*N*-carboxy-benzaldehyde-NeuAc (^{BA}NeuAc) (see **Supplementary Methods**) that is taken up by cells and incorporated into cell surface glycans (Fig. 1a). For optimal incorporation of sialic acids we used a hyposialylated B cell line, BJA-B subclone K20 (K20), that cannot synthesize its own sialic acids¹⁵. As a control we used the BJA-B subclone K88 (K88), which has wild type levels of sialic acids.

The reaction of periodate-treated K88 cells with aminooxy-biotin (100 μM) was dramatically enhanced upon addition of aniline (10 mM), as detected by DTAF-streptavidin staining (Fig. 1b), yielding 8–10 fold increased biotinylation over that without aniline, and 1,000 fold increase over that without periodate within the same period of time. Aniline similarly enhanced ligation of aminooxy-biotin to K20 cells metabolically labeled with ^{BA}NeuAc (**Supplementary Fig. 2**). We also compared the extent of aniline-catalyzed oxime ligation in K88 and K20 B cells subjected to periodate oxidation or metabolic labeling. Following periodate oxidation, K88 cells showed high levels of biotinylation, regardless of whether the growth medium was supplemented with NeuAc or ^{BA}NeuAc (Fig. 1c). Similar enhancement of ligation following periodate treatment was seen for K20 cells cultured with NeuAc, but not with ^{BA}NeuAc, since substitution at C-9 prevents periodate cleavage of vicinal hydroxyls at C-7 and C-8 (data not shown). In all cases, efficient biotinylation was only seen in the presence of 10 mM aniline. In contrast, without periodate oxidation, only K20 cells cultured with ^{BA}NeuAc showed substantial biotinylation as detected by DTAF-Streptavidin (Fig. 1c). K88 cells cultured with ^{BA}NeuAc were not biotinylated, presumably because this sialic acid analog was not incorporated due to competition with the endogenous pool of NeuAc, a problem encountered using metabolic labeling techniques. These results emphasize the generality and efficiency of PAL for introduction of aminooxy-labeled biotin tags onto glycoproteins of native cells.

We found that PAL can also be adapted to a convenient one-pot reaction, with little loss of labeling efficiency when compared to sequential periodate oxidation and oxime ligation Fig. 1d, making it useful for applications that do not require optimal labeling of glycoproteins. Viability of the cells from the combined periodate and oxime ligation reactions was ~93% compared to cells incubated with buffer alone (**Supplementary Fig. 3**). PAL was equally efficient with the oxime ligation conducted for 90 minutes at room temperature and 4 °C (**Supplementary Fig. 4**).

To determine the degree of cell surface glycoprotein labeling by PAL, we examined the biotinylation of glycoproteins of K20 cells labeled with ^3H -NeuAc using CMP- ^3H -NeuAc and sialyltransferase ST6Gal I (**Supplementary Methods**). Glycoproteins from cell lysates of ^3H -NeuAc-labeled K20 cells before and after PAL were quantitatively precipitated with streptavidin, resolved by gel electrophoresis and visualized by fluorography (Fig. 2a). Without PAL, no glycoproteins were precipitated by streptavidin. In contrast, all ^3H -NeuAc labeled glycoproteins were precipitated by streptavidin from lysates of PAL treated cells. In triplicate independent experiments, ~40–55% of ^3H -labeled glycoproteins from PAL treated cells were precipitated by streptavidin, demonstrating the high efficiency of biotin labeling.

In separate experiments, western blot analysis of CD45, a major glycoprotein of B cells (Fig. 2b) showed biotinylation of 40% and 100% of CD45 in lysates of PAL-treated K20 cells using 100 μM and 250 μM aminooxy-biotin, respectively. Other glycoproteins examined were precipitated by streptavidin to varying extents (10–100%), presumably reflecting different degrees of sialylation (**Supplemental Fig. 5**).

In accord with the nature and localization of the glycosylation machinery, sialylated glycoproteins are restricted to the plasma membrane and the subcellular organelles of the secretory and endocytic pathways of cells. To assess the subcellular localization of labeling we used confocal microscopy analysis on Chinese hamster ovary (CHO) cells. After PAL labeling at 4 $^{\circ}\text{C}$, cells were permeabilized and stained with DTAF-streptavidin. CHO cells showed intense staining on the cell surface, with no apparent intracellular staining (Fig. 2c). Staining was completely dependent on periodate oxidation and only weak staining was evident after labeling with aminooxy-biotin in the absence of aniline (Fig. 2c). Staining was also completely dependent on the presence of sialic acids, as demonstrated by comparison of staining on Id1D-CHO cells grown in the presence and absence of galactose required for sialylation (**Supplementary Fig. 6**).

To determine if labeled proteins would be endocytosed, cells were either left at 4 $^{\circ}\text{C}$, or were warmed to 37 $^{\circ}\text{C}$ for 1 hour following PAL labeling at 4 $^{\circ}\text{C}$. Cells were permeabilized prior to staining with DTAF-streptavidin and antibodies to markers of endosomes (EEA1) or lysosomes (UH3). Before warming to 37 $^{\circ}\text{C}$, labeling was found exclusively on the cell surface, with no discernable co-localization with antibody markers of endosomes or lysosomes (Fig. 2d and **Supplementary Fig. 7**). Within 1 hour after cells were warmed to 37 $^{\circ}\text{C}$ (Fig. 2d), or if PAL was conducted at room temperature instead of 4 $^{\circ}\text{C}$ (**Supplementary Fig. 7**), biotin label co-localized with both endosomes and lysosomes. Similar results were obtained with PAL treated K20 cells containing sialic acids introduced with the sialyltransferase ST6Gal I, that does not use glycolipids as substrates, confirming that biotin labeled glycoproteins are internalized (**Supplementary Fig. 8**). Taken together, the results suggest that periodate and/or aminooxy-biotin are cell impermeable at 4 $^{\circ}\text{C}$ yielding labeling exclusively on the cell surface, and that endocytosis of biotinylated glycoproteins occurs only at elevated temperatures.

Combining periodate oxidation with aniline-catalyzed oxime ligation (PAL) offers a simple bio-orthogonal approach for chemically introducing molecular tags into glycoproteins on the surface of living cells. It combines the stringent selectivity of the periodate oxidation of sialic acids with a vastly accelerated oxime ligation that allows reaction at neutral pH with low concentrations of inexpensive and commercially available reagents. While metabolic labeling of cellular glycans with unnatural sugars containing bio-orthogonal functional groups has been a major advance and has become a mainstay in glycobiology^{1–4}, a major advantage of using periodate oxidation to introduce aldehydes into cell surface glycans is that it can be used for labeling glycoproteins of most any native cell that contains sialic acids.

The high efficiency of PAL for ligation of a molecular tag into glycoprotein glycans under mild conditions is another advantage of the method. We therefore anticipate that this method will be useful not only for studies aimed at visualizing, monitoring, and tracking cell surface glycoproteins, but also for the rapid labeling, enrichment, isolation, and identification of sialoglycoconjugates as required for glycoproteomics.

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Figure 1. Labeling cell surface glycoproteins by aniline-catalyzed oxime ligation. **(a)** Scheme for aminoxy-biotin labeling of aldehydes on cell surface sialic acids introduced by periodate oxidation (top panel) or metabolically labeling with aldehyde-substituted NeuAc (^{BA}NeuAc) (bottom panel). **(b)** Aniline increases labeling with aminoxy-biotin. BJA-B K88 cells subjected to periodate oxidation (1 mM at 4 °C for 30 minutes) were reacted with 100 μM aminoxy-biotin in the presence or absence of 10 mM aniline (pH 6.7, at 4 °C for 90 minutes). Incorporated biotin was detected by flow cytometry with DTAF-streptavidin. Control refers to untreated BJA-B K88 cells. P: periodate, BO: aminoxy-biotin, A: aniline **(c)** Comparison of aminoxy-biotin labeling of aldehydes introduced by periodate or culture with ^{BA}NeuAc.

BJA-B K88 and sialic acid deficient BJA-B K20 cells were cultured in serum-free medium with or without NeuAc or ^{BA}NeuAc. Cells were subjected to ligation with aminooxy-biotin ± 10 mM aniline, stained with DTAF-streptavidin and subjected to flow cytometry. Values represent the mean and s.d. ($n=3$). MCF: Mean Channel Fluorescence. **(d)** One-pot labeling of BJA-B K20 cells. Cells were subjected to one-pot or sequential labeling of surface sialic acids. For the one-pot reaction cells were incubated with 1 mM NaIO₄ and 250 μ M aminooxy-biotin with 10 mM aniline at pH 6.7 at 4 °C for 30 minutes. The sequential reaction involved periodate oxidation at pH 7.4 for 30 minutes, followed by the aniline-catalyzed oxime ligation at pH 6.7 for 30 minutes. Results in b–d are representative of 2 or more experiments.

Figure 2.

Efficient biotinylation of cell surface glycoproteins using PAL. **(a)** PAL uniformly labels sialylated glycoproteins. BJA-B K20 cells with ³H-NeuAc labeled glycoproteins were subjected to PAL with 250 μ M aminooxy-biotin. The efficiency of PAL was assessed by fluorography of sialylated proteins in cell lysates (Total) and streptavidin pull-downs (IP) resolved by gel electrophoresis. **(b)** Quantitation of biotin-labeled CD45 from PAL labeled cells. BJA-B K20 cells were subjected to oxime ligation using PAL followed by quantitative anti-CD45 and streptavidin pull downs, and immunoblotting with anti-CD45 antibody. **(c)** Confocal microscopy of PAL labeled cells. Untreated and periodate treated (1 mM at 4 °C for 5 min) CHO cells were subjected to ligation with aminooxy-biotin (100 μ M) $\pm$ 10 mM aniline at 4 °C for 90 min. Cells were permeabilized and stained with DTAF-streptavidin (green). Nuclei were stained with DAPI (blue). **(d)** Endocytosis of PAL labeled proteins following warming to 37 °C. CHO cells, labeled by PAL at 4 °C, were returned to culture medium at 37 °C for the indicated time period (0 or 1 hour), then permeabilized and stained with DTAF-streptavidin (green), and anti-EEA (endosomes) or anti-UH3 (lysosomes) antibody followed by anti-mouse IgG-AF555 (red). Images were obtained by confocal microscopy. Scale bars, 15 μ m **(c)** and 7.5 μ m **(d)**.