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Glycoprotein labeling with click chemistry (GLCC) and carbohydrate detection

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ABSTRACT

Molecular labeling and detection techniques are essential to research in life science. Here, a method for glycoprotein labeling/carbohydrate detection through glycan replacement, termed **g**lycoprotein **l**abeling with **c**lick **c**hemistry (GLCC), is described. In this method, a glycoprotein is first treated with specific glycosidases to remove certain sugar residues, a procedure that creates acceptor sites for a specific glycosyltransferase. A 'clickable' monosaccharide is then installed onto these sites by the glycosyltransferase. This modified glycoprotein is then conjugated to a reporter molecule using a click chemistry reaction. For glycoproteins that already contain vacant glycosylation sites, deglycosylation is not needed before the labeling step. As a demonstration, labeling on fetal bovine fetuin, mouse immunoglobulin IgG and bacterial expressed human TNFα and TNFβ are shown. Compared to traditional ways of protein labeling, labeling at glycosylation sites with GLCC is considerably more specific and less likely to have adverse effects, and, when utilized as a method for carbohydrate detection, this method is also highly specific and sensitive.

1. Introduction

Proteins and carbohydrates are fundamentally important molecules in all kinds of living organisms. They can exist independently but can also form conjugates in the form of glycoproteins or proteoglycans.[1] In fact, protein glycosylation is the most common type of post-translational modification, and the majority of proteins expressed by human cells may be glycosylated.[2,3] Here, we introduce a click chemistry-based labeling method for glycoproteins/proteins, termed **g**lycoprotein/protein **l**abeling with **c**lick **c**hemistry (or GLCC).

Labeling and detection techniques are essential to protein and carbohydrate research, and by far the most popular tools for research are antibody based. Methods for protein labeling include the use of reactive groups such as, N-hydroxysuccinimide (NHS)[4] and isothiocyanate,[5] both of which rely on reacting with either the primary amine or sulfhydryl groups on the target protein. As primary amines and sulfhydryl groups are common on proteins, it is difficult to achieve high level of specificity, optimal stoichiometry, and desired localization of the labeling with these methods. When carbohydrates are the focus of research, the task actually becomes more challenging as they are not particularly antigenic, and carbohydrate-specific antibodies usually have a low affinity for their antigens. A common method for carbohydrate labeling is the use of hydrazine to form a hydrazone of an aldehyde on a glycoprotein.[6] In addition, several enzymatic labeling methods, such as biotin ligase based biotinylation,[7] transglutaminase based labeling,[8] and mutant glycosyltranferase based labeling[9,10] have also been reported. However, these methods have limitations in that they can only be applied to a restricted number of target proteins, as the acceptor sites on natural proteins are usually rare and the numbers of enzymes available for labeling are limited.

Click chemistry is a way to quickly and reliably join small units together.[11] One of the simple click methodologies is the azide-alkyne Huisgen cycloaddition using a copper (Cu) catalyst at room temperature.[12] Click chemistry has been successfully applied to glycan metabolic labeling[13–15] and *exo*-enzymatic labeling of N-glycans on living cells.[16] In GLCC, a specific glycosyltransferase incorporates a clickable monosaccharide into a glycosylation site, that

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is, generated by the removal of existing carbohydrate moieties with specific glycosidases, therefore allowing specific labeling or detection of particular glycans on a target protein.

2. Materials and methods

Bovine serum fetuin and CTP were from Sigma Aldrich. Recombinant human enzymes ST3Gal1, ST3Gal5, ST6Gal1, ST6GalNAc4, GALNT1, GALNT2, GALNT3, GALNT7, CD73, CD39L3, recombinant human TNFα and TNFβ, biotinylated recombinant human TNFα, recombinant yeast pyrophosphatase *S cerevisiae* PPA1, recombinant *N. meningitidis* SiaB, recombinant *E. faecalis* Endo-EF, recombinant *C. perfringens* neuraminidase, mouse *anti*-human IL1 IgG and its biotinylated version, streptavidin conjugated horse-radish peroxidase (strep-HRP) and its ECL substrate were from Bio-Techne/R&D Systems. Click-IT® biotin DIBO Alkyne was from Life Technologies.

2.1. Generating glycosyltransferase acceptor sites through glycosidase treatment

To remove the existing terminal sialic acid, a glycoprotein sample was mixed with *C. perfringens* neuraminidase at the mass ratio of 100:1 in a buffer of 25 mM Tris, 150 mM NaCl at pH 7.5, and at room temperature for 20 min. The treated sample was then separated on a gel filtration column to remove the neuraminidase. For O-glycan replacement labeling, the sample was first treated with both neuraminidase and *E. faecalis* Endo-EF under otherwise identical conditions. The glycosidases in the reactions were then inactivated by heat treatment at 95 °C for 2 min.

2.2. Glycosyltransferase labeling reaction

Glycosyltransferase reactions were carried out according to previously described methods.[17] For the sialic acid replacement reaction, a sample of 10 μg protein was mixed with 0.3 nmol of CMP-azido-Sialic acid, 0.1 μg of CD73 and 2 μg of sialyltransferase in 50 μL of 25 mM Tris, supplemented with 10 mM of $MnCl_2$ and 150 mM NaCl at pH 7.5, and incubated at 37 °C for minimum of 20 min. For the O-glycan replacement reaction, 10 μg glycoprotein sample was mixed with 0.3 nmol of UDP-azido-GalNAc, 2 μg of a ppGalNAcT (or GALNT), and 0.1 μg rhCD39L3 in 50 μL of 25 mM Tris supplemented with 10 mM of $MnCl_2$ and 150 mM NaCl at pH 7.5 and incubated at 37 °C for minimum of 20 min.

2.3. Click chemistry reaction

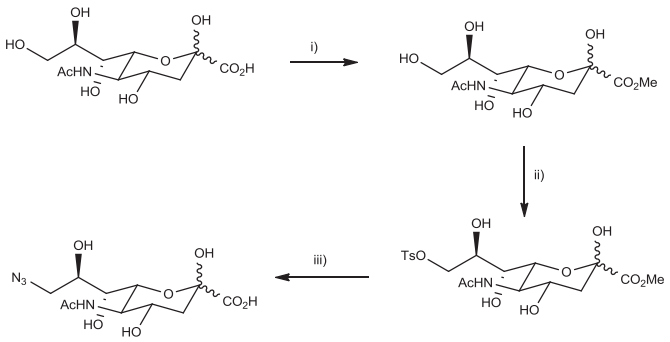
For the click chemistry reactions, ascorbic acid, $CuCl_2$ and biotin alkyne adduct D were directly added to the glycosyltransferase reaction with the final concentration of 2 mM, 0.1 mM and 0.1 mM, respectively. The mixture was incubated at room temperature for a minimum of 30 min. For copper free click chemistry reactions, 30 μM of Click-IT® biotin DIBO Alkyne was added into each reaction and incubated at room temperature for 5 h.

2.4. SDS-PAGE and gel blotting

Once the click chemistry reaction was complete, the samples were separated on 12% SDS-PAGE gel. The gels were visualized with UV in the presence of trichlorethanol (TCE staining), which reacts with the indole ring of the amino acid tryptophan.[18] Next, the gels were blotted to nitrocellulose paper under 25 V for 30 min. The blots were then blocked with 10% fat-free milk for 10 min, followed by probing with strep-HRP at 30 ng/mL for 30 min. The blots were then washed three times with TBST buffer containing 25 mM Tris, pH 7.6, 137 mM NaCl and 0.01% Tween (TBS) for 30 min. The membrane was finally visualized with enhanced chemiluminescence (ECL) peroxidase substrate.

2.5. Synthesis of CMP-azido-sialic acid

N-acetyl neuraminic acid methylester was prepared as follows. N-acetyl neuraminic acid (10.00 g, 32.33 mmol) was dissolved in methanol (100 mL) and trifluoroacetic acid (4.00 mL, 5.96 g, 52.24 mmol) was subsequently added. The solution was stirred overnight and then evaporated to dryness to give the required product (10.44 g, 99%). 9-Azido sialic acid was first synthesized according to a known procedure (Scheme 1).[19] Analysis was in accordance with the previously published data. CMP-azido-sialic acid was then synthesized using an enzymatic method.[20] Briefly, 2 μmol CTP plus 2 μmol 9-azido sialic acid were mixed with 20 μg of r*N. meningitidis* Sia B and 5 μg of yeast inorganic pyrophosphatase *S. cerevisiae* PPA1 in 0.5 mL of buffer containing 25 mM Tris and 10 mM $MgCl_2$ at pH 7.5. The mixture was incubated at 37 °C for 1 h. The formed CMP-azido-sialic acid was directly used for subsequent labeling reactions. CMP-azido-sialic acid may be purified through cation-exchange purification if necessary.



Scheme 1. Synthesis of 9-azido sialic acid.

2.6. Synthesis of biotin alkyne adduct D (3-(2′-(2″-(2‴-Amide-D-biotin-ethoxy)ethoxy)ethoxy)prop-1-yne)

The 2-(2-(2-(prop-2-yn-1-yloxy)ethoxy)ethoxy)ethanamine was synthesized following a known procedure[21] and converted into the required 3-(2′-(2″-(2‴-Amide-D-biotin-ethoxy)ethoxy)ethoxy) prop-1-yne via reaction with biotin, again via modification of a published procedure (Scheme 2).[22] Analysis was in accordance with the previously published data.

2.7. Synthesis of UDP-azido-GalNAc

Uridine 5′-(trihydrogen diphosphate), 2′-deoxy-, P′-[2-[(2-azidoacetyl)amino]-2-deoxy-α-D-galactopyranosyl] ester ammonium salt was synthesized by modification of a known procedure.[23] Thus α-galactosamine 1-phosphate was treated with *NHS*-2-azidoacetate and the crude intermediate, after conversion to the triethylammonium salt, was reacted directly with uridine 5′-monophosphomorpholidate 4-morpholine-N,N-dicyclohexylcarboxamidine salt. Crude purification using HILIC preparative chromatography and lyophilization gave a mixture containing 66% of uridine 5′-(trihydrogen diphosphate), 2′-deoxy-, P′-[2-[(2-azidoacetyl)amino]-2-deoxy-α-D-galactopyranosyl] ester ammonium salt (Scheme 3). Analysis was in accordance with the previously published data.