Glycosylation Analysis

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Assessing the Terminal Glycosylation of a Glycoprotein by the Naked Eye**

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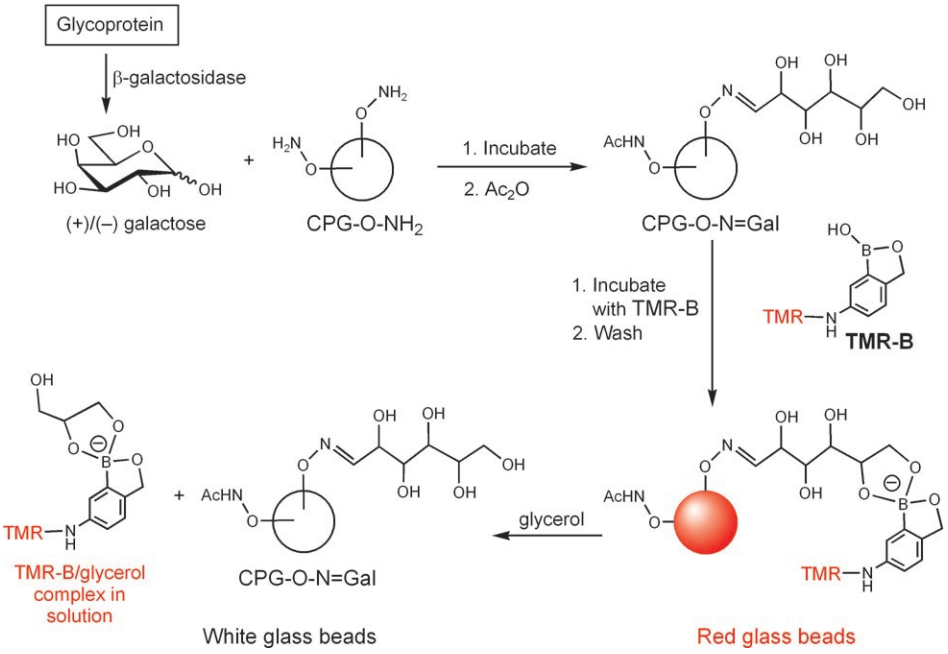
Analysis of the glycosylation pattern of a glycoprotein is generally considered a complex undertaking that is best performed by experienced and highly specialized researchers. The most commonly used techniques involve releasing the oligosaccharides from the protein followed by analysis using mass spectrometry[1] (either with or without derivatization) or by chromatographic analysis (often after fluorescence tagging) assisted by the availability of reference standards.[2]

A second method involves the use of carbohydrate-binding proteins of known specificity, especially plant lectins. Lectins are available that are specific for most of the terminal oligosaccharide sequences expected on mammalian glycoproteins. The analytical approach can involve sequential lectin affinity chromatography[3] or, more recently, the binding of intact glycoproteins to immobilized lectin arrays,[4] yielding at least qualitative information on glycosylation patterns.

We sought a general method that could provide information on the terminal glycosylation state of a glycoprotein and that would not require any sophisticated equipment or experience in glycoanalysis. One immediate area of application might be in the analysis of recombinant glycoprotein therapeutics whose biological activity can be strongly affected by their glycosylation.[5] In particular, glycoforms that bear terminal non-sialylated galactosyl residues are highly undesirable as they are quickly cleared by the liver. We therefore chose to specifically develop a visual technique for assessing terminal galactosylation but with the expectation that it could be extended to other types of terminal glycosylation (e.g. *N*-acetylglucosamine, fucose, etc).

The strategy we pursued is presented in Scheme 1. The glycoprotein to be investigated is incubated with an exo-glycosidase (e.g. β-galactosidase) to probe for the presence of terminal β-galactosylated glycans. If terminal β-galactose residues are present, reducing galactose (Gal) will be released which can then be covalently captured on glass beads functionalized with hydroxylamine groups. We used controlled pore glass (CPG) beads that we have previously described,[6] here designated CPG-O-NH_2. The Gal is captured on the beads as predominantly the acyclic oxime, designated CPG-O-N=Gal (Scheme 1). Brief treatment with acetic anhydride then "caps" any unreacted hydroxylamine groups.



Scheme 1. Schematic representation of the strategy for the visual detection of the terminal glycosylation state of a glycoprotein. The complete structure of TMR-B is shown in Scheme 2.

Aromatic boronic acids are known to bind polyalcohols even in water, often with a strong preference for acyclic polyols such as glycerol or sorbitol.[7] The reagent chosen for the detection of captured Gal on CPG-O-N=Gal was therefore an arylboronate attached to a fluorescent dye. Specifically, we prepared TMR-B (Scheme 2), where the novel *o*-hydroxymethylphenyl boronic acid recently reported by Dowlut and Hall[8] was conjugated to tetramethylrhodamine

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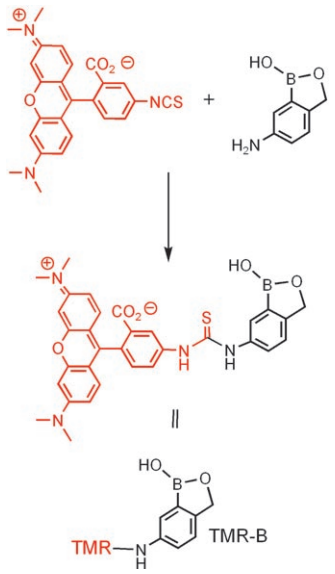

Scheme 2. Synthesis and structure of TMR-B.

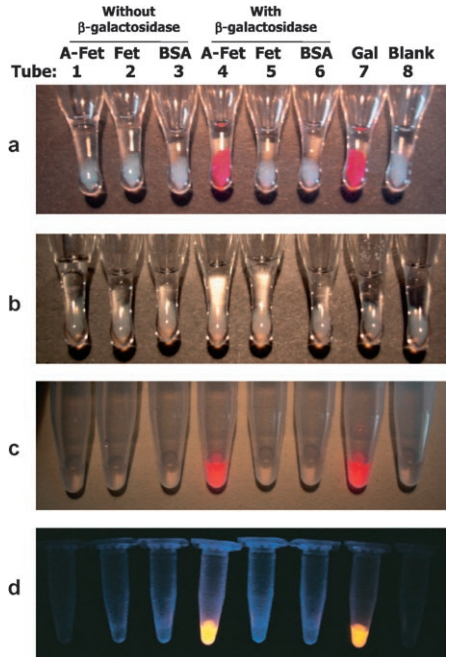

Figure 1. Visual detection of Gal captured on glass beads. a) Capture beads (CPG-O-NH_2, 2 mg) that have been exposed to ultrafiltered solutions derived from A-Fet (tube 1), Fet (tube 2), BSA (tube 3), A-Fet + β-galactosidase (tube 4), Fet + β-galactosidase (tube 5), BSA + β-galactosidase (tube 6), 2 mM Gal (tube 7), and buffer alone (tube 8), followed by staining with TMR-B and washing. The solution above the beads is capture buffer. b) The beads in tubes 1–8 (panel a) after they have been treated with glycerol/methanol/water. c) The glycerol/methanol/water solutions derived from tubes 1–8 (panel a). d) The tubes in panel c viewed under the light of a handheld long-wavelength UV lamp.

(TMR), a brilliant red fluorescent dye. The preparation of TMR-B, wherein B is used to indicate the boronic acid moiety, required a single step from commercial starting materials (Scheme 2, see the Supporting Information). TMR-B has good solubility in aqueous solutions.

Incubation of CPG-O-N=Gal with a solution of TMR-B should result in the formation of a covalent complex (Scheme 1, where the boronate is hypothetically shown binding to a single terminal diol), from which unbound boronate can be removed by washing. CPG without the captured Gal cannot form such a complex. The prediction is therefore that beads derived from a glycoprotein bearing glycan chains terminating in β-Gal residues will turn red following the process of Scheme 1, while those without terminal Gal will remain colorless.

The strategy of Scheme 1 was evaluated on two model glycoproteins. Bovine fetuin (Fet) has three N-linked and three O-linked glycosylation sites[9] with a high, but not complete, degree of site occupancy,[1h] and where essentially all of the glycan chains terminate in sialic acid. Asialo-fetuin (A-Fet) is derived from Fet by removal of terminal sialic acid residues using mild acid hydrolysis, resulting in the exposure of terminal β-Gal residues.[9a] Bovine serum albumin (BSA), a non-glycosylated protein, was included as a control.

Concentrated solutions of A-Fet, Fet, and BSA (1 mg per 100 μL) were processed according to Scheme 1, each without and with prior incubation with β-galactosidase (bovine testes[10]). After incubation, the protein solutions were centrifuged in a microcon tube; the filtrate devoid of protein was incubated with the CPG-O-NH_2 at 60 °C overnight, then processed according to Scheme 1. The resulting beads are shown in Figure 1, where tubes 1–3 are derived from A-Fet, Fet, and BSA, respectively, that had not been exposed to β-galactosidase. Tubes 4–6 show the beads derived from A-Fet, Fet, and BSA, respectively, that had been exposed to β-galactosidase. Included for controls are a solution of pure Gal (tube 7, 2 mM in the enzyme incubation buffer) and a blank (tube 8, the enzyme incubation buffer alone). The photograph of the resulting beads (2 mg), taken with a small handheld digital camera, is shown in Figure 1 a. It is completely clear that only the beads in tube 4 (A-Fet that had been treated with β-galactosidase) and tube 7 (the reference standard Gal) are red. There was no visually detectable background for the other beads.

Incubation of the beads in tubes 4 and 7 (Figure 1 a) with a glycerol-containing solution (glycerol/methanol/water, 1:2:2) eluted the red TMR-B/glycerol complex back into solution (shown schematically in Scheme 1, lower left). The supernatant glycerol solutions were transferred to microfuge tubes (Figure 1 c) where the red color is again readily detected by eye. Visualization of the same microfuge tubes under a handheld long-wavelength UV lamp further highlights the presence of the fluorescent compound in solution (Figure 1 d).

The absence of a visually detectable background binding in the absence of terminal galactosylation prompted us to evaluate this simple technique with respect to sensitivity of detection and possible quantification. To this end, a series of solutions containing Gal in the concentration range 0–160 μM were processed according to Scheme 1. The red color of the beads decreased progressively to light pink with decreasing Gal concentration (Figure 2 a). Even more impressive was the

distinction that could clearly be made between the blank beads (tube 1, Figure 2b, no Gal) and the beads that had been incubated with 5 μM Gal (tube 2, Figure 2b) when the beads

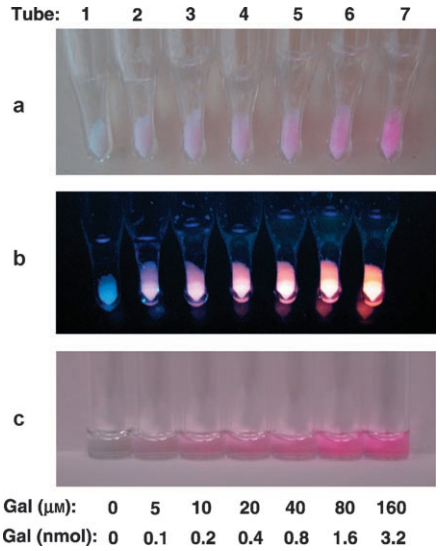

Figure 2. Variation of visual response as a function of Gal concentration. a) Capture beads (CPG-O-NH_2, 2 mg) that have been exposed to solutions of Gal (0–160 μM, corresponding to 0–3.2 nmol), followed by staining with TMR-B and washing. b) The tubes in panel a viewed under the light of a handheld long-wavelength UV lamp. c) The glycerol/methanol/water solutions derived from tubes 1–7.

were observed under long-wavelength UV light (Figure 2b). Assuming that the Gal capture had been quantitative, the 2 mg of beads shown in tube 2 (Figure 2) carry only 100 picomoles (18 ng) of Gal.

The beads in tubes 1–7 (Figure 2) were incubated with glycerol/methanol/water (1:2:2) and the supernatants transferred to vials providing a set of visual standards (Figure 2c) that allow correlation of the intensity of the red color to the concentration of Gal used in the capture experiments. By comparison with this set of solutions, the concentration of Gal in a capture solution derived from a glycoprotein can be visually estimated as being, for example, "near 40 μM" or "less than 5 μM".

The fluorescence of the eluted TMR-B/glycerol solutions (Figure 2c) was measured using a NanoDrop fluorimeter, a cuvetteless device requiring only 1 μL of sample. The standard curve obtained for the concentration range 0–160 μM Gal, using the solutions shown in Figure 2c is shown in Figure 3. The nonlinearity of the curve is a common feature of rhodamine and fluorescein dyes at micromolar concentrations and is attributed to the stacking of the molecules with accompanying changes in their spectroscopic properties.[11] A quantitative estimation of the Gal concentration can still be obtained fluorometrically, provided that the experimental point of interest falls on the standard curve.

In a final experiment with glycoproteins, solutions of A-Fet and Fet at a concentration of 20 μg per 100 μL (1/50th of that used in Figure 1) were run through the method of Scheme 1 using 10 mg of capture beads. As expected, only the

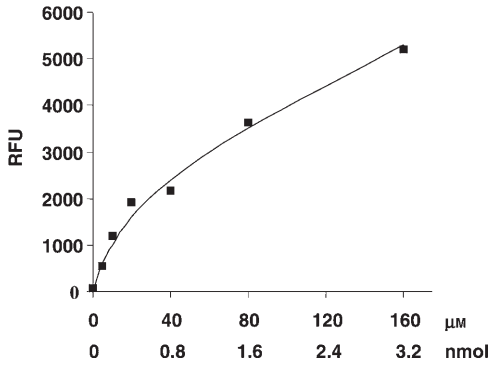

Figure 3. Fluorescence of TMR-B/glycerol solutions (100 μL) released from beads (2 mg) as a function of the concentration (μM) and absolute quantity (nmol) of Gal for solutions used in the capture experiments. RFU = relative fluorescence units.

β-galactosidase-treated A-Fet sample yielded red beads, which were visually of approximately the same intensity as those derived from the capture of "between 20 and 40 μM solution of Gal" (Figure 2c). The fluorescence of the TMR-B/glycerol solution eluted from 2 mg of beads was 2074 RFU, suggesting a concentration near 35 μM using the standard curve of Figure 3, thus confirming the visual estimation.

It was of interest to examine whether the remaining classes of potential terminal sugar residues could also be captured and form complexes with TMR-B, thus potentially expanding the utility beyond the analysis of terminal galactosylation. To this end, we incubated solutions of Gal (a hexose), fucose (Fuc, a deoxyhexose), sialic acid (*N*-acetylneuraminic acid, Neu5Ac) and *N*-acetylglucosamine (GlcNAc, an aminodeoxyhexose), each at 40 μM, with the CPG-O-NH_2 capture beads under the conditions described above for Gal. After capping, staining with TMR-B, and washing, the beads all became red to the naked eye. Elution of the dye using the glycerol solution allowed quantification of the relative efficiency of the entire capture/dye-binding/washing sequence for each sugar (Figure 4). The relative responses were Gal/Fuc/Neu5Ac/GlcNAc 1:0.67:0.59:0.36. The especially low value for GlcNAc likely reflects the known low reactivity of this molecule in oxime formation (and

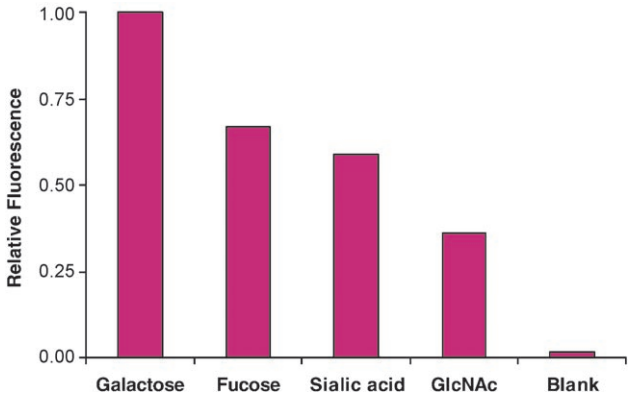

Figure 4. Relative response of different sugars in the capture/stain/release procedure.

reductive amination).[12] More experimentation will be required to optimize each of the steps of the capture/dye-binding/washing sequence for the entire set of mammalian monosaccharides, a process expected to yield more reliable response factors and to establish their reproducibility. This work is in progress.

The novelty of the present method is the ability to detect and semiquantify the terminal glycosylation of a glycoprotein by the naked eye. The method has been demonstrated here for terminal β-galactosylation. It should, however, be applicable to any terminal glycosylation provided that 1) a specific glycosidase is available that can cleave the terminal sugar residue in question, 2) the released monosaccharide can be captured on the hydroxylamine beads, and 3) the captured monosaccharide can form a complex with TMR-B that is stable to washing. For mammalian glycoproteins, the required exo-glycosidases (α-sialidases, α-fucosidases, α- and β-galactosidases, α- and β-hexosaminidases, α-mannosidases, β-glucuronidases, and others), all with anomeric specificity and some with linkage specificity, have been described.[1a,c,13] These glycosidases are in fact routinely applied in the enzyme-assisted sequencing of glycans. In the present research we have additionally confirmed that Gal, Fuc, Neu5Ac, and GlcNAc can be captured and detected by the same procedure, though each with a different response factor. Thus all classes of monosaccharide present in mammalian glycoproteins should be detectable by this method.

In the present method, the required exo-glycosidases act on the intact glycoprotein and not on the released glycans, so caution must be exercised regarding the steric accessibility of the glycan chains to the enzyme active sites. A partial protease digestion may therefore be required prior to the exo-glycosidase digestions. The present method should, however, be ideally suited for the routine monitoring of the terminal glycosylation of a known glycoprotein, for example, a biopharmaceutical in development, as the behavior of the glycoprotein towards exo-glycosidase digestion can be established prior to the evaluation of, for example, different cell-culture conditions for glycoprotein expression.

The key points that were specifically addressed in this work are whether hydroxylamine-functionalized glass is a suitable matrix for the capture and visualization of monosaccharides and whether a single reagent (TMR-B) can be used for broad detection of the captured monosaccharides. The answer to both questions is "yes". The potential limit of detection was not directly addressed in the present work, as no effort was made to minimize sample volumes. From Figure 2 it appears that stained beads derived from solutions of Gal that are more dilute than micromolar are difficult to visually detect. When reference solutions of pure TMR-B/glycerol complex were viewed by various members of the research laboratory, the lower limit of detection spanned the range 0.1–1 μM Gal (under the conditions used in Figure 2). Even with this limitation imposed by the human eye, microgram quantities of glycoproteins can be visually evaluated. Tube 5 (Figure 2), for example, reports on the approximately 0.7-nmol of Gal released from 4 μg (ca. 0.1 nmol) of A-Fet.

Finally, the most important feature that justifies future development of the method is that a single reagent, TMR-B, is used for the detection and potential quantification of all sugars without the need for chromatographic separations. Extensive efforts are therefore underway to optimize the capture/staining/washing procedures as well as miniaturizing the assay with the expectation of increased speed, efficiency, and sensitivity.

Experimental Section

General procedures are as described.[6] The synthesis of TMR-B and the preparation of CPG-ONH_2 and protein samples are reported in detail in the Supporting Information. "Buffer" in the following refers to 0.1M citrate/phosphate buffer, pH 5.0.

Enzymatic release of terminal galactose from A-Fet: A solution of dialyzed A-Fet (12 μL, ca. 60 μg) was added to a solution of dialyzed β-galactosidase (18 μL, 2.6 U mL^{-1}) and buffer (270 μL). The solution was incubated at 37°C. After 24 h, 130 μL of solution was removed placed in a prewashed Microcon YM-10 (10000 molecular-weight cutoff dialysis membrane) and centrifuged (25 min at 25°C and 14000 × g). The ultrafiltrate was used directly in the capture experiments.

General procedure for the capture of sugars: A solution (100 μL) containing a sugar in buffer, either as a reference standard or as the ultrafiltrate from a β-galactosidase-digested glycoprotein, was added under argon to CPG-O-NH_2 (10 mg) in a Teflon-fritted plastic 1 mL syringe, and the syringe was closed at both ends and incubated at 60°C in a heating block over night. The beads were cooled to room temperature, and 50% Ac_2O in anhydrous MeOH (100 μL) was added to cap remaining hydroxylamine groups. After 20 min the manipulations were no longer performed under an argon atmosphere, and the beads were washed with H_2O (2 × 200 μL), 5% *N*,*N*-diisopropylethylamine (DIPEA)/DMF (200 μL) and MeOH (2 × 200 μL). The beads were dried under vacuum. When the sugar used was Gal, the product was designated CPG-O-N=Gal (Scheme 1).

Visual detection of captured galactose by complexation of TMR-B from solution: A sample of CPG-O-N=Gal (2 mg) was washed with DMF (1 × 0.5 mL) and treated with a mixture of TMR-B in DMF (0.5 mM, 100 μL) and 0.1M $NaHCO_3/Na_2CO_3$, pH 9 (100 μL). The beads were gently shaken for 1 h and then washed with DMF (2 × 200 μL) and 0.1M $NaHCO_3/Na_2CO_3$, pH 9 (2 × 200 μL).

Release of TMR-B from stained beads: The stained beads obtained above (2 mg) were used directly after washing and without drying. A solution of glycerol/MeOH/H_2O (1:2:2, 100 μL) was added to the beads to release bound TMR-B. The beads were gently shaken for 1 h. The supernatant, now containing the fluorescent TMR-B/glycerol complex, was removed from the beads for fluorescence measurement using a NanoDrop ND-3300 fluorospectrometer. Irradiation was with a white light-emitting diode (500–650 nm) with emission measured at 579 nm. The quantity of released fluorescent boronate was then estimated using a standard curve.

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