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Generation of a natural glycan microarray using 9-fluorenylmethyl chloroformate (FmocCl) as a cleavable fluorescent tag

Xuezheng Song, Yi Lasanajak, Carlos Rivera-Marrero, Anthony Luyai, Margaret Willard, David F. Smith, Richard D. Cummings *

Department of Biochemistry, Emory University School of Medicine, O. Wayne Rollins Research Center, 1510 Clifton Road, Suite 4001, Atlanta, GA 30322, USA

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ABSTRACT

Glycan microarray technology has become a successful tool for studying protein–carbohydrate interactions, but a limitation has been the laborious synthesis of glycan structures by enzymatic and chemical methods. Here we describe a new method to generate quantifiable glycan libraries from natural sources by combining widely used protease digestion of glycoproteins and Fmoc chemistry. Glycoproteins including chicken ovalbumin, bovine fetuin, and horseradish peroxidase (HRP) were digested by Pronase, protected by FmocCl, and efficiently separated by 2D-HPLC. We show that glycans from HRP glycopeptides separated by HPLC and fluorescence monitoring retained their natural reducing end structures, mostly core α1,3-fucose and core α1,2-xylose. After simple Fmoc deprotection, the glycans were printed on NHS-activated glass slides. The glycans were interrogated using plant lectins and antibodies in sera from mice infected with *Schistosoma mansoni*, which revealed the presence of both IgM and IgG antibody responses to HRP glycopeptides. This simple approach to glycopeptide purification and conjugation allows for the development of natural glycopeptide microarrays without the need to remove and derivatize glycans and potentially compromise their reducing end determinants.

The field of functional glycomics [1,2] has recently seen enormous progress by the development of high-throughput methods such as glycan microarrays [3–8]. Glycan structures are immobilized onto activated glass surfaces and interrogated with glycoproteins, antibodies, or whole microorganisms. Many methodologies enabling solid-phase immobilization of glycan derivatives onto various activated surfaces have been developed for the purpose of microarrays. Noncovalent attachment mechanisms include TLC plate–protein overlay [9,10], neoglycolipids–nitrocellulose membrane [11,12], and biotinylated glycoconjugate–streptavidin-coated substrates [3]. Covalent attachment generally utilizes interreactive pairs, such as activated ester-primary amine [5,13], epoxy-amine [14–16], sulfhydryl-maleimide [17–19], and azide-alkyne [20]. While each methodology has its own advantages and disadvantages, their preference depends on the availability of the solid-phase substrate and the convenience of the chemistry. The glycan microarray of the Consortium of Functional Glycomics, utilizing commercially available *N*-hydroxysuccinimide (NHS)[1]-activated glass slides and including more than 400 glycans, has been one of the most successful glycan microarrays.

Despite the enormous success, the progress of glycan microarray methodology has been limited by the relatively slow expansion of glycan libraries relative to the large size of the glycome. An alternative approach is to develop natural glycan libraries, where glycans from natural sources are released, tagged, and separated. These tagged glycan libraries (TGLs) can be prepared directly from any biological system such as cells, tissues, and microorganisms, and can be interrogated with relevant carbohydrate binding proteins (GBPs), antibodies, and microorganisms. The most crucial component in TGL methodology is the glycan derivatization chemistry, which needs not only to facilitate the separation by adding a sensitive detectable tag but also to functionalize the glycan for further conjugation such as solid-phase immobilization. Widely used fluorescent labels such as 2-aminobenzamide (2-AB) and 2-aminobenzoic acid (2-AA) [21] are good tags for HPLC separation, but are not suitable for further reaction. The bifunctional alkoxyamine linker [22]

* Corresponding author. Fax: +1 404 727 2738.
E-mail address: rdcummi@emory.edu (R.D. Cummings).

[1] *Abbreviations used*: AAL, *Aleuria aurantia* Lectin; AEAB, 2-amino-*N*-(2-aminoethyl)-benzamide; ConA, concanavalin A; c.v., column volumes; DAP, 2,6-diaminopyridine; DMF, dimethylformamide; FmocCL, 9-fluorenylmethyl chloroformate; GBP, glycan binding protein; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; NHS, *N*-hydroxysuccinimide; RCA I, *Ricinus communis* agglutinin I; RFU, relative fluorescence unit; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TGL, tagged glycan library; THF, tetrahydrofuran.

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enables further solid-phase immobilization, but it is not fluorescent or UV active, which limits its application to structurally defined glycans at relatively large scale. To address these questions, we have developed new methods utilizing fluorescent homo- or heterobifunctional linkers, i.e., 2,6-diaminopyridine (DAP) [15,23] and *N*-aminoethyl 2-aminobenzamide (AEAB) [24] for the generation of TGLs and successfully applied them for the identification of galectin ligands. In this paper, we report a new strategy utilizing well-known 9-fluorenylmethyl chloroformate (FmocCl) chemistry to generate TGLs for functional study. Arsequell et al. reported the derivatization of glycosylamine derivatives of free oligosaccharides with Fmoc glycine for oligosaccharide fractionation [25]. Here we show that Fmoc serves as a fluorescent tag for glycoamino acids and glycopeptides and facilitates their separation, characterization, and quantification. Most importantly, Fmoc is conveniently removed to expose amino groups on the peptide moiety for further reaction such as solid-phase immobilization. This new strategy avoids the release of glycans from glycoproteins by hazardous chemicals and expensive enzymes, can be applied to both *N*- and *O*-linked glycans as glycopeptides, and serves as a powerful tool for functional glycomics studies.

Materials and methods

Materials

HPLC solvents were purchased from Fisher Scientific (Pittsburgh, PA). An Ultraflex-II TOF/TOF system from Bruker Daltonics was used for MALDI-TOF mass spectrometry analysis of glycan conjugates. Pronase was purchased from EMD Chemicals Inc. (Darmstadt, Germany). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Chicken egg glycopeptide was prepared as described previously [26].

Pronase digestion of glycoproteins

The chicken egg glycopeptide and glycoproteins (200 mg) were dissolved in 0.1 M Tris buffer, pH 8.0, so that the final concentration was 20 mg/mL. Pronase was added to a final concentration of 0.1 mg/mL. At 24 and 48 h of incubation at 37 °C, an equivalent amount of Pronase was added, respectively. The final digestion was passed through a 2-g C18 Sep-Pak, from which the flowthrough was passed through a 1-g Carbograph and washed by 6 column volumes (c.v.) water. The glycoamino acids and glycopeptides were eluted from the Carbograph by 3 c.v. 50% acetonitrile with 0.1% trifluoroacetic acid (TFA). The elution was dried by Speed-vac for Fmoc derivatization. Chicken ovalbumin, bovine fetuin, and horseradish peroxidase were digested in a similar fashion.

Fmoc derivatization of glycopeptides

The lyophilized glycopeptides were reconstituted into cold water so that the concentration was 100 mg/mL. An equal volume of 50 mg/mL sodium bicarbonate ($NaHCO_3$) solution, an equal volume of water, and an equal volume of 20 mg/mL FmocCl in tetrahydrofuran (THF) were added. The mixture was then agitated vigorously by vortexing for 30 min, after which 3×1 vol of ethyl acetate was used to extract the mixture. The aqueous phase was applied on C18 Sep-Pak and washed by 6 c.v. water. The Fmoc glycopeptides were eluted by 50% acetonitrile with 0.1% TFA.

High-performance liquid chromatography (HPLC)

A Shimadzu HPLC CBM-20A system was used for HPLC analysis and separation of Fmoc glycopeptides, which was coupled with a UV detector SPD-20A and a fluorescence detector RF-10Axl. UV absorption at 254 nm or fluorescence at 254 nm excitation (Ex) and 340 nm emission (Em) were used to detect and quantify Fmoc-tagged glycopeptides.

For normal-phase HPLC separation, a Zorbax NH_2 column (250×4.6 mm) was used; the mobile phases were acetonitrile, water, and 250 mM ammonium acetate (pH 4.5). The concentration of water increased from 16% to 40% and the concentration of ammonium acetate buffer increased from 4% to 50% over 60 min.

For reverse-phase HPLC with a C18 column, a Vydec C18 column was used. The mobile phase was acetonitrile and water with 0.1% TFA. The concentration of acetonitrile increased from 15% to 45% in 30 min.

Generation of normal and 20-week Schistosoma mansoni-infected mice sera

Ten 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and infected with 50–60 *Schistosoma mansoni* cercariae (Puerto Rican strain) subcutaneously. Ten age-matched uninfected C57BL/6 mice served as controls. Both groups of mice were housed at the Emory University laboratory animal facility. After 20 weeks, the mice were bled via a retro-orbital route and blood was collected in microtainer serum separator tubes (BD and Company, Franklin Lakes, NJ). The microtainer tubes with blood were centrifuged at 6000 rpm in an Eppendorf bench-top microcentrifuge (Westbury, NY) for 10 min at room temperature. The sera were aspirated, pooled separately as either infected or uninfected normal mouse sera, and stored at −20 °C in small aliquots.

Printing, binding assay, and scanning

NHS-activated slides were purchased from Schott (Louisville, KY). Epoxy slides were purchased from Corning (Lowell, MA). Noncontact printing was performed using a Piezoarray printer from Perkin Elmer. The average spot volume was within 10% variation of 1/3 nL. All the samples were printed in phosphate buffer (300 mM sodium phosphates, pH 8.5). After printing, the slides were boxed loosely and put in a high-moisture chamber at 50 °C and incubated for 1 h. The slides were then washed and blocked with 50 mM ethanolamine in 0.1 M Tris buffer (pH 9.0) for 1 h. The slides were dried by centrifugation and stored desiccated at −20 °C for future use. Before assay, the slides were rehydrated for 5 min in TSM buffer (20 mM Tris–HCl, 150 mM sodium chloride (NaCl), 0.2 mM calcium chloride ($CaCl_2$), and 0.2 mM magnesium chloride ($MgCl_2$). Biotinylated lectins were used in the binding assay and the bound lectins were detected by a secondary incubation with cyanine 5-streptavidin. For multipanel experiments on a single slide, the array layout was designed using Piezoarray software according to the dimension of a standard 16-chamber adaptor. The adaptor was applied on the slide to separate a single slide into 16 chambers sealed from each other during the assay.

The slides were scanned with a Perkin Elmer ProScanarray microarray scanner equipped with 4 lasers covering an excitation range from 488 to 637 nm. The scanned images were analyzed with the ScanArray Express software. Detection of bound biotinylated lectins was carried out by incubation with cyanine5-streptavidin. Detection of bound mouse sera antibodies was carried out by incubation with Alexa568-labeled goat anti-mouse IgG and Alexa488-labeled goat anti-mouse IgM. For cyanine5 fluorescence, 649 nm (Ex) and 670 nm (Em) were used. For Alexa488, 495 nm (Ex) and 519 nm (Em) were used. For Alexa568, 579 nm (Ex) and 604 nm (Em) were used. All images obtained from the scanner were in grayscale and colored for easy discrimination.