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ARTICLE *in* ANALYTICAL CHEMISTRY · APRIL 2015

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Rapid Preparation of Released *N*-Glycans for HILIC Analysis Using a Labeling Reagent that Facilitates Sensitive Fluorescence and ESI-MS Detection

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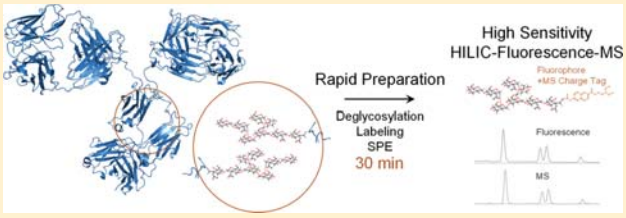
Supporting Information

ABSTRACT: *N*-glycosylation of proteins is now routinely characterized and monitored because of its significance to the detection of disease states and the manufacturing of biopharmaceuticals. At the same time, hydrophilic interaction chromatography (HILIC) has emerged as a powerful technology for *N*-glycan profiling. Sample preparation techniques for *N*-glycan HILIC analyses have however tended to be laborious or require compromises in sensitivity. To address these shortcomings, we have developed an *N*-glycan labeling reagent that provides enhanced fluorescence response and MS sensitivity for glycan detection and have also simplified the process of preparing a sample for analysis. The developed labeling reagent rapidly reacts with glycosylamines upon their release from glycoproteins. Within a 5 min reaction, enzymatically released *N*-glycans are labeled with this reagent comprised of an NHS-carbamate reactive group, a quinoline fluorophore, and a tertiary amine for enhancing ESI+ MS ionization. To further expedite the released *N*-glycan sample preparation, rapid tagging has been integrated with a fast PNGase F deglycosylation procedure that achieves complete deglycosylation of a diverse set of glycoproteins in approximately 10 min. Moreover, a technique for HILIC-SPE of the labeled glycans has been developed to provide quantitative recovery and facilitate immediate HILIC analysis of the prepared samples. The described approach makes it possible to quickly prepare *N*-glycan samples and to incorporate the use of a fluorescence and MS sensitivity enhancing labeling reagent. In demonstration of these new capabilities, we have combined the developed sample preparation techniques with UHPLC HILIC chromatography and high sensitivity mass spectrometry to thoroughly detail the *N*-glycan profile of a monoclonal antibody.



N-glycosylation of proteins is routinely characterized and monitored because of its significance to the detection of disease states[1−3] and the manufacturing of biopharmaceuticals.[4] In particular, the glycan profile of a biopharmaceutical is sometimes defined as a critical quality attribute,[5,6] since it can be a measure of efficacy, immunogenicity, and manufacturing conditions.[4,7] It is therefore beneficial for glycan analytical approaches to exhibit high sensitivity so that profiles can be thoroughly characterized. Additionally, decreasing analysis time and improving sample throughput would be valuable in situations where it is necessary to perform routine monitoring. Results from glycosylation assays are routinely used to guide the process development of protein-based drug candidates.[8,9] Accelerating *N*-glycan sample preparations and analyses might therefore hasten the commercialization of new protein-based therapeutics.

There are many diverse analytical approaches for the analysis of *N*-linked glycans. A commonality shared by most strategies for evaluating *N*-glycans is that they involve deglycosylation of glycoproteins with an enzyme, such as peptide-*N*-glycosidase F (PNGase F).[10] The resulting, free *N*-glycans can thereby be analyzed without interference from the heterogeneity of the protein. Numerous protein glycosylation studies have been performed without any further modification of these released *N*-glycans.[11,12] In an emerging approach, Bynum et al. characterized native *N*-glycans from monoclonal antibodies using an online PNGase F microreactor and an LC-MS technique based on porous graphitized carbon.[13] More often, released *N*-glycans are derivatized to enhance the sensitivity of an analysis. For example, permethylation of *N*-glycans has long been used to improve glycan MS detectability.[12,14−16] In yet another, highly effective approach, *N*-glycans can be labeled with a fluorescent tag.[12,17] Rudd and others have made pioneering contributions over the last two decades toward developing techniques wherein fluorescently labeled *N*-glycans are profiled using hydrophilic interaction chromatography (HILIC).[17,18] With recent advances in ultra high pressure liquid chromatography and sub-2 μm amide-bonded stationary

Received: February 25, 2015
Accepted: April 30, 2015
Published: April 30, 2015

DOI: 10.1021/acs.analchem.5b00758
Anal. Chem. 2015, 87, 5401−5409

phases, labeled *N*-glycans can now be separated with high resolution and detected by fluorescence (FLR) and potentially even MS.[19−23]

Unfortunately, approaches to the preparation of *N*-glycans for HILIC-FLR-MS have tended to be laborious or require compromises in MS sensitivity.[11,12] For instance, a traditional deglycosylation procedure requires that a glycoprotein sample be incubated for a minimum of 30 min, while many researchers conventionally employ an overnight incubation.[24,25] Combined with this process is a lengthy, 2 to 4 h labeling step based on reductive amination of aldehyde termini that form on *N*-glycans only after they hydrolyze from their glycosylamine forms.[11] Additionally, in the case of one of the most frequently employed labeling compounds, 2-aminobenzamide (2-AB), the resulting glycans are readily detected by fluorescence but are challenging to detect by electrospray ionization (ESI)-MS becuase of their poor ionization efficiency.

Variations to conventional approaches for the preparation of *N*-glycan samples that are suitable for HILIC analysis have been explored, but have not, as of yet, presented a practical solution that combines the desired attributes of simplicity, speed and high sensitivity. Alternative labeling reagents that have functional groups to enhance electrospray ionization efficiency have been used,[26,27] but this does not address the time-consuming nature of reductive amination reactions. Rapid tagging procedures that yield labeled glycans in a matter of minutes have consequently been investigated. In this approach, glycosylamines are modified with electrophilic reactive groups before they hydrolyze to reducing, aldehyde terminated glycans. Cook and co-workers have presented the use of a rapid tagging analog of aminobenzamide (AB).[28] Although such a rapid tagging reagent accelerates the labeling procedure, it does not provide the enhanced ionization efficiency needed to simplify glycan characterization and peak identification. Gong and researchers have, in addition, demonstrated the use of a rapid tagging labeling reagent, constructed with a basic tertiary amine, for the rapid preparation of labeled glycosylamines that are amenable to MS analysis.[29] However, the proposed labeling reagent does not impart a chromophore onto the glycan, thus limiting its utility for many analyses, wherein fluorescence-based profiling is desirable.[22,30]

To address the above shortcomings, we have developed an approach that both decreases the time required for *N*-glycan sample preparation and enhances the sensitivity of glycan detection. A labeling reagent, named RapiFluor-MS (or RFMS), has been synthesized that rapidly reacts (in <5 min) with glycosylamines upon their release from glycoproteins. RFMS is comprised of an *N*-hydroxysuccinimide (NHS) carbamate reactive group, a quinoline fluorophore, and a basic tertiary amine. Consequently, RFMS-labeled glycans can be detected by both fluorescence and positive ion mode ESI-MS with high sensitivity. The entire *N*-glycan sample preparation procedure has also been accelerated by directly integrating rapid tagging with a fast deglycosylation procedure and a HILIC-solid phase extraction (SPE) cleanup step that provides quantitative recovery of glycans and allows for immediate analysis of samples. In demonstration of these capabilities, we have combined the new sample preparation techniques with HILIC chromatography and high sensitivity mass spectrometry to thoroughly characterize the *N*-glycan profile of a monoclonal antibody (mAb). Rapid preparation of RFMS-labeled *N*-glycans has, in particular, been combined with high sensitivity MS/MS, to yield valuable structural information for low abundance glycans.

EXPERIMENTAL SECTION

Deglycosylation of Glycoproteins. Samples of anticitrinin murine monoclonal IgG1, pooled human IgG (from serum), bovine fetuin, or combinations thereof were deglycosylated using the same technique. Glycoproteins (15 μg) were diluted or reconstituted to a concentration of 0.52 mg/mL into a 28.8 μL solution of 1% (w/v) RG surfactant (RapiGest SF, Waters, Milford, MA) and 50 mM HEPES (pH 7.9). These solutions were heated to approximately 95 °C over 2 min, allowed to cool to 50 °C, and mixed with 1.2 μL of PNGase F solution (GlycoWorks Rapid PNGase F, Waters, Milford, MA). Deglycosylation was completed by incubating the samples at 50 °C for 5 min. It is worth noting that some glycoproteins, other than IgGs, must be subjected to reducing conditions in order for complete deglycosylation to be achieved. In such cases, use of 4 mM TCEP along with 1% RG surfactant has proven to be effective (see Supporting Information). Moreover, heating of samples to ≥80 °C is critical to ensuring that glycoproteins are sufficiently denatured and that *N*-glycans are readily accessible for enzymatic deglycosylation.

An important feature of these deglycosylation conditions is that they are quick and relatively stabilizing for the *N*-glycosylamine structures. An analysis on the stability of *N*-glycosylamines shows that they have a half-life of approximately 2 h at a temperature of 50 °C in the described buffer conditions (Supporting Information Figure S1).

Labeling of *N*-Glycosylamines with Rapid Tagging Reagents. Deglycosylation mixtures were allowed to cool to room temperature following their incubation at 50 °C and were then reacted with RFMS (*Rapi*Fluor-MS, Waters, Milford, MA) or IAB (Instant AB, Prozyme, Hayward, CA) without a protein depletion step. RFMS or IAB was dissolved in anhydrous dimethylformamide (DMF) to a concentration of 127 mM then mixed with the deglycosylation mixture in a 1:2.5 volumetric ratio to produce a reaction comprised of 36 mM labeling reagent and 0.36 mg/mL of deglycosylated protein. The molar excess of reagent over modifiable amine in these reactions was estimated to be approximately 50−300-fold (assuming 1 mg of glycoprotein will produce 0.3−0.7 μmol of released *N*-glycosylamines and proteinaceous amines, in addition to any other free amines in the mixture). Labeling reactions were allowed to proceed at room temperature for 5 min then diluted 1:9 with acetonitrile (ACN) in preparation for HILIC SPE.

Extraction of Labeled *N*-Glycosylamines Using HILIC SPE. The ACN-diluted samples of labeled *N*-glycosylamines were thereafter subjected to SPE using vacuum aspiration and a silica based aminopropyl sorbent (GlycoWorks μElution Plate, Waters, Milford, MA). Wells containing 5 mg of sorbent were conditioned with water (200 μL), equilibrated with 85% (v/v) ACN (200 μL), and then loaded with sample. Adsorbed samples were subsequently washed with two, 600 μL volumes of a solution containing 1% formic acid in 90% acetonitrile (ACN). Lastly, enriched, labeled glycosylamines were eluted in three 30 μL elution volumes from the SPE sorbent using an eluent composed of 200 mM ammonium acetate (pH 7), 5% ACN. The obtained labeled *N*-glycosylamines were immediately analyzed in the form of the SPE eluate (diluted to 22:25:53 (v/v/v) eluate/DMF/ACN). A sample dry-down step

DOI: 10.1021/acs.analchem.5b00758
Anal. Chem. 2015, 87, 5401−5409

was not needed with this preparation. Instead, samples were analyzed directly from this mixture. RFMS derivatized glycans were observed to be very stable in this sample diluent. Analysis of a sample before and after 3 days of storage at 10 °C showed there to be no changes in the glycan profile, as assessed by the techniques described below (Supporting Information Figure S2).

HILIC-Fluorescence-ESI-MS (MS/MS) Analysis of Labeled *N*-Glycans. To evaluate response factors, labeled *N*-glycans were analyzed via HILIC separations combined with fluorescence and mass spectrometric detection using a UHPLC chromatograph (ACQUITY UPLC H-Class Bio, Waters, Milford, MA). Either a 2.1 × 50 mm or a 2.1 × 150 mm column packed with 1.7 μm amide-bonded organosilica particles (ACQUITY UPLC Glycan BEH Amide 130 Å, Waters, Milford, MA) was employed along with an aqueous mobile phase comprised of 50 mM ammonium formate (pH 4.4) and another of ACN. Samples were injected as 1 μL aqueous volumes or 10 μL ACN/DMF volumes and separated at 60 °C according to the gradients shown in Supporting Information Tables S1 or S2. The quantities of *N*-glycans injected in each analysis are provided within the figure captions. Labeled *N*-glycans were detected using a fluorescence detector (5 Hz scan rate, Gain = 1, ACQUITY UPLC FLR, Waters, Milford, MA) using the excitation and emission wavelengths provided in Supporting Information Table S3. Eluting glycans were also detected by positive ion mode electrospray ionization mass spectrometry using an ion mobility capable QTof mass spectrometer (Synapt G2-S, Waters, Milford, MA) operating with a capillary voltage of 3.0 kV, source temperature of 120 °C, desolvation temperature of 350 °C, and sample cone voltage of 80 V. Mass spectra were acquired at a rate of 1 Hz with a resolution of approximately 20,000 over a range of 500−2500 *m*/*z*.

Detailed characterization of the anticitrinin murine IgG1 monoclonal antibody was performed with a QTof mass spectrometer outfitted with a high transmission efficiency collision cell (Xevo G2-XS QTof, Waters, Milford, MA). The mass spectrometer was operated with a capillary voltage of 3.0 kV, source temperature of 120 °C, desolvation temperature of 250 °C, and sample cone voltage of 80 V. Mass spectra were acquired at a rate of 2 Hz in MS and MS/MS modes with a resolution of approximately 30 000 over a range of 750−2000 *m*/*z*. MS/MS analyses were performed in continuum mode from 100−2000 *m*/*z* with collision induced dissociation (CID) to generate glycan fragmentation data. Ions with 2+ and 3+ charge states were selected for fragmentation. Customized collision energy (CE) tables that were charge state and mass specific were used for optimized fragmentations; the approximate CE range was between 15 and 40 eV. Data dependent acquisition (DDA) was used with duty cycle times of 1.6 and 0.5 s for MS and MS/MS modes. The two most abundant precursors were selected for fragmentation.

HILIC-Fluorescence-ESI-MS Analysis of Intact IgG. Deglycosylation of anticitrinin murine IgG1 was assayed via intact protein HILIC separations using columns packed with prototype sub-2 μm amide-bonded (300 Å average pore diameter) organosilica stationary phase and a UHPLC chromatograph (ACQUITY UPLC H-Class Bio, Waters, Milford, MA). Solutions of native or deglycosylated (see above) IgG were diluted to 0.14 mg/mL into solutions of 0.9% TFA, 73% ACN, and injected in 10 μL volumes onto two 2.1 × 150 mm columns coupled with a high pressure, low dead volume connector. Separations were performed at 80 °C using the mobile phases and gradient described in Supporting Information Table S4. Eluting species were detected serially via fluorescence detection (2 Hz, excitation 280 nm/emission 320 nm) followed by online ESI-MS with a QToF mass spectrometer (Waters Xevo G2 QTof, Milford, MA) operating with a capillary voltage of 3.0 kV, source temperature of 150 °C, desolvation temperature of 350 °C, and sample cone voltage of 45 V. Mass spectra were acquired at a rate of 2 Hz with a resolution of approximately 20 000 over a range of 500−5000 *m*/*z*.

RESULTS AND DISCUSSION

Rational Design of a New *N*-Glycan Labeling Reagent. A new labeling reagent (RFMS) for facilitating *N*-glycan analysis has been synthesized based on rational design considerations that would afford rapid labeling kinetics, high fluorescence quantum yields, and enhanced MS detectability (Figure 1). Conventional *N*-glycan sample preparation is

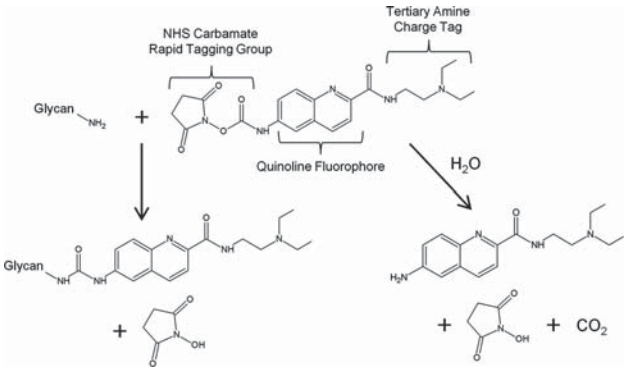


Figure 1. RFMS derivatization of an *N*-glycosylamine. The pathway on the left shows the derivatization of an *N*-glycosylamine, which produces an *N*-glycan with a urea (NH−CO−NH) linked RFMS label. Hydrolysis of RFMS is shown in the pathway on the right.

dependent on reductive amination of aldehyde terminated saccharides, a process that requires glycans to undergo multiple chemical conversions and a lengthy high temperature incubation step.[12] Moreover, glycans must be reductively aminated in anhydrous conditions to minimize desialylation.[31] Sample preparations are therefore burdened with transitioning a sample from aqueous to anhydrous conditions. For these reasons, the newly designed labeling reagent foregoes reductive amination and instead takes advantage of an aqueous rapid tagging reaction. An NHS-carbamate reactive group was chosen for its rapid reaction kinetics (Figure 1, left-side). Electrophilic reactive groups activated with NHS leaving groups have long been used to modify proteinaceous amine residues.[32] The NHS-carbamate reactive group has, in particular, been used for decades to derivatize free amino acids.[33,34] Notably, NHS-carbamate reagents hydrolyze to form amine byproducts and thereby undergo self-quenching reactions (Figure 1, right-side), making reaction steps simple to implement. The NHS-carbamate reactive group of this reagent rapidly modifies glycosylamine-bearing *N*-glycans after their enzymatic release from glycoproteins. Within a 5 min reaction, *N*-glycans are labeled with the new reagent under ambient, aqueous conditions to yield a highly stable urea linkage (Supporting Information Figure S2). In addition to rapid tagging

DOI: 10.1021/acs.analchem.5b00758
Anal. Chem. 2015, 87, 5401−5409

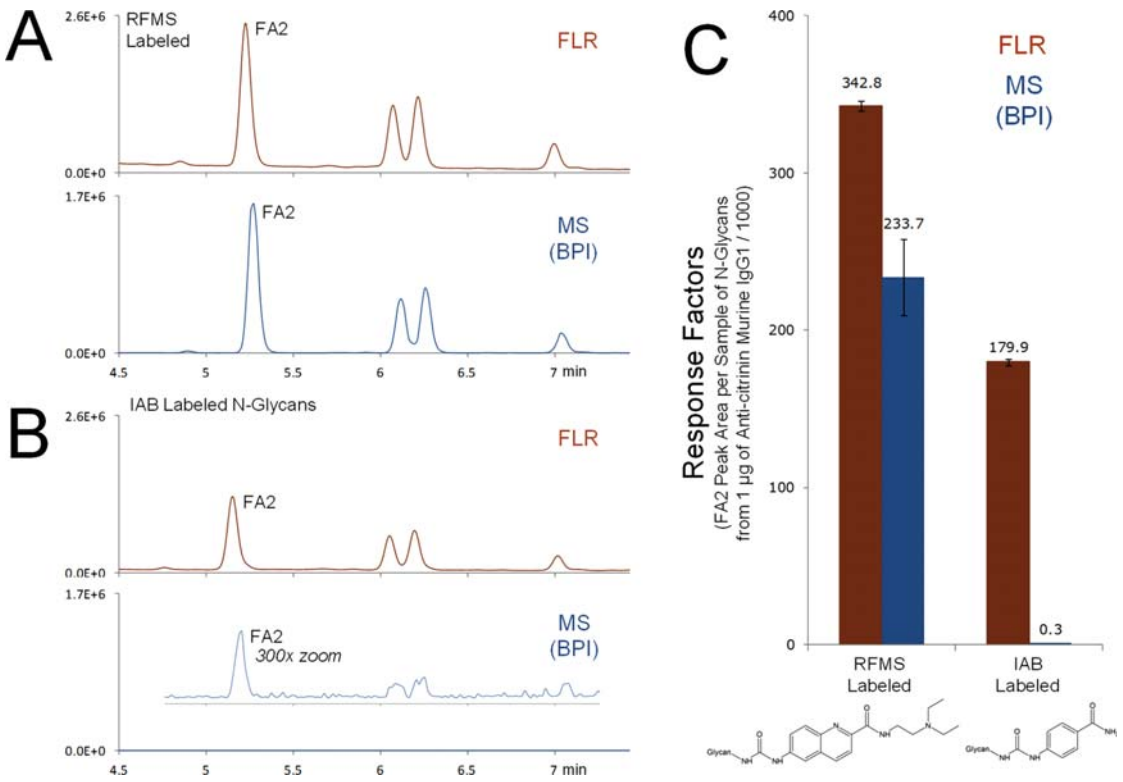


Figure 2. HILIC-FLR-MS of (A) RFMS- and (B) IAB-labeled *N*-glycans from anticitrinin murine IgG1. Fluorescence (FLR) chromatograms are shown in orange and base peak intensity (BPI) MS chromatograms are shown in blue. Labeled glycans (from 0.4 μg of glycoprotein, 1 μL of aqueous injection) were separated using a 2.1 × 50 mm column packed with 1.7 μm amide bonded organosilica (130 Å) stationary phase. (C) Response factors for RFMS and IAB labeled glycans (measured as the FA2 peak area per sample of *N*-glycans resulting from 1 μg of anticitrinin murine IgG1). Fluorescence (FLR) and MS (base peak intensity) response factors are shown in orange and blue, respectively. Analyses were performed in duplicate.

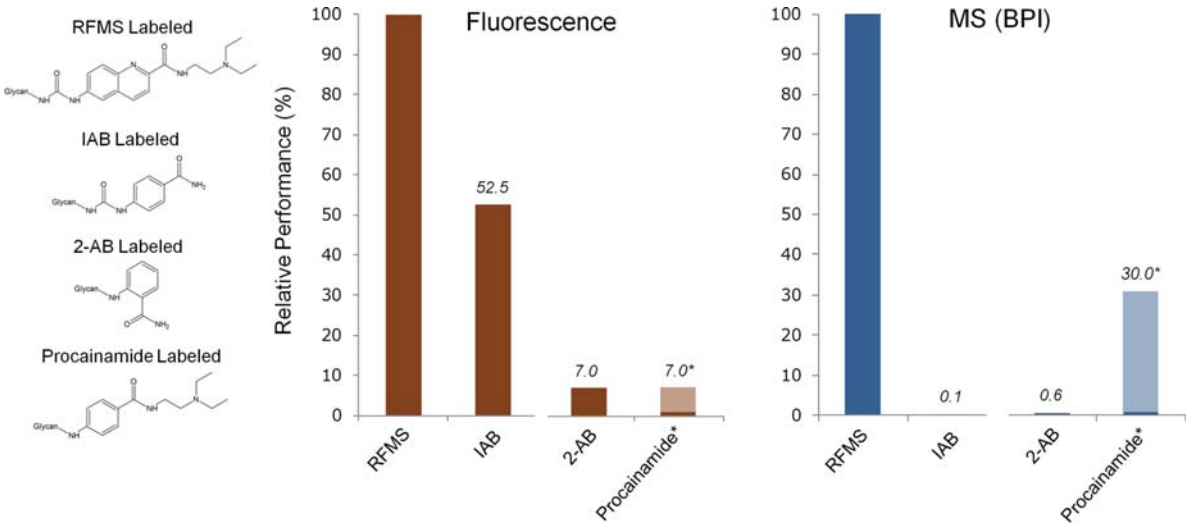


Figure 3. Relative (%) performance of glycan labels. Response factors shown as percentages versus the fluorescence and MS response factors of RFMS labeled *N*-glycans. (*) Comparative result extrapolated from a published comparison of *N*-glycans, wherein it was found that procainamide provided comparable fluorescence and up to 50-fold greater ESI-MS sensitivity when compared to 2-AB.[17]

capabilities, the new labeling reagent also supports high sensitivity fluorescence and MS detection. A highly efficient quinoline fluorophore[33,34] serves as the central functionality of the new reagent, though the quinoline group of RFMS has been derivatized to bear a tertiary amine side chain as a means to improve MS signal upon positive ion mode electrospray ionization (ESI+). RFMS has, in brief, been purposefully designed with three important chemical attributes, a rapid tagging reactive group, an efficient fluorophore, and a basic MS charge tag.

High Sensitivity Fluorescence and MS Detection. The sensitivity that RFMS labeling provides to *N*-glycan analyses

DOI: 10.1021/acs.analchem.5b00758
Anal. Chem. 2015, 87, 5401−5409

has been evaluated. Specifically, the response factors of RFMS labeled glycans have been benchmarked against response factors for glycans labeled with alternative reagents. The most closely related, commercially available alternative to RFMS is an NHS carbamate analog of aminobenzamide that will be referred to as IAB.[28] Figures 2A and B present HILIC fluorescence and base peak intensity (BPI) MS chromatograms for equivalent quantities of *N*-glycans released from a murine IgG1 monoclonal antibody and labeled with RFMS and IAB, respectively. On the basis of the observed chromatographic peak areas, response factors for fluorescence and MS detection were determined for the most abundant glycan in the IgG profile, the fucosylated, biantennary FA2 glycan (*Oxford* notation[35,36]) (Figure 2C). Our results for the FA2 glycan indicate that RFMS labeled glycans produce 2 times higher fluorescence signal and, more astoundingly, nearly 800 times greater MS signal than *N*-glycans labeled with IAB. In a similar fashion, RFMS labeling has also been compared to conventional 2-AB labeling. To draw such a comparison, *N*-glycans prepared from pooled human IgG with either RFMS or 2-AB were analyzed by HILIC-FLR-MS at equivalent mass loads (Supporting Information Figures S3A and S3B, respectively). Given that rapid tagging and reductive amination are performed by significantly different procedures, external calibrations were established using quantitative standards to determine the amounts of FA2 glycan loaded and eluted from the HILIC column (Supporting Information Figure S4 and S5). Response factors calculated using these calibrated amounts of FA2 glycan are provided in Supporting Information Figure S3C. It was determined that RFMS labeled glycans were detected with superior sensitivity, specifically with 14 times higher fluorescence and 160 times greater MS signal versus 2-AB labeled glycans.

To summarize the above observations, we have plotted the response factors of IAB and 2-AB as percentages against the response factors of RFMS (Figure 3). The gains in fluorescence and MS sensitivity are apparent in this plot, since it portrays response factors for IAB and 2-AB normalized to those for RFMS. In Figure 3, the relative performance of reductive amination with another alternative labeling reagent, procainamide, is also provided. Procainamide is a chemical analog to aminobenzamide that has recently been shown to enhance the ionization of reductively aminated glycans when they are analyzed by HILIC-ESI(+)-MS. Previous studies have shown that procainamide labeled glycans yield comparable fluorescence signal and 10 to 50 times greater MS signal when compared to 2-AB labeled glycans,[26] an observation corroborated by our own analyses of 2-AB and procainamide labeled *N*-glycans (Supporting Information Figure S6). Compared to procainamide, RFMS is thus predicted to provide, at a minimum, a 3-fold gain in MS sensitivity. Since both these labels contain a tertiary amine moiety, it is reasonable to suggest that the superior ionization of RFMS labeled glycans originates from the RFMS label being more hydrophobic than the procainamide label (Supporting Information Figure S7). Previous studies have shown that the addition of hydrophobic surface area to a glycan label leads to increased electrospray ionization.[37,38] The fact that permethylation enhances the MS detectability of glycans corroborates this observation.[12,14−16] It is therefore noteworthy that the RFMS label has a basic side chain in addition to a relatively hydrophobic core structure. These characteristics of the label give some explanation as to why RFMS labeled glycans exhibit high ionization efficiencies, though it should also be noted that the observed gains in MS sensitivity are likely to vary according to instrumentation. While reviewing previous studies, we have noticed that the MS gains reported for tertiary amine containing glycan labels have been highly variable. When using procainamide in place of 2-AB, Klapoetke et al. observed 10- to 50-fold increases in MS signals. When making the same comparison, Pabst and co-workers, in contrast, observed increases of less than 2-fold.[39] These observations suggest that gains in MS sensitivity are likely to vary as a function of the settings and designs of the employed mass spectrometers.

Rapid Deglycosylation. Since *N*-glycan sample preparation is equally dependent on deglycosylation as it is on a labeling step (Supporting Information Figure S8), we have also looked to optimize deglycosylation reactions. Although any mammalian *N*-glycan is a potential substrate for PNGase F, optimal enzymatic activity requires unrestricted access to the asparagine linkage site. Some protein *N*-glycan sites are sterically protected by protein structure, while others are generally accessible.[40] Also, because different *N*-glycan structures have varying rotational freedom, different glycan species can reshape local protein structure into distinct conformations.[41,42] Consequently, every *N*-glycan moiety-linkage site combination has a unique susceptibility or resistance to deglycosylation. Unless deglycosylation is complete, a biased *N*-glycan profile will be obtained. An optimized glycan sample preparation workflow benefits from a short PNGase F reaction that is both complete and compatible with fluorescent labeling reactions. Numerous approaches have been explored to reduce deglycosylation times.[29,43−47] In this work, we have investigated the combined use of 50 °C incubation temperatures and a high concentration of enzyme-friendly surfactant[46] in order to achieve complete deglycosylation in an approximately 10 min procedure.

To assay the completeness of glycan release, we have resolved deglycosylation products using intact protein HILIC separations and a prototype widepore, amide-bonded organosilica stationary phase. Figure 4 presents HILIC-FLR chromatograms resulting from such an assay for a murine IgG1 mAb. Figure 4A specifically shows a chromatogram obtained for the mAb before it had been subjected to rapid deglycosylation (a negative control). Figure 4B meanwhile presents the chromatogram observed for this mAb after it had been subjected to 1% (w/v) RG surfactant at 95 °C for 2 min followed by incubation with PNGase F in the presence of the surfactant at 50 °C for 5 min. As can be seen, HILIC retention of these samples was found to be dramatically different. Online mass spectrometric detection has confirmed that the peaks in these profiles correspond to the mAb with different states of glycan occupancy. The control sample is represented by masses corresponding to the doubly glycosylated, native forms of the mAb. In contrast, the sample subjected to rapid deglycosylation is represented by a single mass that is in agreement with the molecular weight predicted for the mAb after complete loss of its *N*-glycans (145.3 kDa).

In addition to releasing *N*-glycans from IgG Fc domains, it has been confirmed that the proposed rapid deglycosylation approach produces complete release of Fab domain *N*-glycans, as is evidenced by a comparison of a HILIC profile for cetuximab subunits versus an *N*-glycan HILIC profile generated for cetuximab using rapid deglycosylation and RFMS labeling (Supporting Information Figure S9). Furthermore, using a gel shift assay,[48,49] we have confirmed that this approach produces

DOI: 10.1021/acs.analchem.5b00758
Anal. Chem. 2015, 87, 5401−5409

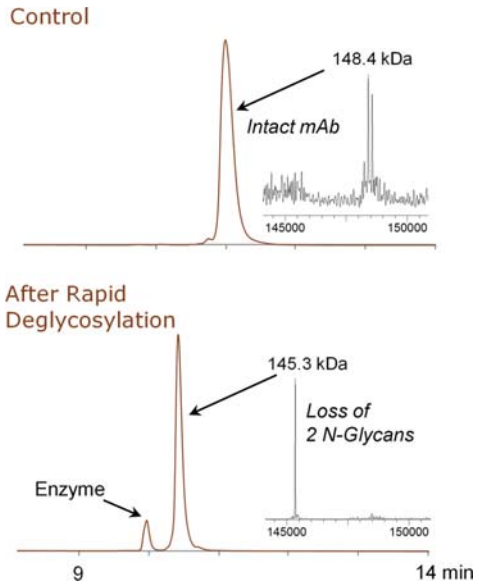


Figure 4. Assaying deglycosylation by intact protein HILIC-FLR-MS. HILIC fluorescence profiles obtained for two different conditions are shown: anticitrinin murine IgG1 mAb (A) before being subjected to rapid deglycosylation (a negative control) and (B) after being subjected to 1% (w/v) RG surfactant at 95 °C for 2 min followed by incubation with PNGase F and the surfactant at 50 °C for 5 min (a 2 step approach). Samples of the mAb (1.5 μg) were separated using two coupled 2.1 × 150 mm columns packed with prototype sub-2 μm (300 Å average pore diameter) amide bonded organosilica stationary phase.

deglycosylation of a diverse set of glycoproteins (Supporting Information Figure S10). As has been hypothesized before, it is believed that some surfactants, such as RG, enhance deglycosylation by relaxing protein structure and ensuring that *N*-glycans are accessible for efficient deglycosylation.[46] Here, use of such a surfactant is combined with elevated temperature incubations in a 2-step sample preparation that can be performed in approximately 10 min. And as described in the Experimental Section, this rapid deglycosylation procedure can be directly integrated with RFMS glycan labeling.

Quantitative HILIC SPE and Sample Preparation Recovery. The final step in an *N*-glycan sample preparation aims to extract the labeled glycans in preparation for their analysis. An effective approach for extraction of labeled glycans from reaction byproducts has been achieved using SPE designed to selectively extract RFMS labeled *N*-glycans from labeling reaction byproducts, which can otherwise interfere with analysis of the labeled glycans by HILIC column chromatography (Figure 5A). A highly polar, aminopropyl silica-based sorbent was selected for this application since it possesses useful hydrophilic interaction retentivity for glycans as well as a weakly basic surface that provides further selectivity advantages based on ion exchange and electrostatic repulsion. In the employed SPE process, glycans were adsorbed to the sorbent via a HILIC mechanism, then the sample was washed to remove sample matrix. An acidic wash solvent comprised of 1% formic acid in 90% acetonitrile was employed in this step to introduce electrostatic repulsion between the aminopropyl HILIC sorbent and labeling reaction byproducts and to enhance the solubility of the matrix components. After washing, the RFMS labeled *N*-glycans were eluted from the aminopropyl sorbent using an eluent comprised of a pH 7 solution of 200 mM ammonium acetate in 5% acetonitrile. Upon their elution, the RFMS labeled glycans were diluted with a mixture of organic solvents (ACN and DMF) and directly analyzed by HILIC column chromatography, as shown in Figure 5B.

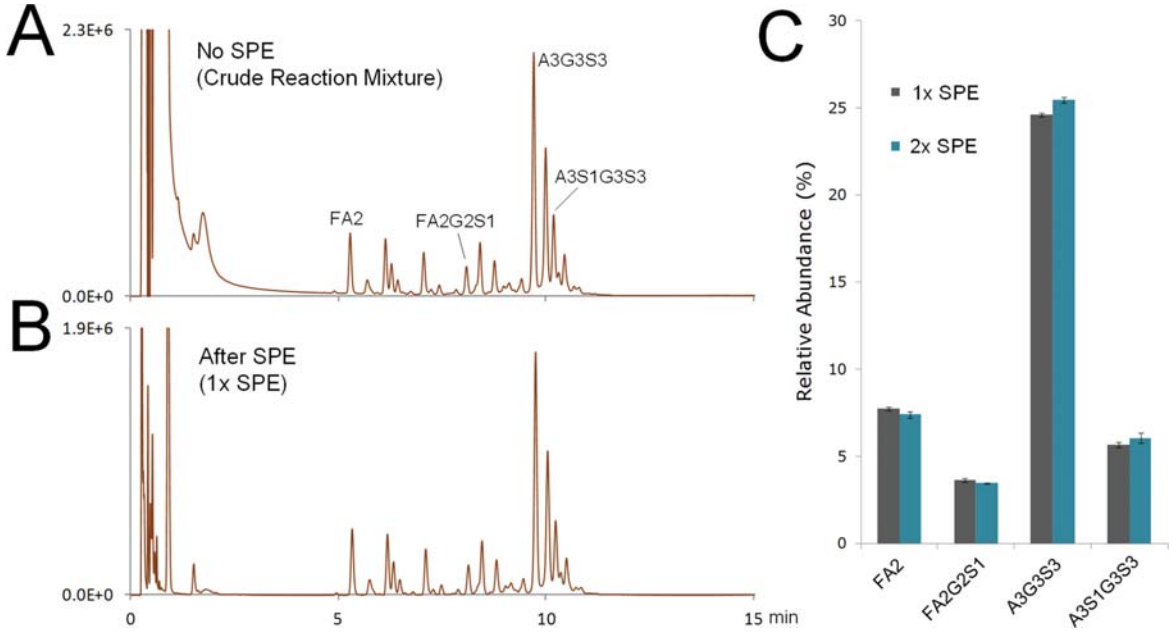


Figure 5. Extraction of RFMS labeled *N*-glycans by HILIC SPE. (A) A test mixture comprised of RFMS labeled glycans from pooled human IgG and bovine fetuin separated on a 2.1 × 50 mm column packed with 1.7 μm amide bonded organosilica (130 Å) stationary phase and detected via fluorescence (labeled *N*-glycans from 0.4 μg glycoprotein, 10 μL injection of ACN/DMF diluted sample). (B) The test mixture after extraction by HILIC SPE (one pass/1× SPE). (C) Relative abundances of four *N*-glycans from the test mixture as measured after 1 and 2 extractions by HILIC SPE (1× SPE versus 2× SPE). Samples were analyzed in triplicate.

DOI: 10.1021/acs.analchem.5b00758
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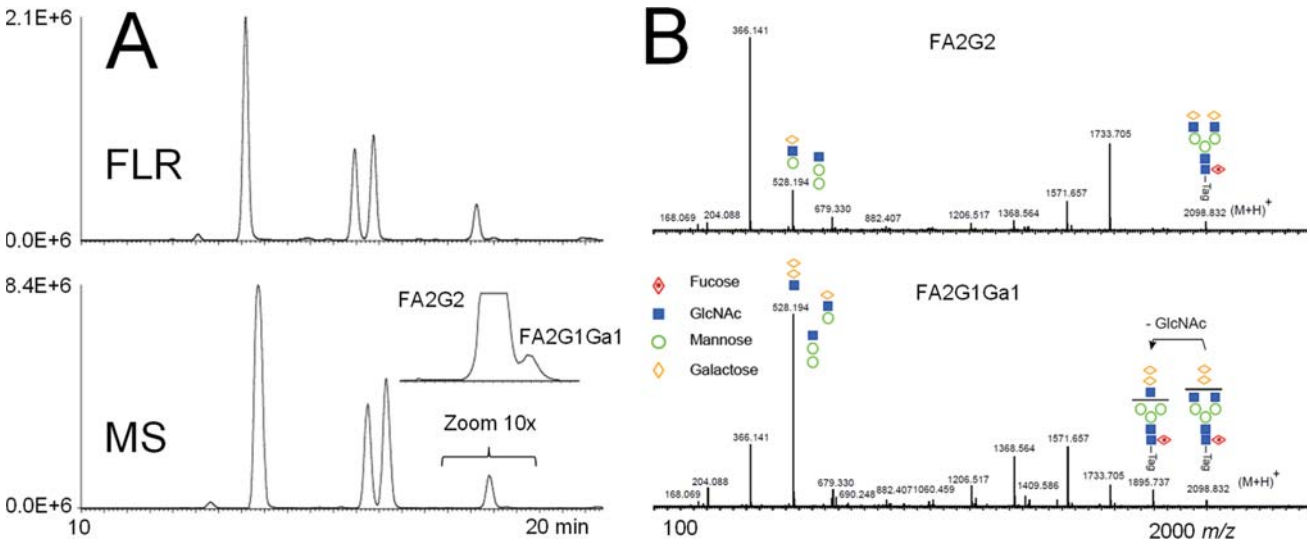

Figure 6. RFMS labeled *N*-glycans from 0.9 μg of anticitrinin murine IgG1 mAb. (A) Fluorescence (FLR) chromatogram and based peak intensity (BPI) MS chromatograms obtained using a 2.1 × 150 mm column packed with 1.7 μm amide bonded organosilica (130 Å) particles. The magnified peaks show the separation of two isobaric glycans, FA2G2 and FA2G1Ga1, that are present in the sample at significantly different abundances. (B) MS/MS spectra of FA2G2 and FA2G1Ga1: Structurally diagnostic ions for FA2G1Ga1 are displayed in the bottom spectrum. MS/MS spectra were charge deconvoluted and deisotoped using MaxEnt 3.

Absolute and relative recoveries for this novel HILIC SPE process have been studied. RFMS labeled *N*-glycans prepared from a mixture of pooled human IgG and bovine fetuin were processed by multiple passes through the HILIC SPE steps and recoveries across the serial processing were measured. Specifically, recoveries were measured for species representing extremes in glycan properties, including an asialo FA2 glycan and a glycan with a tetrasialylated, triantennary structure (A3S1G3S3). Absolute recovery through the SPE process was found to be approximately 74% (Supporting Information Table S5), which is a relatively high recovery for an oligosaccharide enrichment strategy. Zhang and co-workers recently studied oligosaccharide SPE and reported that recoveries between 60% and 80% are obtained with optimized protocols and the best performing sorbents.[50] With the SPE strategy we have proposed, the majority of the observed sample losses appear to be a consequence of elution volume. Increasingly higher SPE recoveries were achieved when increasingly larger elution volumes were employed (Supporting Information Figure S11). To facilitate direct analyses, however, a compromise was made with the technique such that a 90 μL elution volume was used to obtain a relatively concentrated glycan eluate.

Of greater significance is the fact that the observed sample losses appear to be nonspecific in nature, since this SPE has been found to exhibit highly accurate relative yields. Figure 5C shows the relative abundances for four glycans (FA2, FA2G2S1, A3G3S3, and A3S1G3S3) as determined after one pass or two passes of the SPE process, respectively. The largest deviation in relative abundance was observed for the tetrasialylated A3S1G3S3 glycan, in which case a relative abundance of 5.7% was determined after a single pass of SPE versus 6.1% after two passes of SPE. With these results, it is demonstrated that this SPE technique, combining HILIC and electrostatic repulsion, provides a mechanism to immediately analyze a sample of extracted RFMS labeled glycans and does so without significant compromise to the accuracy of the relative abundances determined for a wide range of *N*-glycans.

The percent yield of the entire *N*-glycan sample preparation workflow also was evaluated to measure the collective efficiency of combining fast deglycosylation, rapid labeling, and HILIC SPE extraction of RFMS labeled glycans. RFMS labeled *N*-glycans from a murine IgG1 were prepared, analyzed by HILIC-FLR-MS, and quantified by means of an external calibration (Supporting Information Figures S12A and S12B). Using a theoretical yield (Supporting Information Figure S12C) and duplicate analyses, it was determined that the percent yield of the entire *N*-glycan sample preparation was approximately 73%, which indicates that the described approach is essentially lossless save for the SPE processing.

Detailing the *N*-Glycan Profile of a mAb. Because of the poor ionization efficiency of glycans labeled with conventional fluorescent labels, it is often difficult to detect, let alone characterize, low abundance glycans species during HILIC-ESI-MS analysis. To demonstrate the capability of RFMS glycan labeling to improve the quality of MS data, the *N*-glycan profile of the anticitrinin murine IgG1 mAb previously shown in Figure 2 was reanalyzed using a higher resolution HILIC separation and MS/MS analysis with a QTof mass spectrometer outfitted with a high transmission efficiency collision cell.

Figure 6A shows fluorescence and base peak intensity (BPI) MS chromatograms obtained in this study for RFMS labeled *N*-linked glycans resulting from only 0.9 μg of the mAb. Notably, the BPI MS chromatogram in this analysis presented signal-to-noise comparable to that of the fluorescence chromatogram, making assignment of glycan species via accurate mass measurement straightforward (Supporting Information Table S6). The magnified peaks in Figure 6A additionally show two isobaric glycans that are partially resolved by the HILIC separation. From historical data and abundances of glycans in typical mAb samples,[30,51] the first peak was assigned to a common FA2G2 glycan. A later yet closely eluting species was also detected with the same m/z value as the FA2G2 peak, albeit at a relative abundance of only 0.7%. MS/MS spectra of

DOI: 10.1021/acs.analchem.5b00758
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these RFMS labeled, isobaric glycans were thus contrasted to elucidate their structural differences.

With the enhanced MS signal afforded by the RFMS label, structurally diagnostic fragmentation data were obtained for the FA2G2 glycan and it isobaric analog. Figure 6B shows a side-by-side comparison of the fragment ions generated from the high abundance FA2G2 glycan versus its isobaric, low abundance counterpart that eluted in the shoulder peak. A 528 m/z ion was found to be prominent in the spectrum for the low abundance, isobaric species. High yields of such an ion have previously been reported as being diagnostic of an α-Gal configuration in glycans,[52] wherein two galactose residues are linked through an α-1,3 bond. Also observed in the spectrum for the low abundance, isobaric species was a very prominent loss of GlcNAc, which is observed when an *N*-glycan branch terminates with a GlcNAc residue. Collectively, these high signal-to-noise fragmentation data support the identification of the isobaric, low abundance species as an α-Gal containing FA2G1Gal glycan. By showing that low abundance glycans from a pool of released *N*-glycans can be readily detected and characterized with minimal ambiguity, this case study underscores the significant promise that RFMS labeling holds for *N*-glycan profiling and structure elucidation.

CONCLUSIONS

Hydrophilic interaction chromatography has matured over the last two decades into a powerful technology for *N*-glycan profiling. The methodology described in this work strengthens this approach to glycan characterization by addressing several shortcomings corresponding to sample preparation techniques. With the described developments, we have enhanced the sensitivity of glycan detection and have also decreased the time required to prepare *N*-glycans for analysis. In this approach, glycoproteins are deglycosylated in approximately 10 min to produce *N*-glycosylamines. These glycans are then rapidly reacted with the RFMS reagent and are thereby labeled with a tag comprised of an efficient fluorophore and a basic tertiary amine. In a final step, RFMS-labeled glycans are extracted from reaction byproducts by means of a robust HILIC SPE method that facilitates immediate analysis of samples. These techniques make it possible to quickly prepare *N*-glycan samples and to incorporate the use of a fluorescent labeling reagent that enhances the sensitivity of ESI-MS detection. To demonstrate these properties, the RFMS-labeled *N*-glycan profile of a monoclonal antibody was elucidated through the combined use of HILIC, accurate mass measurements and MS/MS fragmentation. As has been indicated with this work, it is believed that the described approach will facilitate future studies of *N*-glycosylation by allowing samples to be more quickly analyzed and to be more easily characterized to greater levels of detail. This should, at a minimum, help accelerate process development of biopharmaceuticals. With this work, we have clearly demonstrated the utility of the methodology for profiling the *N*-glycans of monoclonal antibodies, the most frequently exploited modality for therapeutic applications. Nevertheless, it is interesting to consider the use of these sample preparation techniques for the analysis of *N*-glycans from complex matrices, such as human blood serum,[53] though additional work will be required to investigate their applicability to such studies.

ASSOCIATED CONTENT

Supporting Information

Supplemental experimental information, supplemental results and discussion on the stability of RFMS glycan derivatives, the stability of *N*-glycosylamines, evaluation of response factors, deglycosylation, SPE recovery, SPE recovery as a function of elution volume, sample preparation yield, and IdeS-digested cetuximab, tables provides LC chromatographic conditions, a figure showing a comparison of HILIC-FLR-MS with RFMS labeled versus 2-AB labeled *N*-glycans, figures presenting the quantitative analysis of RFMS and 2-AB labeled *N*-glycans, a figure showing a comparison of HILIC-FLR-MS signal from procainamide versus 2-AB labeled glycans, a figure depicting the described *N*-glycan sample preparation workflow, a figure presenting the comparison of HILIC profiling of cetuximab subunits versus HILIC profiling of RFMS labeled *N*-glycans prepared from cetuximab using rapid deglycosylation, a figure showing a gel shift assay of a diverse set of glycoproteins deglycosylated using rapid deglycosylation, a figure outlining the yield calculation for the entire sample preparation technique, a table listing the fluorescence peak areas and relative abundances of RFMS labeled glycans as measured after one and two passes through HILIC SPE, a figure demonstrating the charge states of RFMS labeled glycans, a figure displaying an MS/MS spectrum for RFMS labeled FA2, a figure showing NMR spectra obtained for RFMS derivatized propylamine, and a figure presenting HPLC impurity assays obtained for RFMS derivatized propylamine. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b00758.

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Notes

The authors declare the following competing financial interest(s):The authors of this manuscript are employed by Waters Corporation and New England Biolabs. Equipment and products from Waters and New England Biolabs were used for this research.

ACKNOWLEDGMENTS

We would like to thank Pamela Iraneta, Scott McCall, Bonnie Alden, Weiqiang Gu, Daniel Walsh, and Kevin Wyndham for their research on wide pore amide stationary phases. We would also like to thank Michael Morris and Xiang Jin Song for their help with the synthesis and characterization of RFMS derivatized propylamine. Finally, we would like to acknowledge Vera Ivleva for providing comparative data on procainamide versus 2-AB labeled glycans and Weibin Chen for providing a critical review of our manuscript.

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DOI: 10.1021/acs.analchem.5b00758
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