

# Development of a PNGase Rc Column for Online Deglycosylation of Complex Glycoproteins during HDX-MS

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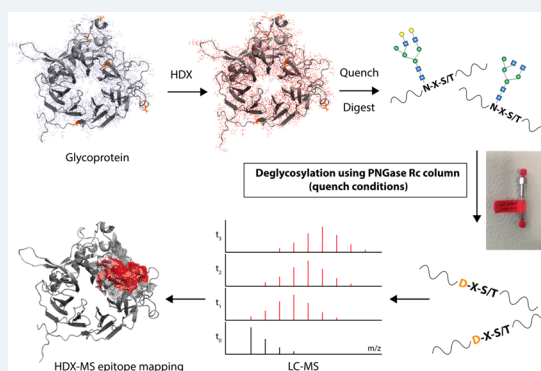
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**ABSTRACT:** Protein glycosylation is one of the most common PTMs and many cell surface receptors, extracellular proteins, and biopharmaceuticals are glycosylated. However, HDX-MS analysis of such important glycoproteins has so far been limited by difficulties in determining the HDX of the protein segments that contain glycans. We have developed a column containing immobilized PNGase Rc (from *Rudaea cellulosilytica*) that can readily be implemented into a conventional HDX-MS setup to allow improved analysis of glycoproteins. We show that HDX-MS with the PNGase Rc column enables efficient online removal of N-linked glycans and the determination of the HDX of glycosylated regions in several complex glycoproteins. Additionally, we use the PNGase Rc column to perform a comprehensive HDX-MS mapping of the binding epitope of a mAb to c-Met, a complex glycoprotein drug target. Importantly, the column retains high activity in the presence of common quench-buffer additives like TCEP and urea and performed consistent across 114 days of extensive use. Overall, our work shows that HDX-MS with the integrated PNGase Rc column can enable fast and efficient online deglycosylation at harsh quench conditions to provide comprehensive analysis of complex glycoproteins.



## INTRODUCTION

Glycosylation of Asn residues (N-glycosylation) is a predominant post-translation modification of proteins exposed to the extracellular environment.<sup>1,2</sup> N-glycosylation offers structural stability, improved solubility, and binding partner recognition mechanisms that allow proteins to carry out their function outside of cells.<sup>3</sup> Also, many extracellular proteins of interest as targets for drugs or as drug modalities (biopharmaceuticals) are N-glycosylated.<sup>1,2</sup> For these reasons, there is a growing need to be able to study the structure and interactions of proteins in their natively glycosylated state.<sup>4</sup> However, traditional methods for structural analysis of proteins like X-ray crystallography and NMR are often not readily compatible with natively glycosylated proteins due to the increased dynamics and structural heterogeneity afforded by the presence of N-glycosylations.

Hydrogen–deuterium exchange mass spectrometry (HDX-MS) can provide a powerful alternative method to study the conformation and interactions of dynamic or conformationally heterogeneous protein systems in solution.<sup>5</sup> In HDX-MS, a protein is subjected to deuterated solvent and the exchange of the backbone amide hydrogens for heavier deuterium atoms of the solvent can be measured by MS. Involvement in hydrogen-bond networks, which define protein higher-order structure,

heavily influences the exchange rate of backbone amide hydrogens, making HDX-MS a sensitive probe of protein conformation and dynamics. The HDX of proteins can be resolved to the peptide-level using online proteolysis. The sensitivity of HDX-MS and its intrinsic ability to tolerate sample complexity makes the method helpful for studying a variety of protein states, like glycoproteins, that can be incompatible to conventional methods.<sup>5</sup> Common applications of HDX-MS include analysis of conformational changes in proteins during function and regulation, including those of membrane proteins, protein–ligand/drug interactions, mode of action of biopharmaceuticals, and epitope mapping of therapeutic monoclonal antibodies.<sup>5–8</sup>

Glycosylation can however present particular difficulties for HDX-MS. Different glycoforms of a protein can coexist when various heterogeneous glycan structures occupy a single site<sup>9,10</sup> and as a result cause glycan-induced signal spread, which

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results in a weaker signal during MS analysis and thus gaps in the sequence coverage in regions containing N-linked glycosites. Additionally, glycan HDX interpretation may be impacted by the retention of deuterium by acetamido groups within glycan structures which introduces error when quantifying deuterium retention.<sup>9,11</sup>

One solution is to use fragmentation in MS/MS analysis as a means of overcoming these difficulties.<sup>12,13</sup> However, collision-induced dissociation (CID) MS/MS is especially difficult to use for this purpose. This is due to the preferential fragmentation of glycosidic links in glycopeptides<sup>12,13</sup> and the occurrence of H/D scrambling during CID.<sup>14</sup> Other fragmentation methods that occur without scrambling, such as electron transfer dissociation (ETD),<sup>15</sup> have demonstrated selective cleavage of the peptide backbone—while keeping the glycosidic bond intact. However, these options require MS systems having this capability and ETD suffers from markedly lower fragmentation efficiency than CID.<sup>12,13</sup>

N-Glycan removal during HDX-MS has also been explored using acid-stable endoglycosidases. The first enzyme to be used for this purpose was PNGase A, which was used in an off-line manner to facilitate deglycosylation at quench conditions during HDX-MS of glycoproteins.<sup>9</sup> However, the relatively high pH optimum of 5 of PNGase A and its low tolerance to common quench buffer additives like TCEP and urea, prompted the exploration of other acidic PNGases, like PNGase H+ (from *Terriglobus roseus*).<sup>10</sup> Similar to PNGase A, PNGase H+ can release a broad variety of N-glycans and has the advantage over PNGase A that it can also deglycosylate glycoproteins and not just glycopeptides.<sup>9</sup> Another advantage over PNGase A is its pH optimum at 2.6 and high tolerance to reducing agents like TCEP. However, PNGase H+ showed low expression yields and purity. Later on, a few PNGase H+ variants were found and some of them showed high deglycosylation activities. One of the promising PNGase H+ variants was PNGase Dj originating from *Dyella japonica*. To facilitate the use of such rare enzymes we immobilized PNGase Dj to a microchip and showed its use in HDX-MS during analysis of complex glycoproteins including epitope mapping applications.<sup>16</sup> Building on this work, we have recently shown, in collaboration with the research group of Josef Voglmeir (Nanjing Agriculture University), that another PNGase H+ variant, PNGase Rc originating from *Rudaea cellulositytica*, has higher intrinsic activity and higher expression yields than PNGase Dj, the latter likely due to the presence of only two disulfide bonds in PNGase Rc vs three in PNGase Dj.<sup>17</sup> Furthermore, PNGase Rc can cleave a broad variety of N-glycans (similar to PNGase H+), including core-fucosylated glycan structures (not cleaved by PNGase F) and glycans from plants and invertebrates.<sup>17,18</sup> For these reasons, PNGase Rc represents an attractive enzyme for use in HDX-MS applications of glycoproteins.<sup>17,19</sup>

Here we have developed a column containing immobilized PNGase Rc that can be used in an online HDX-MS workflow to greatly improve analysis of glycoproteins. The immobilization of PNGase Rc offers considerable advantage compared to the usage of PNGase enzymes in solution, including reusage and a higher enzyme to substrate ratio. Additionally, of key advantage to HDX-MS, no preincubation time is needed, resulting in a fast online workflow that increases throughput and limits back-exchange.<sup>16</sup> We show that the PNGase Rc column enables efficient online removal of N-linked glycans and the determination of the HDX of glycosylated regions in

several highly complex/challenging glycoproteins, including in epitope mapping applications. The column retains activity in the presence of common HDX-MS quench-buffer additives like TCEP and urea and maintains high deglycosylation activity after repeated use across >100 days.

## EXPERIMENTAL SECTION

**PNGase Rc Expression and Purification.** See [Supporting Information \(SI\)](#) and the information described previously.<sup>17,19</sup>

**Immobilization of PNGase Rc and Manufacture of the PNGase Rc Column.** See [SI](#).

**HDX-MS of Glycoproteins Using the PNGase Rc Column.** Haptoglobin and transferrin experiments were performed using a Synapt G2Si HDMS coupled to an HDX-MS UPLC system (Waters Corporation, U.K.).

HDX of Haptoglobin (Hp 1–1, Sigma-Aldrich) was initiated by diluting the protein into labeling buffer (20 mM Tris pH(read) 7.0, 90% D<sub>2</sub>O) to a concentration of 1 μM. The HDX reaction was quenched at various time points by transferring reaction aliquots (50 μL) into a 1:1 (v/v) volume of quench buffer (250 mM TCEP and 4 M urea in 0.5 M glycine at pH 2.3). Quenched samples were incubated on ice for 3 min to allow for protein unfolding and reduction and frozen on dry ice. During analysis, HDX samples were rapidly thawed and injected into the cooled (0 °C) HDX-MS UPLC system and subjected to online proteolysis (20 °C) in an in-house built column packed with agarose-immobilized pepsin, followed by online deglycosylation (20 °C) using a column placed immediately downstream packed with immobilized PNGase Rc (2.1 × 20 mm<sup>2</sup>, Wuxi GALAK Chromatography Technology Co, China), and finally desalting on a trap column (Waters Acquity BEH C18, 1.7 μm, 2.1 × 5 mm<sup>2</sup> length) for 3 min with aqueous phase (0.23% FA) at 200 μL/min. Separation was performed on an analytical column (Waters Acquity BEH C18, 1.7 μm, 1 × 100 mm<sup>2</sup>) using a 9 min gradient from 2 to 40% organic phase (0.23% FA in acetonitrile) at 0 °C. Peptides were identified by both data-dependent acquisition (DDA) and data-independent acquisition (DIA) mode (i.e., MSE) MS/MS and were filtered using Protein Global Server 3.0 (Waters) and DynamX v3.0 (Waters). The deuterium uptake of respective peptides was determined using DynamX. The HDX experiment was performed in independent triplicates.

Maximum-labeled control samples of Hp 1–1 were generated by online digestion of Hp 1–1 as described above and manual collection of samples after trapping. Peptic peptides of Hp 1–1 were exposed to labeling buffer as above for 5 h at to achieve maximal labeling and applied to the HDX-UPLC system configured and operated exactly like those described above, but without the pepsin column and in both the absence and presence of the PNGase Rc column.

Back-exchange (BE) values were calculated by comparing the data from the maximal deuterated control sample against an undeuterated control.

HDX of transferrin was performed by diluting solid apohuman transferrin (Sigma-Aldrich) to a concentration of 10 μM in equilibration buffer (PBS, pH 7.4). Labeling was initiated by diluting 5 μL of each protein aliquot to 50 μL total volume by addition of labeling buffer (PBS prepared in D<sub>2</sub>O, pH (read) 7.0) giving a 90% D<sub>2</sub>O environment. Labeling took place for various time points. The labeling reaction was quenched by a 1:1 addition of ice-cold quench buffer (50 mM KH<sub>2</sub>PO<sub>4</sub>, 4 M

urea, 200 mM TCEP) to a final pH of 2.5. Samples were digested online (20 °C) with a Waters Enzymate BEH pepsin column, followed by online deglycosylation (20 °C) using a column placed immediately downstream packed with immobilized PNGase Rc (2.1 × 20 mm<sup>2</sup>, Wuxi GALAK Chromatography Technology Co, China). Peptides were trapped on a Waters BEH C18 VanGuard precolumn for 3 min at a flow rate of 100 μL/min in buffer A (0.1% formic acid ~ pH 2.5).

Peptides were separated on a Waters BEH C-18 analytical column with a linear gradient of buffer B (0.1% formic acid in acetonitrile ~ pH 2.5) at a flow rate of 40 μL/min. All trapping and separation were performed at ~0 °C to reduce back exchange. All data were collected using the same flow rates for states with and without the PNGase column.

MS data were acquired using MSE workflow. All time points across this experiment were obtained in triplicate. MS was calibrated separately using NaI and the MS data were obtained with lock mass correction using LeuEnk. Peptides were assigned with the ProteinLynx Global Server (PLGS, Waters Corporation, U.K.) software and the isotope uptake of respective peptides was determined using DynamX v3.0.

HDX of VISTA was performed by reconstituting the protein (Human B7–H5, Acro BioSystems) 5× concentrated compared to manufacturer's instructions. The reconstituted solution contained 50% trehalose which inhibited in pre-experiments deglycosylation by PNGase Rc. Therefore, VISTA was buffer-exchanged using Zeba Spin Desalting Columns (7K MWCO Fisher Scientific, U.S.A.) to PBS buffer. The final concentration determined via NanoDrop 2000 spectra photometer (Fisher Scientific, U.S.A.) was 1.65 mg/mL.

Six μL VISTA (90 μM in PBS) were deuterated by incubation with 54 μL PBS HDX buffer (1×, D<sub>2</sub>O pH 7.4) for 0.25, 1, 10, 60, and 600 min at 25 °C. Aliquots of 10 μL were quenched and reduced by adding 1:1 (v/v) ice-cold quenching solution (0.4 M TCEP and 4 M urea in 100 mM ammonium formate, pH 2.4) resulting in a final pH of 2.6 and a final concentration of 0.2 M TCEP and 2 M urea. Samples were immediately snap-frozen in liquid nitrogen and stored until analysis at –80 °C. All experiments were performed in independent triplicates.

Full deuteration labeling was attempted by deuteration of the above-described solution for 50 h and by incubation of VISTA in the presence of 2 M urea-d<sub>4</sub> prepared in D<sub>2</sub>O. Samples were incubated at 20 °C and quenched by adding 1:1 (v/v) ice-cold quenching solution (0.4 M TCEP dissolved in 100 mM ammonium formate buffer, pH 2.6). Full deuteration was however not achieved under the tested conditions.

**HDX-MS Epitope Mapping of c-Met.** C-Met (Human, Proteros Biostructures GmbH, Germany) experiments were performed using an Orbitrap Fusion Lumos (Thermo Fisher Scientific, U.S.A.) coupled to an Acquity UPLC system with HDX (Waters Corporation, U.K.). Sample preparation was performed by the LEAP HDX autosampler (Trajan).

HDX of unbound and bound c-Met (1:2.2 ratio with mAb (Roche Diagnostics GmbH, Germany)) was performed by diluting c-Met into labeling buffer (20 mM Histidine, 140 μM NaCl, 90% D<sub>2</sub>O, pH(read) 6.0) to a concentration of 0.35 μM. The labeling reaction (60 μL) was quenched at various time-intervals by a 1:1 addition of ice-cold quench buffer (170 mM KH<sub>2</sub>PO<sub>4</sub>, 250 mM TCEP 4 M urea, pH 2.8). HDX samples were injected into the cooled (0 °C) HDX-MS UPLC system and subjected to online proteolysis (20 °C) with a

Nepenthesin-2 column (AffiPro), followed by online deglycosylation (20 °C) using a column placed immediately downstream packed with immobilized PNGase Rc and trapped and desalted on a Waters BEH C18 VanGuard precolumn for 3 min at a flow rate of 200 μL/min in buffer A (0.23% formic acid ~ pH 2.5). Peptides were separated on a Waters BEH C-18 analytical column using a linear gradient of buffer B (0.23% formic acid in acetonitrile ~ pH 2.5) at a flow rate of 40 μL/min. All trapping and separation were performed at ~0 °C to reduce back exchange. All data were collected using the same flow rates for states with and without the PNGase Rc enzymatic column.

Peptide identification was performed in three runs by DDA fragmentation using HCD, ETD and EThcD and the following criteria in the PEAKS Studio 11 (Bioinformatic Solutions, inc): Peptide tolerance: 5 ppm, MS/MS tolerance: 0.5 Da. The deuterium uptake of respective peptides was determined using HDExaminer 3.0 (Sierra Analytics).

To allow access to HDX data of this study, HDX Summary Table and HDX Data Tables (Table S1) are included in the SI according to the community-based recommendations.<sup>8</sup>

**Tryptic Digestion of Trastuzumab (TZ).** See the SI and information described previously.<sup>16</sup>

**LC-MS Assay for Monitoring the Online Deglycosylation Activity of the PNGase Rc Column.** Assessment of PNGase Rc activity was done in a similar manner as described previously using samples from a tryptic digest of Trastuzumab (TZ).<sup>16</sup> The PNGase Rc column was interfaced with a cooled HDX-UPLC system (Waters, Milford, MA). The LC-MS configuration and columns used were the same as during HDX-MS experiments of Hp 1–1. 50pmol of TZ tryptic digest was injected and run through the column at 200 μL/min of LC mobile phase (0.23% formic acid (FA), pH 2.5) before desalting and separation by reversed-phase chromatography and MS analysis. As described previously,<sup>16</sup> the online deglycosylation activity of the column was evaluated by monitoring the signal intensity of the glycosylated and deglycosylated tryptic peptide EEQYNSTYR normalized to the signal intensity of the ATII (angiotensin II) peptide internal standard. The deglycosylation efficiency was calculated according to the following equation:

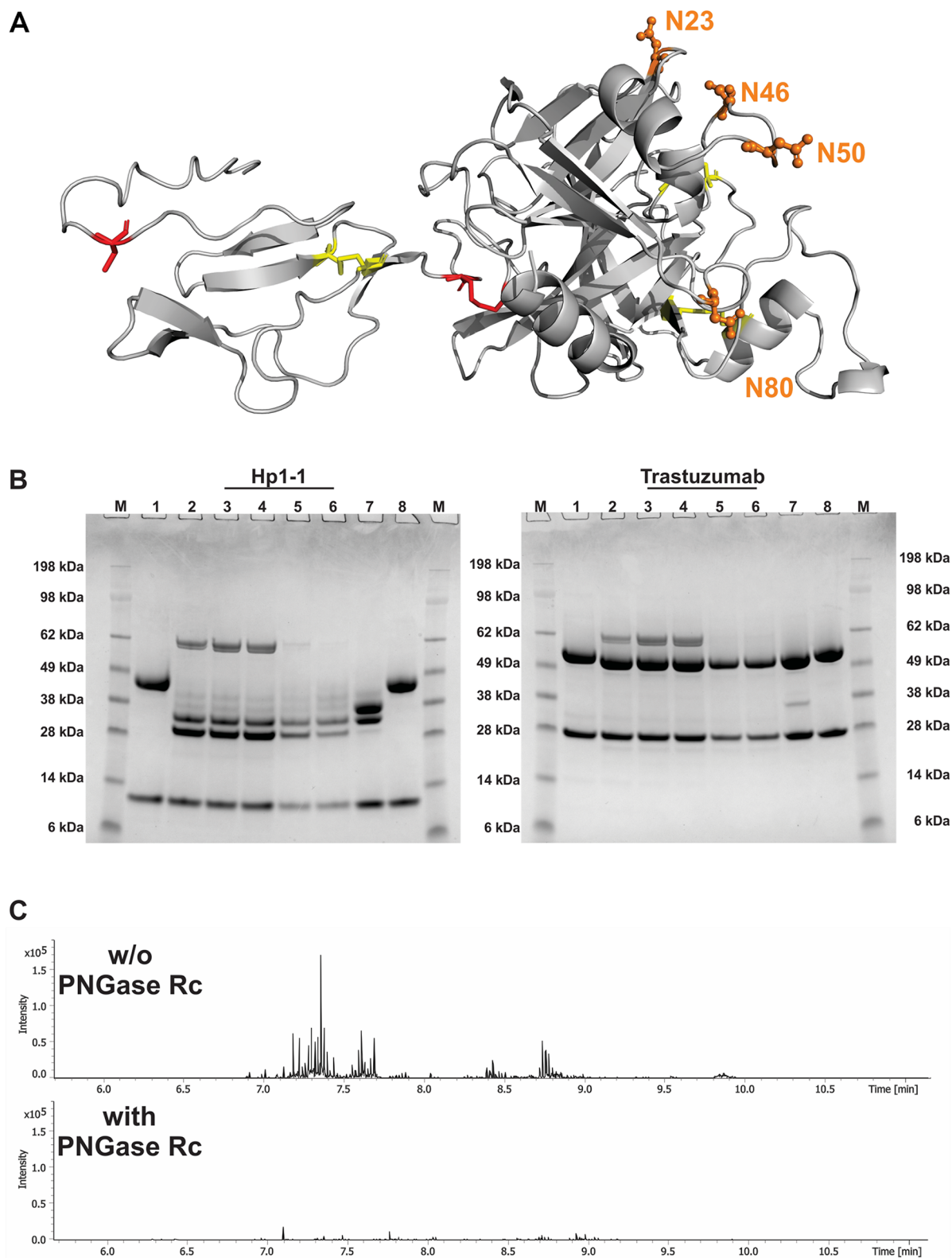
$$\text{Deglycosylation efficiency (\%)} = ((I_0 - I)/I_0) \times 100\%$$

where  $I$  and  $I_0$  is the normalized intensity of the glycopeptide obtained with or without the PNGase Rc column, respectively.

## RESULTS AND DISCUSSION

**Immobilization of PNGase Rc.** PNGase Rc was recombinantly expressed in *E. coli* as described previously at high yield and purity.<sup>17,19</sup> Immobilization was performed on POROS AL beads via Lys residues in PNGase Rc. Investigation of the AlphaFold-generated structure of PNGase Rc revealed that all Lys residues were located outside of the predicted active site (based on looking at active sites of PNGase F and A, Figure S1).<sup>17</sup> The immobilization efficiency ( $\psi$ ) was tested by assessment of PNGase Rc concentration in the supernatant after bead coupling. The concentration was determined from a calibration curve using the UV 280 nm peak area of standard dilutions of PNGase Rc analyzed by HPLC using a C4 column (Figure S2). An enzyme-to-bead (E:B) ratio of 1:10 yielded ~ 39% immobilization efficiency, whereas an E:B ratio of 1:20 yielded 95% immobilization efficiency. Therefore, the E:B ratio of 1:20 results in





**Figure 1.** (A) Structure of the Hp 1-1 heterodimer (AlphaFold) containing both the alpha and beta chain. Disulfide bonds are shown in yellow (intra) and red (inter) sticks, while glycans are shown in orange sticks and labeled. (B) Deglycosylation of Hp 1-1 (left) and Trastuzumab (right) by PNGase Rc analyzed by SDS-PAGE. Lanes 1 and 8 correspond to samples without PNGase Rc at pH 3.5 and 7.5, respectively. Lanes 2 and 7 correspond to samples following in-solution deglycosylation by free PNGase Rc and free PNGase F, respectively. Lanes 3 and 4 correspond to samples from in-solution deglycosylation using PNGase Rc coupled beads made with and without the use of sodium sulfate, respectively. Lanes 5 and 6 correspond to samples collected following online deglycosylation under HDX-MS compatible conditions (pH 2.3) using the PNGase Rc column at a flow rate of 100  $\mu$ L/min. Lane M corresponds to the LMW protein standard. (C) Extracted ion chromatograms of the oxonium ion  $m/z$  366.13 (Hex-Hex-Nac glycan fragment) from LC-MS/MS analysis of a sample of Hp 1-1 (in 0.5 M glycine, 2 M urea, 100 mM TCEP, pH 2.3) following online proteolysis with or without downstream online deglycosylation using the PNGase Rc column at HDX-MS compatible conditions.

quantitative coupling of the enzyme to the beads but most likely not the whole accessible surface is modified with enzyme. The activity of immobilized PNGase Rc was assessed as described previously using an LC-MS assay monitoring the deglycosylation of the intact IgG1 antibody Trastuzumab (TZ) that has a N297 glycosite.<sup>9,16,19</sup> In initial testing, immobilization with 1:10 E:B resulted in beads with higher deglycosylation efficiency than beads produced by immobilization with 1:20 E:B (Figure S4). However, upon further optimization (SI), an immobilization procedure was developed that required less enzyme (1:25 E:B) while producing beads with high deglycosylation efficiency (Figure 1) and the resulting PNGase Rc-functionalized beads were packed into HPLC/UPLC compatible columns (2.1 × 20 mm<sup>2</sup>).

Haptoglobin 1-1 (Hp 1-1) was chosen as another model substrate to assess the deglycosylation activity of immobilized PNGase Rc. Hp 1-1 is one of the three phenotypes (Hp 1-1, Hp 2-1, Hp 2-2) of the human glycoprotein haptoglobin. Hp 1-1 is a heterotetramer composed of two heterodimers, each with a light  $\alpha$ -chain and a heavy  $\beta$ -chain (Figure 1A). The two  $\alpha\beta$  heterodimers are held together by disulfide-bonds.<sup>20</sup> Four glycans are attached to the  $\beta$ -chain and are located at residues N23, N46, N50, and N80.

PNGase Rc immobilized on beads was capable of deglycosylation of all N-linked glycans of Hp 1-1 at pH 3.5 and 2.3 as monitored by SDS-PAGE (Figure 1B) and LC-MS/MS analysis (Figure 1C), respectively.<sup>21</sup> Upon deglycosylation, the band corresponding to glycosylated Hp 1-1 beta chain (~45 kDa) was converted into bands corresponding to removal of 1, 2, 3, and all 4 N-glycans (~30 kDa for fully deglycosylated form). In-solution deglycosylation of Hp 1-1 by PNGase Rc at pH 3.5 was more effective compared to PNGase F at pH 7.4 under otherwise similar conditions. Moreover, efficient deglycosylation of the heavy chain of Trastuzumab could also be achieved by immobilized PNGase Rc (Figure 1B). Furthermore, LC-MS/MS analysis of Hp 1-1 following online proteolysis and deglycosylation at HDX-MS compatible conditions using the PNGase Rc column showed the absence of glycan-specific oxonium ions indicating highly efficient deglycosylation of all Hp 1-1 glycopeptides.

**HDX-MS of Glycoproteins of Varying Complexity.** To evaluate the implementation of the PNGase Rc column into HDX-MS setups in different laboratories, PNGase Rc columns were sent to three separate research laboratories and were used to perform HDX-MS experiments on three different glycoproteins of significant complexity in terms of disulfide-bonds and N-linked glycans. Namely, Hp 1-1, V-domain Ig suppressor of T cell activation transmembrane protein (VISTA) and transferrin.

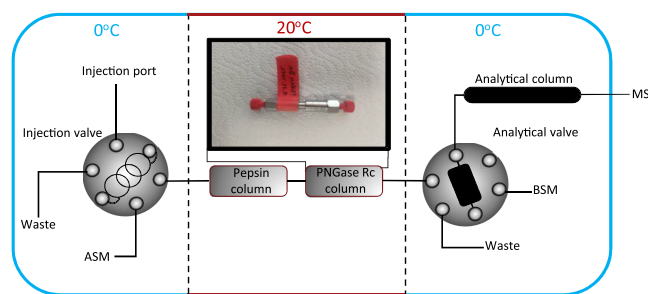
As mentioned previously, Hp 1-1 forms a disulfide-bonded heterotetramer containing two  $\alpha$ -chains and two  $\beta$ -chains with both  $\alpha$ -chains linked to each other and to one  $\beta$ -chain, through disulfide bridges located at residues  $\beta$ C105 and  $\alpha$ C72 for the  $\alpha$ - $\beta$  chain connections, and at residues  $\alpha$ C15 and  $\alpha$ C15 for the  $\alpha$ - $\alpha$  chain connection (Figure 1A). In addition to those interchain disulfide bonds, each  $\beta$ -chain contains two intrachain disulfide bonds at residues C148–C179 and C190–C219, and each  $\alpha$ -chain contains one intrachain disulfide bond at residues C34–C68. Four glycans of Hp 1-1 are attached to each  $\beta$ -chain (N23, N46, N50, and N80).

VISTA consists of 180 residues with 6 N-glycosites at residues N17, N59, N76, N96, N103, and N158. VISTA also

contains 3 intra disulfide bonds located at residues C10–C146, C22–C114, and C51–C113.

Transferrin contains 679 residues with 2 N-glycosites at residues N413 and N614 and 19 disulfide bonds located throughout its structure.

To achieve maximal sequence coverage, HDX-MS analysis of each protein was performed with different concentrations of TCEP and urea in the quench buffer to facilitate denaturation and reduction of disulfide bonds. In all HDX-MS experiments, the PNGase Rc column was placed immediately downstream of the proteolytic column (Figure 2).



**Figure 2.** Integration of the PNGase Rc column within a conventional commercially available HDX-MS setup.

For Hp 1-1, without online deglycosylation, a sequence coverage of 91.6% was achieved for the  $\alpha$ -chain and a sequence coverage of 84.5% was achieved for the  $\beta$ -chain (Table 1, Figures S6). For the  $\beta$ -chain, 66 peptides could be identified, however, without coverage of any of the four N-linked glycosite regions (N23, N46, N50, and N80). At the N80 N-linked glycan site, two peptides were identified corresponding to the nonglycosylated form. In comparison, during HDX-MS with the PNGase Rc column, sequence coverage for the  $\alpha$ -chain was 100%, and the sequence coverage for the  $\beta$ -chain was 98.8% (Figure 3 and Table 1). For the  $\beta$ -chain, 86 peptides with a total redundancy of 5.33 were found. The most significant result was the entire coverage of all four glycosites with substantial redundancy at glycosites N46, N50, and N80 (Figure 3).

The HDX of Hp 1-1 was largely in agreement with its AlphaFold predicted structure. The regions around N46, N50, and N80 demonstrated rapid deuterium uptake, in contrast to the region around N23, which displayed slow HDX across the 10-h time course (Figures S7 and S8). This is also consistent with where the N-glycan sites are situated inside the  $\beta$ -chain structure with N23 being situated within a  $\beta$ -strand. The solid hydrogen bonding of this region creates a more protected and stable area, which slows HDX. Conversely, all other N-glycosites are located in loop regions, which are characterized by being flexible explaining their faster HDX (Figures S7 and S8).

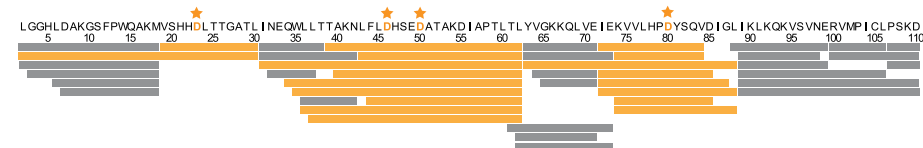
Similarly, HDX-MS analysis with the PNGase Rc column of VISTA and transferrin also showed deglycosylation of all N-glycosites in each protein leading to complete sequence coverage of all glycosylated regions (Figure 4, Figure S9 and Table 1). The HDX of both VISTA and transferrin was in good overall agreement with their crystal structures with N-glycosites located in flexible loops exchanging more rapidly than those located in the more stable  $\beta$ -strands (Figures S10 and S12). Overall, the HDX-MS results on these three disulfide-bonded glycoproteins showed that incorporation of

Table 1. Overview of Glycoproteins Subjected to HDX-MS Analysis with the PNGase Rc Column

glycoprotein	sequence coverage (%) without PNGase Rc column	no. of peptides (without PNGase Rc column)	sequence coverage (%) with PNGase Rc column	no. of peptides (with PNGase Rc column)	no. of N-linked glycosites	no. of deglycosylate d N-linked glycosites	quench conditions (after 1:1 dilution)
c-Met	91	721	98	755	6	6	125 mM TCEP and 1.7 M urea pH 2.8
haptoglobin 1-1 ( $\alpha$ chain)	92	9	100	10	0	0	125 mM TCEP and 2 M urea pH 2.3
haptoglobin 1-1 ( $\beta$ chain)	84.5	66	98	81	4	4	125 mM TCEP and 2 M urea pH 2.3
transferrin	65	87	71	92	2	2	100 mM TCEP and 2 M urea, pH 2.5
VISTA	73	50	97	110	6	6	200 mM TCEP and 2 M urea pH 2.6

Chain  $\alpha$ 

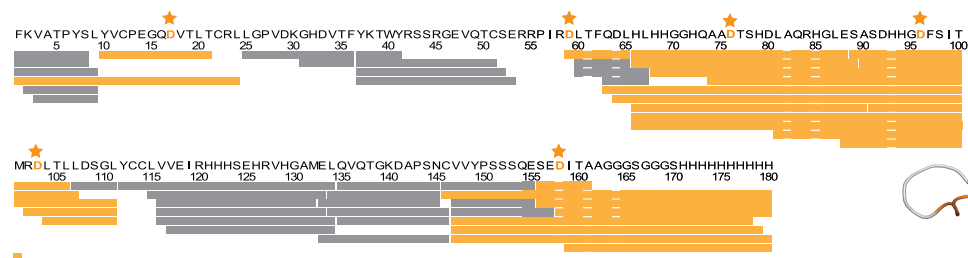
VDSGNDVTDIADDGCPKPEI AHGYVEHSVRYQCKNYYKLRTEGDVYTLNNEKQWINKAVGDKLPECEAVCGKPKNPANPVQ  
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80

Chain  $\beta$ 

YAEVGRVGVVSGMGRNANFKFTDHLKYVMLPVADQDQCIRHYEGSTVPEKKT<sup>\*</sup>PKSPVGVQPI<sup>\*</sup>LNHEHTFCAGMSKYQEDTCYGDGSAF<sup>\*</sup>AVHDL<sup>\*</sup>EEDTWATGI<sup>\*</sup>LSFDKCS  
115 120 125 130 135 140 145 150 155 160 165 170 175 180 185 190 195 200 205 210 215 220

AVAEYGVYKVTSLQDWQKTI AEN  
225 230 235 240 245

**Figure 3.** Sequence coverage map of Hp 1-1 obtained during HDX-MS with online deglycosylation by PNGase Rc. N-Linked glycosites are highlighted in orange and marked by stars. Deglycosylated peptides are colored in orange.



**Figure 4.** Sequence coverage map of VISTA during obtained during HDX-MS with online deglycosylation by PNGase Rc. N-linked glycosites are highlighted in orange and marked by stars. Deglycosylated peptides are colored in orange.

the PNGase Rc column in each HDX-MS experiment resulted in increased sequence coverage, redundancy, and with complete coverage of all N-glycosites in each of the studied glycoproteins.

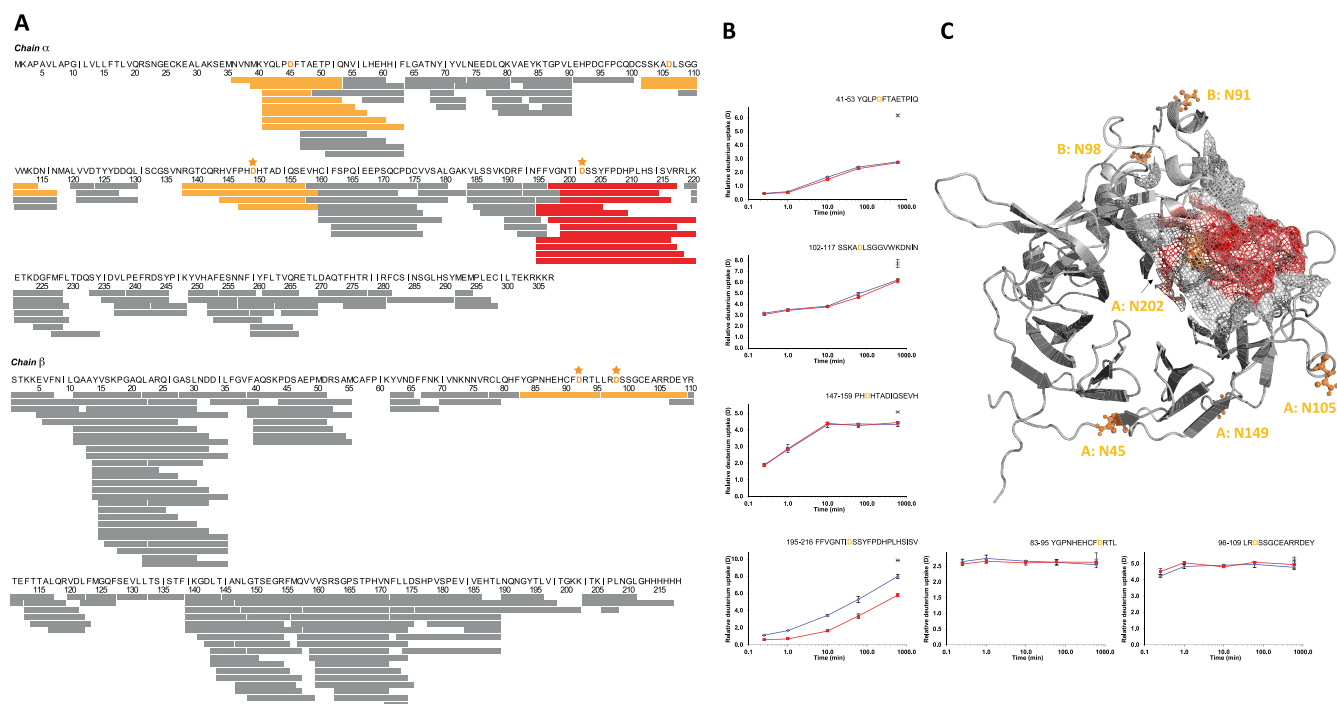
The loss of deuterium label during HDX-MS (back-exchange) was monitored during analysis of Hp 1-1 with and without online deglycosylation (Figure S5). Based on the measurement of the deuterium contents of all detected peptides of Hp 1-1, we found that incorporation of the PNGase Rc column into the HDX-MS setup did not affect back-exchange (<2%).

**Epitope Mapping by HDX-MS with Online Deglycosylation.** We next applied HDX-MS with the integrated PNGase Rc column to map the binding epitope of a mAb on the complex glycoprotein c-Met. c-Met, (Mesenchymal

Epithelial Transition factor), also named Hepatocyte Growth Factor Receptor (HGFR) is generated by the c-Met proto-oncogene and is regarded as a tumor assisted antigen and is an important signaling receptor for the tumor proliferating protein HGF (hepatocyte growth factor).<sup>22</sup> Inhibition of c-Met has been indicated as a promising strategy to treat certain cancer forms.<sup>23</sup>

The epitope mapping experiment was performed on the extracellular domain of c-Met (S25 amino acids) that comprises an  $\alpha$ - and a  $\beta$ -chain, with the  $\alpha$  chain containing 4 N-glycans and 4 intrachain disulfide bonds and the  $\beta$ -chain containing 2 N-glycans and 1 intrachain disulfide bond. The two chains are connected by a disulfide bond between the C274 (chain  $\alpha$ ) and C36 (chain  $\beta$ ).





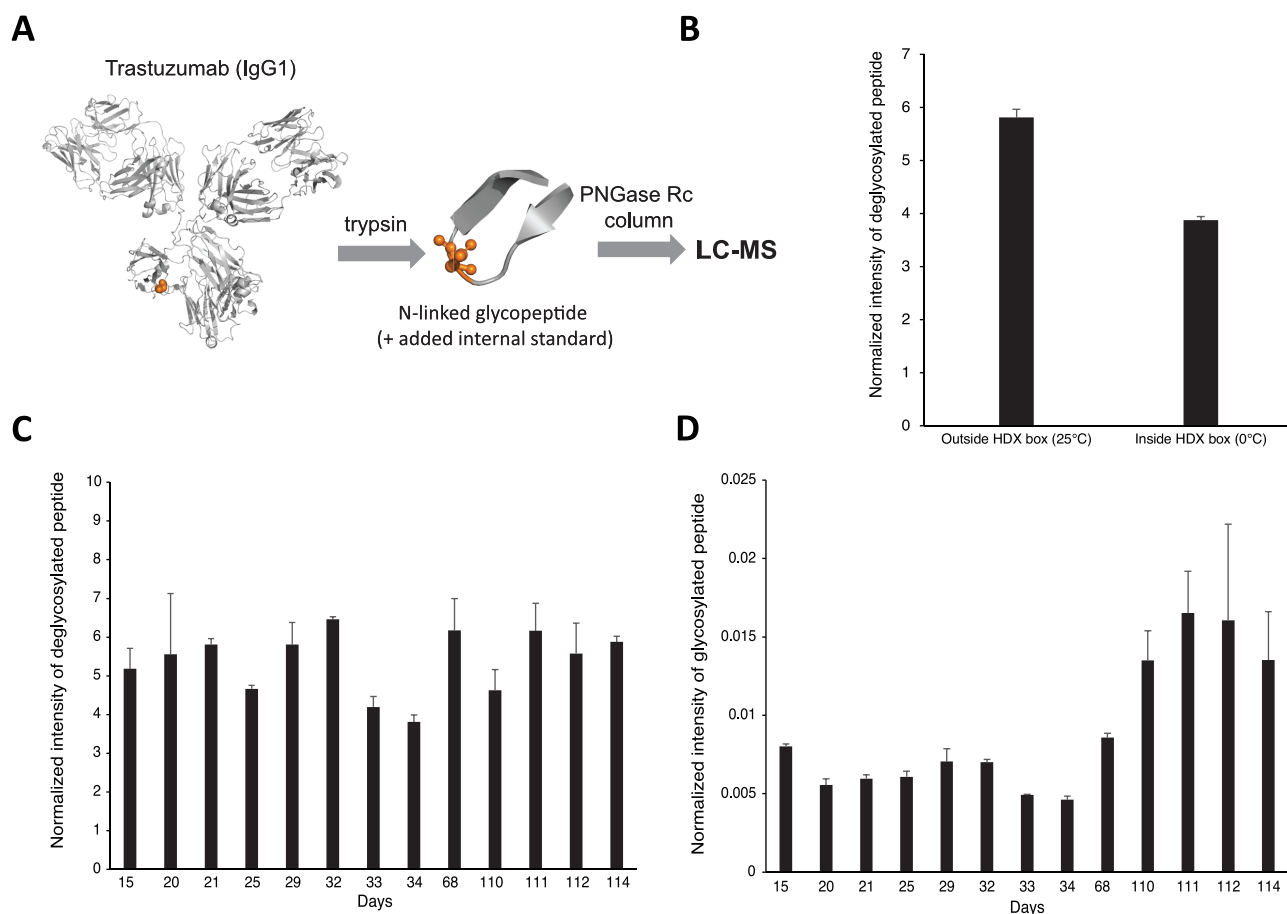
**Figure 5.** HDX-MS epitope mapping of c-Met with online deglycosylation. (A) Sequence coverage map of c-Met, with deglycosylated peptides that contain an N-linked glycosite (denoted by orange star) highlighted in orange. Peptides highlighted in red showed decreased HDX upon mAb binding. (B) HDX uptake plots of all deglycosylated peptides spanning an N-linked glycosite. c-Met with mAb is shown in red curves, c-Met without mAb is shown in blue curves and the maximally labeled control sample is shown as a cross. Error bars represent the standard deviation from three technical replicate measurements. (C) Overlay of the HDX-MS data onto the crystal structure of c-Met (PDB: 1SHY) with the epitope region displaying reduced HDX upon mAb binding (res. 195–216) shown in red and N-glycosites highlighted as orange sticks.

The PNGase Rc-enabled HDX-MS epitope mapping experiment was done using a quench buffer containing TCEP and urea as this had previously been shown to yield the maximum sequence coverage of c-Met.<sup>16</sup> The HDX-MS experiment allowed identification and extraction of HDX information from 124 peptides covering 96.8% of c-Met. Importantly, this included peptides covering all 6 N-glycosites. Overall, the HDX of c-Met was in good agreement with the available crystal structure, with slow HDX in regions of stable structure and faster exchange in loops (Figure S11). For the six glycosylated regions, slow HDX was observed in regions 41–50, 102–117, and 196–218 indicating the presence of significant secondary structure, with fast HDX in regions 83–95, 96–100, and 147–159 corresponding to dynamic loops. Upon binding the mAb, significant reductions in HDX was observed in several peptides spanning the region of the N202 glycosite. None of the other regions of c-Met, including the 5 other N-glycosylated regions showed significant changes in HDX (Figure 5B). Region 196–218 is thus clearly linked to binding of the mAb and it is very likely that the epitope is contained in this region. Notably, this region was previously inaccessible for HDX-MS analysis without the incorporation of the PNGase Rc column to allow efficient deglycosylation. A visual representation of the epitope when overlaid onto the crystal structure of c-Met (Figure 5C) shows that the region is highly solvent exposed and several residues are well-positioned for direct interaction with the mAb.

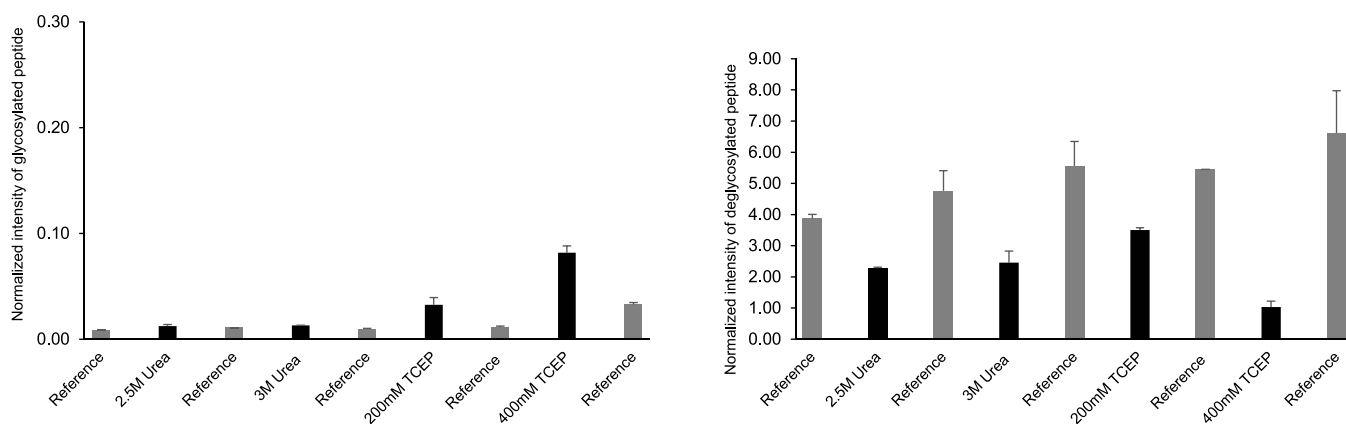
**Reusability and Activity Assessment of the PNGase Rc Column.** Reusability and activity of the PNGase Rc column was measured through the developed LC-MS assay using tryptic glycopeptides from the IgG1 Trastuzumab as

substrate.<sup>16</sup> To investigate the activity of the PNGase Rc column at different temperatures, the efficiency was measured at 0 °C and room temperature. The deglycosylation efficiency at room temperature was 99%, whereas a reduction to an efficiency of 72% was observed at 0 °C (Figure 6B). However, a high degree of deglycosylation could still be obtained at 0 °C, which is suitable for an HDX-MS experiment if required. Notably, the HDX-MS experiments for Hp 1–1, transferrin, and c-Met were all done with the column placed outside the cooling box at room temperature whereas the HDX-MS experiment of VISTA was performed with online deglycosylation at 0 °C.

Next, to investigate the reusability of the PNGase Rc column, the intensity of the glycosylated and deglycosylated form of the glycopeptide was monitored by LC-MS at regular intervals over a time period of 114 days. During this time period, the column was regularly used for other HDX-MS experiments exposing it to TCEP and urea. Overall, the performance and stability of the column was very good, maintaining a deglycosylation efficiency >98%. The fluctuations in intensity of the glycosylated peptide between day 34, 68, and 110 could be caused by use of different substrate batches on day 68 and 110. Nevertheless, from both the intensities of the deglycosylated and the glycosylated peptide, it is clear that even after 114 days of regular usage of the PNGase Rc column, the overall deglycosylation efficiency remains high and largely unchanged (Figure 6C,D). Importantly, over this time period the column was regularly exposed to HDX-MS samples containing urea and TCEP (Table 1). The tolerance of the PNGase Rc column to chaotropic and reducing agents is of high importance, since



**Figure 6.** Monitoring the reusability and temperature dependence of the PNGase Rc column. (A) LC-MS assay used to monitor the activity of the PNGase Rc column. Intensities of the IgG1 N-linked glycopeptide EEQYNSTYR were normalized to intensities of the internal standard Angiotensin II. (B) Activity of the PNGase Rc column when placed inside ( $0^{\circ}\text{C}$ ) and outside the HDX box ( $20^{\circ}\text{C}$ ) at a flow rate of  $200\ \mu\text{L}/\text{min}$ . (C,D) Reusability of the PNGase Rc column over 114 days (monitored by LC-MS assay). During this period, the PNGase Rc column was used regularly in various HDX-MS experiments exposing it to TCEP and urea (see Table 1). The column was used and equilibrated from day 0 to day 15.



**Figure 7.** Deglycosylation efficiency of the PNGase Rc column in the presence of different concentrations of TCEP and urea. The intensity of the glycopeptide EEQYNSTYR was monitored in its glycosylated and deglycosylated form and was normalized to the internal standard. The reference condition represents deglycosylation of the glycopeptide under identical conditions but without the presence of TCEP and urea.

glycoproteins are often extracellular, and thus are very often highly disulfide bonded<sup>24</sup> and thus require both chaotropic and reducing agents in the quench buffer during HDX-MS<sup>5,16</sup>

Furthermore, the same LC-MS assay was used to investigate the deglycosylation efficiency of the PNGase Rc column in

quench buffers with higher concentrations of TCEP and urea (2.5 M and 3 M urea and 200 mM and 400 mM TCEP). The results show that exposure to such high concentrations of TCEP and urea results in some decrease in deglycosylation efficiency compared to the reference (no TCEP and urea)



(Figure 7). Importantly, this decrease in deglycosylation efficiency was fully reversible. Furthermore, as shown in the prior section, the column was fully capable of comprehensive deglycosylation of the different glycoproteins during HDX-MS experiments using quench buffers containing substantial amounts of TCEP and urea (Table 1). Thus, the very good reusability and tolerance to urea and TCEP should make the developed PNGase Rc column of significant utility in improving the HDX-MS analysis of glycoproteins. In a set of limited experiments, we also tested the effect of 1 M GndHCl and the combination of 1 M GndHCl and 50 mM TCEP on the performance of the PNGase Rc column. We observed a similar reduction in activity as that induced by the presence of 400 mM TCEP. Similarly, this reduction in activity was fully reversible (data not shown).

We note that at the same time of this work,<sup>25,26</sup> a paper describing the successful development of a PNGase Dj column for use in HDX-MS has been reported.<sup>27</sup> This work extends our earlier work on immobilization of PNGase Dj in a microchip to a column format. It thus appears that both PNGase Rc and Dj can be immobilized on-column to advance analysis of glycoproteins by HDX-MS. While the current work used the PNGase Rc column in a mode placed downstream of the pepsin column (Figure 2), the PNGase Rc column could also be placed upstream of the pepsin column to allow deglycosylation at the level of the intact glycoprotein as opposed to glycopeptides. In limited experiments, we explored deglycosylation in pre- vs post digestion mode and did not observe a clear difference in terms of no. peptides, sequence coverage, or deglycosylation of N-glycosites. We note, however, that this could be protein dependent.

## CONCLUSIONS

Here we show that the developed PNGase Rc column enables highly efficient online removal of N-linked glycans during HDX-MS of glycoproteins. Implementation of the PNGase Rc column into the conventional HDX-MS setup in each lab was straightforward, placing it immediately downstream from the pepsin column to allow for efficient online digestion and deglycosylation. HDX-MS experiments were performed on four complex glycoproteins that required harsh quench conditions (up to 2 M urea and 200 mM TCEP): Hp 1–1 (4 N-linked glycans, 7 S–S bonds), VISTA (6 N-linked glycans, 3 S–S bonds), transferrin (2 N-linked glycans, 4 S–S bonds), and c-Met (6 N-linked glycans, 5 S–S bonds). In all cases, significantly increased sequence coverage was obtained through use of the PNGase Rc column, enabling measurement of the HDX of all glycosylated regions that were previously inaccessible without the column. We also show how the PNGase Rc column enables mapping of an epitope on c-Met, a challenging glycoprotein drug target. Control experiments showed that use of the PNGase Rc column during HDX-MS resulted in negligible increases (<2%) in the loss of deuterium label (back-exchange).

The column retains high activity in the presence of common quench-buffer additives like TCEP and urea. A moderate reduction in activity was observed at high concentrations of TCEP (200–400 mM TCEP) and urea (2.5–3M), but importantly this reduction was fully reversible. Column performance was consistent across repeated sample injections containing TCEP and urea and stable for >100 days.

We envision that the straightforward incorporation of the PNGase Rc column into conventional HDX-MS setups could

greatly enable HDX-MS analysis of the conformation and ligand interactions of many challenging glycosylated proteins that have so far presented a challenge to HDX-MS. Such proteins could include numerous attractive and unexplored drug targets. Additionally, the column could also be used in other bottom-up proteomic workflows that can benefit from fast online deglycosylation of peptides or proteins prior to LC separation and MS. The developed PNGase Rc column is commercially available (AffiPro, <https://AffiPro.cz/>).

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jasms.3c00268>.

Immobilization protocol, immobilization efficiency test protocol, test of nonspecific adsorption to POROS 20 AL beads, PNGase Rc AlphaFold structure and active site prediction, activity of immobilized PNGase Rc, coverage maps of both glycosylated and deglycosylated Hp 1–1, HDX uptake plots of Hp 1–1, VISTA and transferrin, maximum-labeled control data for Hp 1–1, and HDX uptake plots for the c-Met epitope experiment (PDF)

HDX Summary Table and HDX Data Tables (XLSX)

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### Author Contributions

K.D.R., A.Z., M.K. designed the study. P.K. and D.D. produced PNGase Rc. M.G., P.P., and P.N. performed immobilization with efficiency and enzyme activity tests. P.N. performed SDS-PAGE analysis of deglycosylation of Hp 1–1. Luisa S performed HDX-MS of Hp 1–1. M.G. performed HDX-MS of VISTA. L.S. performed HDX-MS of transferrin. T.L. and C.W. performed HDX-MS epitope mapping of c-Met. C.W., U.R., J.B., A.Z., and K.D.R. supervised the study. The draft of the manuscript was written by T.L. and K.D.R., which was revised through the contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

The authors declare the following competing financial interest(s): Petr Pompach and Petr Novak are founders of the AffiPro s.r.o.

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