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Rapid Magnetic Bead Based Sample Preparation for Automated and High Throughput N-Glycan Analysis of Therapeutic Antibodies

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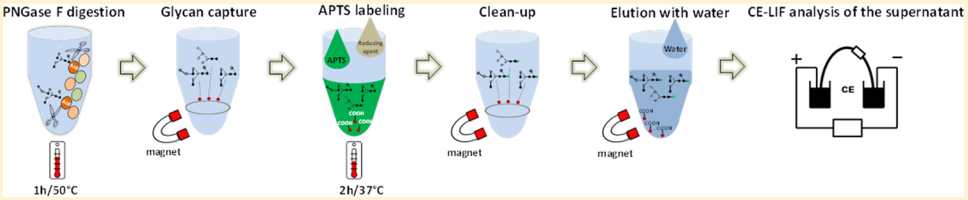

ABSTRACT: Full automation to enable high throughput N-glycosylation profiling and sequencing with good reproducibility is vital to fulfill the contemporary needs of the biopharmaceutical industry and requirements of national regulatory agencies. The most prevalently used glycoanalytical methods of capillary electrophoresis and hydrophilic interaction liquid chromatography, while very efficient, both necessitate extensive sample preparation and cleanup, including glycoprotein capture, N-glycan release, fluorescent derivatization, purification, and preconcentration steps during the process. Currently used protocols to fulfill these tasks require multiple centrifugation and vacuum-centrifugation steps, making liquid handling robot mediated automated sample preparation difficult and expensive. In this paper we report on a rapid magnetic bead based sample preparation approach that enables full automation including all the process phases just in a couple of hours without requiring any centrifugation and/or vacuum centrifugation steps. This novel protocol has been compared to conventional glycan sample preparation strategies using standard glycoproteins (IgG, fetuin, and RNase B) and featured rapid processing time, high release and labeling efficiency, good reproducibility, and the potential of easy automation.

Ever since the importance a protein glycosylation was recognized, development of new glycoanalytical tools has been emerging.[1] Understanding the alterations in protein glycosylation provides essential information for several important aspects of biomedical sciences, the biopharmaceutical field, and regulatory bodies, such as in biomarker discovery for the former and the assessment of the efficacy and safety of protein therapeutics for the latter ones.[2] This challenging task requires high-throughput and highly reproducible screening methods for protein glycosylation.[3] To fulfill this need, automated sample preparation platforms are in great demand to replace the currently used complicated, not easily automatable, and time-consuming manual processes.[4] Examples include shorter incubation times during glycan release and labeling,[5] the option of the use of liquid handling robots for sample preparation, or even multicapillary methods to increase throughput.[6] One of the major handicaps of currently used sample preparation protocols for N-glycosylation analysis (including such steps as enzymatic N-glycan release, carbohydrate purification, fluorophore labeling, and sample cleanup) for subsequent CE or LC analysis is the lack of easy automation options, not requiring high end robotic systems with centrifugation capabilities.

Due to its stability, specificity, and simple sample preparation conditions, the most commonly used enzyme for N-glycan release from glycoproteins is peptide-N4-(*N*-acetyl-*β*-glucosaminyl) asparagine-amidase (PNGase F).[7] Enzymatic deglycosylation by PNGase F is conventionally accomplished at 37 °C with overnight incubation but can be accelerated by means of microwave irradiation,[8] by pressure cycling technology,[9] and with the use of immobilized PNGase F microreactors.[10,11] It has been reported that complete deglycosylation can be obtained in 20 min using a domestic microwave device, where the radiation resulted in the increase of temperature up to 70 °C.[12] Pressure cycling and enzyme immobilization are relatively expensive and complicated technologies, also calling for alternative methods to speed-up the digestion reaction.

Analysis of carbohydrates by capillary electrophoresis with laser-induced fluorescent detection (CE-LIF) requires chemical derivatization of the sugars in order to provide them with adequate charge and fluorescent characteristics. This is usually accomplished by a reductive amination based reaction,[13] most commonly with 1-aminopyrene-3,6,8-trisulfonic acid (APTS).[14,15] Several methods have been reported for APTS-labeling of carbohydrate structures using different reducing agents, different catalysts, or various amounts of the labeling

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dye.[16] Sodium-cyanoborohydride ($NaBH_3CN$) is one of the most frequently used reducing agents, but 2-picoline-borane (pic-BH_3) has also been reported as an equally efficient but nontoxic option.[17] The efficiencies of different acid catalysts in the reductive amination reaction are well-investigated, although in some cases such disadvantages were observed as sialic acid loss.[18] Elevated dye concentration also increases the labeling yield but also requires better cleanup steps.

Magnetic bead based technologies are one of the commonly used approaches to alleviate the centrifugation requirement and have been widely used in the genomics[19] and proteomics[20] fields for many years, ranging from simple sample cleanup to affinity based capture. Carboxyl-coated magnetic microparticles (COOH-beads) are reportedly used to facilitate the isolation and purification of nucleic acid molecules using solid phase reversible immobilization (SPRI) technology.[21] The fact that a plethora of special functional groups can be linked to the surface of magnetic particles resulted in increased attention to their use for the enrichment of biologically important polymers.[22−24]

In this study we have developed and optimized a rapid and high-throughput magnetic bead based sample preparation approach for N-glycosylation analysis, where all preparation steps can be easily automated using simple liquid handling robots. Particular attention was given to avoid any centrifugation steps (both normal and vacuum) and overnight incubations, which are otherwise part of regular protocols of most glycan sample preparation methods.

EXPERIMENTAL SECTION

Chemicals and Reagents. Water, acetonitrile were Chromasolv HPLC grade, IgG, fetuin, RNase B, human serum, acetic acid, sodium-cyanoborohydride, and 2-picoline-borane were from Sigma-Aldrich (St. Louis, MO). 1-Aminopyrene-3,6,8-trisulfonate (APTS), carbohydrate separation gel (NCHO), maltooligosaccharide ladder, and Agencourt Cleanseq magnetic beads were from AB Sciex (Brea, CA, USA). The PNGase F containing deglycosylation kit was purchased from New England Biolabs (Ipswich, MA), and the digestion reaction mixtures were prepared following the manufacturer's protocol.

Capillary Electrophoresis. Capillary electrophoresis profiling of the APTS labeled N-glycans was performed in a PA800+ automated CE instrument (AB Sciex), equipped with a solid state laser-induced fluorescent detector (excitation 488 nm, emission 520 nm). All separations were accomplished in 50 cm effective length (60 cm total) neutral coated, 50 μm i.d. capillary columns filled with N−CHO carbohydrate separation gel buffer (both from AB Sciex). The applied electric field strength was 500 V/cm, with the cathode at the injection side and the anode at the detection side (reversed polarity). Samples were injected by pressure: 1 psi (6.89 kPa) for 5 s. For migration time correction and quantification purposes, APTS labeled maltose (G2) was coinjected with each sample as internal standard. The Karat 32 version 9.1 software package (AB Sciex) was used for data acquisition and analysis.

RESULTS AND DISCUSSION

In this study, a rapid, magnetic bead based sample preparation workflow was developed for CE-LIF based N-glycosylation analysis. Utilization of carboxyl coated magnetic beads alleviated the need for any centrifugation or centrifugal vacuum evaporation steps. The sample preparation protocol is demonstrated via practical examples using representative glycoprotein standards possessing high mannose, neutral, and highly sialylated glycans. All individual processing steps, such as glycan release, fluorescent labeling, and fluorophore label cleanup were optimized to decrease processing time and increase efficiency.

Glycan Release. Our aim at this step was to minimize PNGase F mediated deglycosylation time, while considering easy implementation of this phase for a magnetic bead based platform. Using the liquid-handling, robot-friendly, 96 open-well-plate format, the effect of temperature was important to consider, as >60 °C could cause protein precipitation and buffer evaporation, this latter especially in low volumes (10−50 μL). Digestion efficiency was compared at 50 and 37 °C for the deglycosylation of IgG, fetuin, and RNase B glycoprotein standards using 0.5, 1, 2, 4, 8, and 16 h of incubation with the goal to find the shortest time that results in full deglycosylation. The released glycans were APTS labeled and analyzed by CE-LIF. Three releases were made with each digestion strategy, and three analyses were made with each release, generating nine data points per digestion time and temperature. While peak distribution (measured as peak area percentages) exhibited no differences between the two incubation temperatures, the RFU values represented the changes in the amount of the released glycans. As shown in Figure 1, peak intensities increased

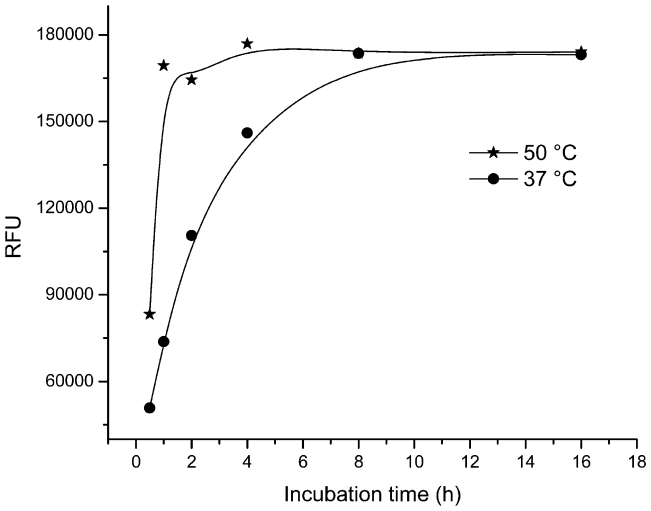

Figure 1. Average fluorescent intensity as a function of PNGase F digestion time at 37 and 50 °C.

significantly more slowly at 37 °C compared to at 50 °C, where the maximum level was already reached after 1 h of incubation time. In addition, no differences were found in peak area percentages when comparing the various digestion times, including the overnight reaction, suggesting that the same glycosylation pattern was obtained using shorter incubations with the main difference in the amount of the released sugars. As expected, accelerated glycan release was observed at the higher temperature; thus, 1 h/50 °C PNGase F digestion was used in all consequent experiments.

APTS Labeling. The goal of the labeling optimization part was to identify conditions that would support automation using magnetic beads and simple liquid handling robots, while providing the same labeling efficiency as that of the conventional centrifugation based method, especially with

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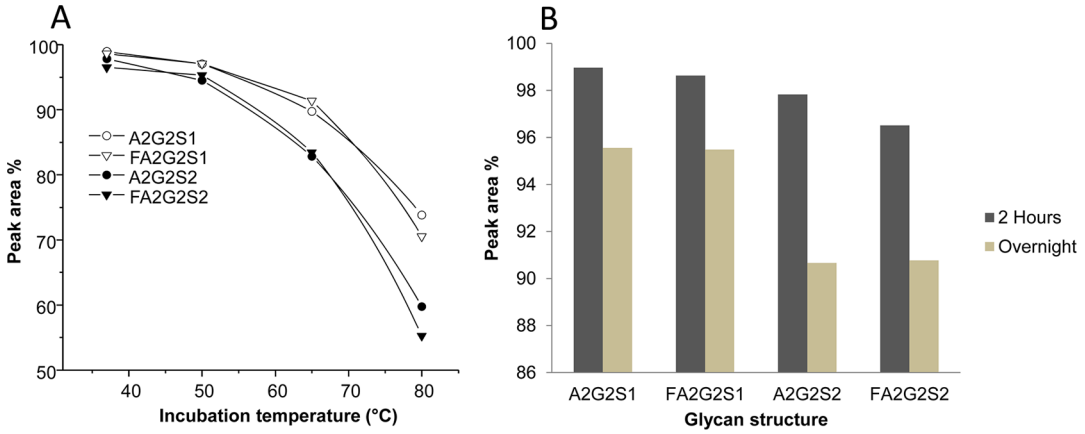

Figure 2. Optimization of fluorophore labeling conditions.

respect to peak intensity and area distribution. Another important aspect of this part of the work was to achieve high labeling efficiency without the need for overnight incubation and vacuum-centrifugation based sample concentration. First, the mono- and disialo glycan standards of A2G2S1, FA2G2S1, A2G2S2, and FA2G2S2 were labeled in duplicate with 20 mM APTS in 15% acetic acid for 2 h at 37, 50, 65, and 80 °C. The nonsialylated counterparts of these glycans (A2G2 and FA2G2) were also labeled and used for spiking the higher temperature reaction mixtures to reveal possible temperature induced desialylation. As one can see in Figure 2A, the increase in the reaction temperature significantly elevated the desialylation process for all sialylated glycan standards. It is interesting to note that disialo standards exhibited more enhanced sialic acid loss. On average, 2% sialylation loss was observed at 50 °C, 11% at 65 °C, and 33% at 80 °C, suggesting that a carefully chosen derivatization temperature is crucial during glycan labeling when sialylated structures are expected in the sample.

The effect of incubation time on fluorophore labeling efficiency was examined at 37 °C. As Figure 2B depicts, significant differences were observed between overnight and 2 h long incubation. The mono- and disialo structures showed on average 2−3% and 9−10% sialic acid loss, respectively, using overnight incubation, signifying the importance of the length of the incubation time. As shorter APTS labeling times (2 h) resulted in lower signal intensity, while longer (overnight) incubation times caused noticeable desialylation, considering the fact that reliable sample preparation and data generation are more important than signal intensity, a 2 h labeling at 37 °C was chosen as a good compromise and was used in all consequent experiments.

In an effort to compensate for the lower signal intensity at shorter incubation times, the effect of catalyst (acetic acid) and fluorophore (APTS) concentration was also investigated (Table 1, sections A, B). Using 20 mM APTS in 15, 20, and 25% acetic acid and mono- and disialo glycan standards (A2G2S1, FA2G2S1, A2G2S2, and FA2G2S2) were labeled to assess the labeling efficiency, while trying to minimize sialylation loss. With the use of 20% acetic acid, the resulting peak pattern and area percentages showed no detectable desialylation, while significantly higher peak intensities were observed (Table 1, section A). Table 1, section B shows the effect of APTS concentration on the derivatization yield of the maltooligosaccharide ladder standard using 20, 40, and 80 mM APTS in 20% acetic acid at 37 °C for 2 h (triplicates). As one can see,

Table 1. APTS Labeling Efficiency Study[a]

Section A			
AcOH conc	15%	20%	25%
A2G2S1	1.9	10.0	10.9
A2G2S2	1.1	5.3	5.9
FA2G2S1	6.6	14.9	15.5
FA2G2S2	3.2	13.0	13.5
Section B			
APTS conc	20 mM	40 mM	80 mM
3 Ladders avg	14.6	39.3	46.0
Section C			
Labeling strategy (37 °C)	2h, 15% AcOH, 20 mM APTS	2h, 20% AcOH, 40 mM APTS	O/N, 15% AcOH,20 mM APTS
IgG	9.5	24.2	52.9
Fetuin	18.9	28.2	66.3
RNase B	13.2	23.5	63.3

[a]The numbers in the table represent the average signal intensity of 3 labeling reactions.

the use of higher APTS concentration lead to increased labeling efficiency. In spite of the fact that the use of 80 mM APTS resulted in the highest intensity, a compromise was suggested here too, to use 40 mM APTS in the higher volume labeling reaction that was required by the automation consideration (minimum of 20 μL volume).

Using the above suggested dye and catalyst concentration, released glycans from 100 μg IgG, fetuin, and RNase B glycoprotein standards were labeled in duplicates using 40 mM APTS in 20% acetic acid at 37 °C for 2 h and compared to the traditionally used 20 mM APTS in 15% acetic acid labeling strategy with 2 h and overnight incubation (Table 1, section C). The combination of higher dye and catalyst concentration resulted in approximately 20% better labeling compared to the original 2 h labeling strategy without any desialylation. Please note that signal intensity was still >50% lower than that with the overnight reaction where, on the other hand, noticeable sialic acid loss was detected. As a first approximation, we suggest APTS labeling at 37 °C for 2 h with 40 mM APTS in 20% acetic acid to obtain adequate labeling efficiency and still minimize desialylation.

Carboxyl Coated Magnetic Beads for N-Glycan Sample Preparation. As emphasized earlier, the key aspect of this work was to find optimal PNGase F based glycan release and APTS labeling parameters for easy downstream automa-

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tion, with the main goal to avoid any centrifugation steps during the sample preparation process, including vacuum centrifugation. Carboxyl coated magnetic beads were apparently capable of capturing both complex carbohydrates in their native (after glycan release) and fluorophore labeled (after APTS labeling) forms. As a first approximation, we consider two possible interaction types between the carboxyl coated beads and the released native glycans as well as the APTS labeled carbohydrates. The first one is with the APTS labeled oligosaccharides, which supposedly repel from the carboxylated beads due to their strong negative charges. However, in the crowded environment[25] with >80% acetonitrile (which acts as crowding reagent), hydrophilic interactions prevail and the carboxylated magnetic beads bind the sugars. Elution is simply accomplished by washing the beads with water, breaking in this way the hydrophilic interactions. The second possible mechanism after the PNGase F digestion is between the glycosylamine form of the released sugars and the carboxyl coated beads. At the working pH of PNGase F digestion (pH 7−8), the resulting glycosylamines[26] are positively charged. The carboxylated beads, on the other, hand are negatively charged at that pH range, prone to ionic interaction between the oppositely charged glycosylamines. During the elution step with aqueous APTS solution in 20% acetic acid, the glycosylamines immediately turn into aldehydes,[27] lose their positive charge, repel from the beads, and start the reaction with the APTS dye.

Considering the above, magnetic beads were first applied to cleanup the APTS labeling reaction mixture, i.e., to remove the nonconjugated, excess APTS. The fluorophore labeled hIgG, fetuin, and RNase B glycans were purified in triplicate after the derivatization reaction using a 200 μL magnetic bead suspension. Binding and washing steps were accomplished by using 150 μL of 87.5% acetonitrile. The elution step used 25 μL of water. Please note that $\geq$20 μL volumes of magnetic bead suspension and binding/elution solutions were readily handled by automatic pipettors, suggesting the utilization option with the same volumes in simple liquid handling robots with regular pipet tips or syringes. The eluate was directly analyzed by CE-LIF without any further processing. The second and third washing fractions were also analyzed to assess the efficiency of the first elution. When the cleanup mixture was properly suspended and 25 μL of water was used for elution, no detectable sample remained on the beads; that is, the second and third washing steps gave negative results. On the other hand, when only 15 μL of water was used in the first elution, traces of remaining APTS labeled glycans were detected with consecutive washing. However, no differences were found in peak area distribution using the magnetic bead based cleanup protocol; that is, the resulting profile was identical to the one that was obtained with conventional sample cleanup methods, suggesting no apparent bias for the different glycan structures (neutral, sialylated, high mannose) toward the carboxylated beads, while most of the free APTS was removed as shown in Figure 3 panels A, B, and C for IgG, RNase B, and fetuin glycans, respectively.

Based on the successful implementation of this centrifugation free APTS cleanup protocol with the use of carboxyl coated magnetic beads, a similar approach was evaluated to capture the released glycans after PNGase F digestion. Magnetic beads were added to the PNGase F reaction mixture after the incubation step, first to bind the released glycans in 87.5% acetonitrile solution. In this case, however, instead of water, the

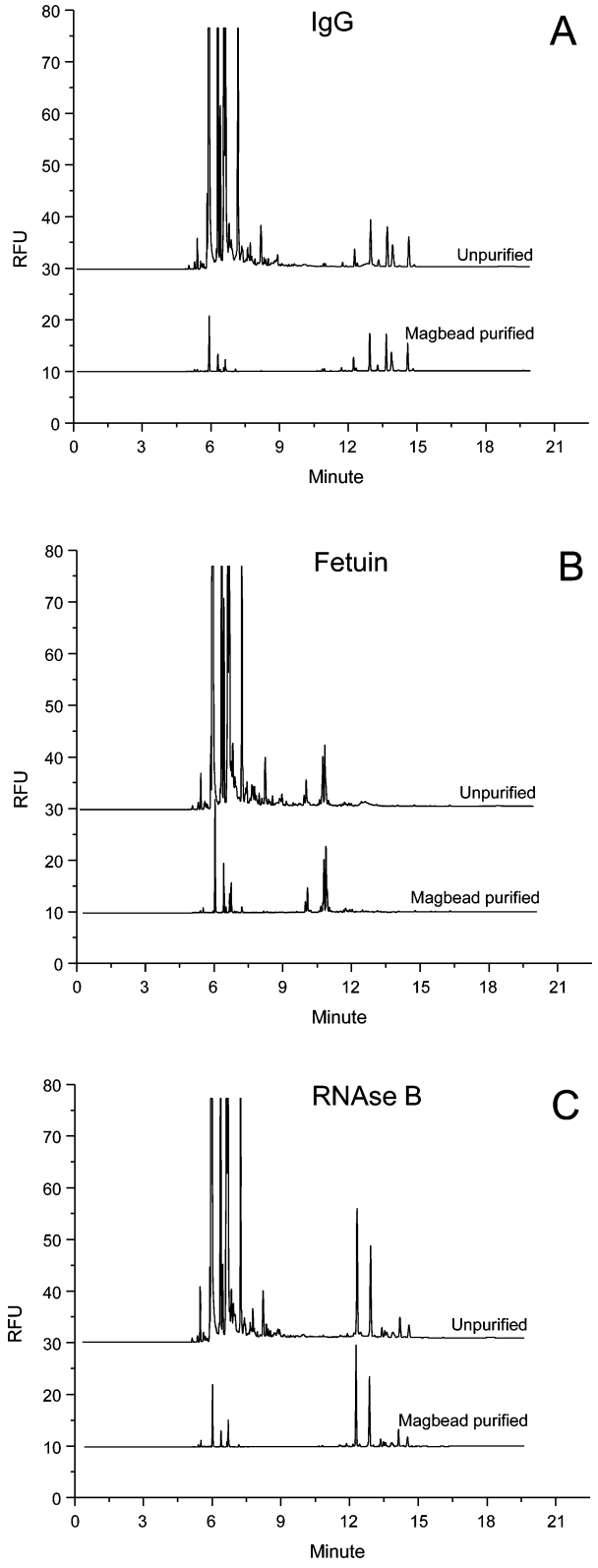

Figure 3. Use of carboxylated magnetic beads for APTS cleanup.

free glycans were eluted with aqueous APTS solution (40 mM in 20% acetic acid) followed by the addition of the reducing agent (1 M pic-BH_3 in MeCN) to immediately initiate the labeling reaction without any interim concentration steps. Please note that this approach, while very effective, did not require any vacuum centrifugation based sample preconcentra-

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Scheme 1. Full Magnetic Bead Based Sample Preparation Workflow for N-Glycosylation Analysis

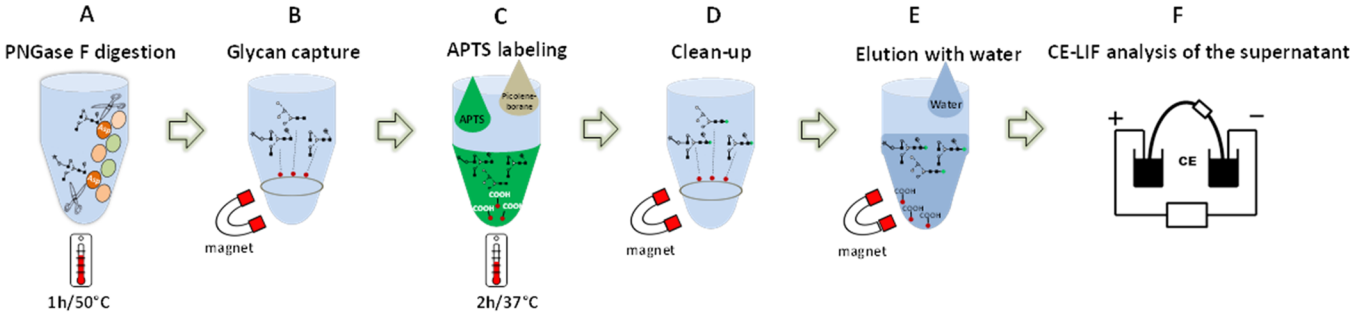

Table 2. Measured Differences between the Two Methods Examining the Peak Area Percentages of 28 N-Glycans

Panel A	Magbead protocol			Overnight protocol			
IgG	Avg Area %	STDEV	RSD%	Avg Area %	STDEV	RSD%	Mann–Whitney significance level
FA2G2S2	1.19	0.04	3.7	1.21	0.09	7.0	0.937
FA2BG2S2	1.23	0.03	2.2	1.20	0.03	2.6	0.132
FA2(3)G1S1	1.71	0.06	3.8	1.73	0.10	5.8	0.818
FA2G2S1	7.45	0.25	3.3	7.48	0.31	4.1	0.937
FA2BG2S1	1.74	0.16	9.0	1.65	0.17	10.4	0.485
FA2	22.12	0.47	2.1	22.23	0.15	0.7	0.699
FA2B	3.97	0.11	2.8	3.97	0.04	1.1	0.589
FA2(6)G1	22.93	0.40	1.8	23.01	0.60	2.6	1.000
FA2(3)G1	11.59	0.06	0.5	11.57	0.17	1.5	0.589
FA2B(6)G1	4.86	0.41	8.5	4.69	0.14	3.1	0.818
FA2B(3)G1	1.02	0.09	9.1	1.07	0.06	5.4	0.132
FA2G2	18.13	0.47	2.6	18.30	0.12	0.6	0.589
FA2BG2	1.46	0.04	3.0	1.40	0.04	2.6	0.065
Panel B	Magbead protocol			Overnight protocol			
RNase B	Avg Area %	STDEV	RSD%	Avg Area %	STDEV	RSD%	Mann–Whitney significance level
Man5	43.45	0.62	1.4	43.90	0.37	0.8	0.132
Man6	33.39	0.36	1.1	33.50	0.25	0.7	0.699
Man7*	3.56	0.13	3.6	3.51	0.04	1.0	0.394
Man7**	2.58	0.08	3.2	2.52	0.07	2.7	0.240
Man7***	2.18	0.03	1.2	2.22	0.11	5.1	0.699
Man8	8.60	0.30	3.5	8.22	0.13	1.6	0.065
Man9	6.00	0.35	5.8	6.04	0.23	3.8	0.699
Panel C	Magbead protocol			Overnight protocol			
Fetuin	Avg Area %	STDEV	RSD%	Avg Area %	STDEV	RSD%	Mann–Whitney significance level
Peak 1	6.95	0.28	4.0	5.08	0.25	4.9	<0.01
Peak 2	15.46	0.58	3.7	11.43	0.47	4.1	<0.01
Peak 3	3.19	0.07	2.1	3.24	0.10	3.1	0.485
Peak 4	3.97	0.11	2.8	4.11	0.18	4.3	0.065
Peak 5	25.93	0.59	2.3	27.90	0.32	1.2	<0.01
Peak 6	32.57	0.30	0.9	32.67	0.21	0.7	0.589
Peak 7	8.45	0.14	1.6	12.73	0.67	5.3	<0.01
Peak 8	3.32	0.12	3.5	3.17	0.14	4.5	0.093

tion or any other purification steps to remove the remaining polypeptide chain and PNGase F enzyme from the digestion reaction mixture.

Magnetic Bead Based Sample Preparation Protocol. The fully extended and optimized glycan sample preparation protocol comprising five individual steps was compared to the conventional centrifugation based and overnight digestion/labeling protocols. As Scheme 1 depicts, the first step was a 1 h PNGase F digestion at 50 °C (Step A). This was followed by magnetic bead based partitioning of the released glycans from the remaining polypeptide chains and the releasing enzyme using a 200 μL magnetic bead suspension in 87.5% final acetonitrile concentration. After thorough mixing, the tube was placed on the magnet (Step B) for partitioning. The supernatant was discarded, and the captured glycans were eluted from the beads into the same tube by 40 mM APTS in 20% acetic acid (21 μL). After the elution step, the reductive amination reaction was immediately started with the addition of pic-BH_3 (Step C, 7 μL, 1 M). The reaction mixture was incubated at 37 °C for 2 h, and then the excess labeling dye was removed (Step D) by the same beads which were used after the digestion in 87.5% final acetonitrile concentration (similar to Step B). After discarding the supernatant, the captured APTS-labeled glycans were eluted from the beads with water (25 μL)

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and partitioned by placing the tube on a magnet (Step E). The supernatant was then analyzed by CE-LIF (Step F). Using this magnetic bead based sample preparation protocol, the full workflow of glycoprotein sample preparation takes approximately 4 h without the need for any centrifugation and or vacuum centrifugation steps, enabling full automation with simple liquid handling robots.

The reliability and reproducibility of the method was evaluated by preparing six IgG, RNase B, and fetuin samples with the magnetic beads and comparing the results with the conventional overnight incubation and centrifugation based protocol.[28] Three repetitions of each release generated 54 data set/preparation platforms. The excellent reproducibility of the approach is depicted in Table 2, showing <5% and <10% RSD for major and minor glycan structures, respectively. The samples analyzed represented neutral and slightly sialylated (Panel A), high mannose (Panel B), and highly sialylated (Panel C) glycans. Mann−Whitney pairwise comparison was applied to explore the differences in peak area percentages. Integrating 28 peaks, the significance (p) level was examined between the 2 methods where only 4 peaks showed significant differences ($p < 0.05$). All of the different peaks were highly sialylated fetuin glycans, and similarly to our previous findings (labeling optimization), the overnight method produced lower sialylation levels, emphasizing the importance of incubation time during reductive amination. The significantly higher area % of peaks 1 and 2 with the use of the magnetic beads correlates with the lower peak area values of peaks 5 and 7, suggesting that decomposition of the highly sialylated species (tetra- and trisialylated) increased the amount low sialylated species (bi- and monosialylated).

■ CONCLUSIONS

A magnetic bead based protocol was developed for N-glycosylation analysis of glycoproteins not requiring difficult-to-automate centrifugation and vacuum-centrifugation steps. Glycan release, APTS-labeling, and cleanup steps were optimized, resulting in a 3−4 h process with excellent yield and high reproducibility. The next step of this work is to apply this protocol with all steps from PNGase F digestion, through fluorophore labeling and cleanup to high throughput sample processing in 96 well plates format with simple liquid handling robots allowing full automation.

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Notes

The authors declare no competing financial interest.

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