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A lectin array-based methodology for the analysis of protein glycosylation

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Abstract

Glycosylation is the most versatile and one of the most abundant protein modifications. It has a structural role as well as diverse functional roles in many specific biological functions, including cancer development, viral and bacterial infections, and autoimmunity. The diverse roles of glycosylation in biological processes are rapidly growing areas of research, however, Glycobiology research is limited by the lack of a technology for rapid analysis of glycan composition of glycoproteins. Currently used methods for glycoanalysis are complex, typically requiring high levels of expertise and days to provide answers, and are not readily available to all researcher.

We have developed a lectin array-based method, Qproteome™ GlycoArray kits, for rapid analysis of glycosylation profiles of glycoproteins. Glycoanalysis is performed on intact glycoproteins, requiring only 4–6 h for most analysis types. The method, demonstrated in this manuscript by several examples, is based on binding of an intact glycoprotein to the arrayed lectins, resulting in a characteristic fingerprint that is highly sensitive to changes in the protein's glycan composition. The large number of lectins, each with its specific recognition pattern, ensures high sensitivity to changes in the glycosylation pattern. A set of proprietary algorithms automatically interpret the fingerprint signals to provide a comprehensive glycan profile output.
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1. Introduction

Glycosylation is the most versatile and one of the most abundant co- and posttranslational modifications of proteins [1]. Glycosylation has a structural role, increasing the protein stability, protecting the protein from proteolysis, and improving the protein solubility. In addition, glycosylation plays important functional roles in many specific biological functions, including immune defense, fertilization, viral replication, parasitic infection, cell growth, inflammation, and cell–cell adhesion. Glycosylation sites on glycoproteins commonly display microheterogeneity as they can be occupied by ensembles of structurally related oligosaccharides. *In vivo*, proteins are glycosylated by the actions of a series of glycosidases and glycosyltransferases resulting in a variety of glycan structures that may vary according to the physiological status of the individual [2]. In *ex vivo* systems, different cell lines and different fermentation conditions can produce significantly different glycosylation patterns [3–5].

Glycosylation is not template-driven, and is currently impossible to predict. Glycoanalysis is a relatively complicated process due to the complexity of the glycans and due to their biophysical properties. Current glycoanalytical methods include mainly chromatography and mass spectrometry-based methods, but also ^{1}H-NMR spectroscopy is applied. These

Abbreviations: Asn, Aspargine; CHO, Chinese hamster ovary; Cy3, Cyanine 3 bihexanoic acid dye; Gal, Galactose; GalNAc, *N*-acetyl galactoseamine; Glc, Glucose; GlcNAc, *N*-acetyl glucoseamine; HPLC, High performance liquid chromatography; Man, Mannose; Neu5Ac, *N*-Acetylneuraminic acid; MS, Mass spectrometry; N, Aspargine; O, Oxygen; PSA, Prostate specific antigen; rHuEPO, Recombinant human erythropoietin; RNase B, Ribonuclease B; *S*, Sulfate; Ser, Serine; THp, Tamm Horsfall glycoprotein.

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methodologies usually require significant labor and expertise in sample preparation and analysis of results. Most methods involve removal of the glycans from the protein [6–8]. Glycoanalysis of complex glycoproteins is therefore often time consuming, and may demand combination of several methods and high expertise [7].

Lectins are a family of carbohydrate-recognizing proteins that are classified into a number of specificity groups based on the monosaccharides and/or disaccharides for which they exhibit the highest affinity [9–11]. They are often not affected by other features of the glycan in which these epitopes reside [12], and therefore demonstrate broad specificities towards

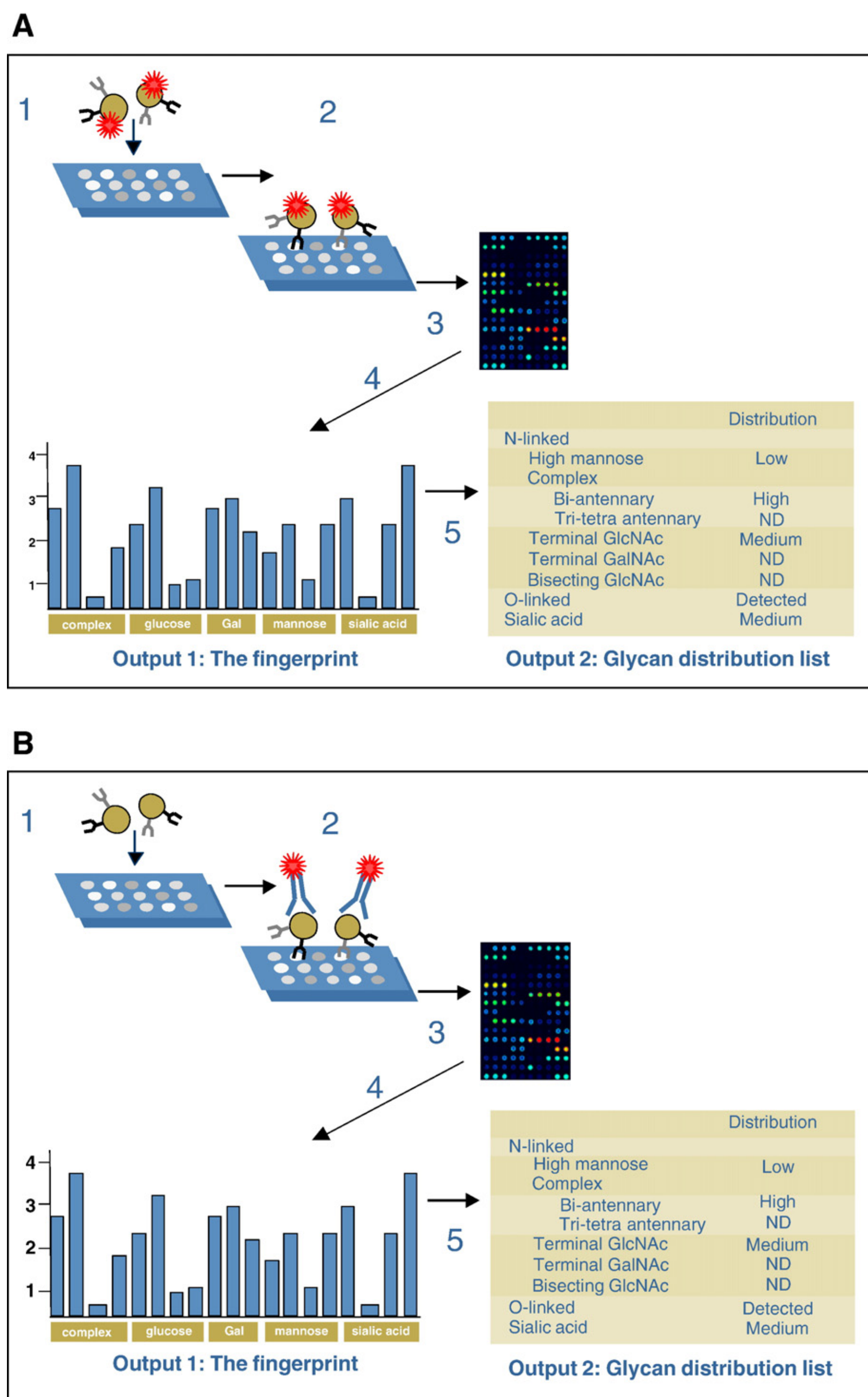

Fig. 1. Schematic representation of the Qproteome™ GlycoArray glycoanalysis process. A. Glycoanalysis process of pre-labeled sample; B. Glycoanalysis process of unlabeled sample. Process steps are: 1). Intact glycoprotein sample is incubated with the lectin array. The sample can be either pre-labeled with a fluorescent dye (A) or unlabeled (B); 2). The glycoprotein binds the array through its glycans, and binding is detected either by a direct fluorescence labeling of the sample prior to incubation with the array (A) or by applying a fluorescently-labeled antibody probe to unlabeled sample (B); 3). Array is scanned; 4). Fluorescent signals are presented as a histogram (the fingerprint), each bar represents a lectin. Lectins are grouped according to their specificity; 5). Proprietary algorithms are used to interpret the fingerprint signals into a glycan distribution list.

glycans. It is therefore a challenging undertake to develop lectin array-based methods for determining the pattern and relative abundance of specific mammalian glycosylation epitopes in a glycosylated protein.

In the present report we describe a lectin array-based glycoanalysis method that is simple, rapid, kit-based, and that can be performed on intact glycoproteins, thereby eliminating the need for time-consuming glycan sample preparation steps. The technology consists of arrays of 24 plant lectins with overlapping specificities. Binding of a glycoprotein to the array results in a characteristic fingerprint that is highly sensitive to changes in the protein's glycan composition. The large number of lectins, each with its specific recognition pattern, ensures high sensitivity to changes in the glycosylation pattern. Since the signal intensities on the array do not correspond to the relative abundance of the respective epitopes, we have constructed an automatic algorithm for calculating these abundances from the array signals. The generated array-binding pattern provides information on the proportion of the various features within a glycoform population. The technology will be illustrated with several examples of well-characterized glycoproteins.

2. Materials and methods

2.1. Glycoprotein labeling

Proteins were labeled either with NHS-CY3 (Amersham Cat. PA13101) or with NHS-Fluos (Molecular Probes Cat. 1311). The labeling was carried out in the dark for 2 h at room temperature using a head over head shaker. Labeled proteins were separated from the un-reacted dye using Sephadex G-25 desalting columns (BIORAD DG10 Cat. 732-2010). Labeled Protein concentration and D/P ratios were calculated according to the instructions included with the dyes.

2.2. Sample/probe preparation

All samples and probes were diluted to the required final concentration of 0.2 μM using the Qiagen Qproteome™ GlycoArray kit (Qiagen Cat. 37702) wash solution. The final dilution contained 1:20 (v/v) Qiagen Qproteome™ GlycoArray kit (Qiagen Cat. 37702) blocking solution. Primary labeled/unlabeled antibodies were of rabbit IgG origin and used at the final concentration of 0.06 μM. Secondary antibodies were CY3-labeled goat anti-rabbit IgGs (Jackson Cat. 111-165-045) at a final concentration of 0.3 μg/ml or FITC-labeled (Jackson Cat. 111-095-045) at 1.5 μg/ml.

2.3. Slide processing

The slide processing was performed according to instructions supplied in the Qiagen Qproteome™ GlycoArray kit (Qiagen Cat. 37702). Labeled glycoproteins were assayed using the "Labeled sample" protocol provided in the kit, and unlabeled samples were assayed using the "Labeled probe" Protocol provided in the kit:

"Labeled samples" protocol: arrays were blocked in 25 ml of "complete blocking solution" for 1 h. Slides were washed 3×5 min with 25 ml of "Complete wash solution". After removal of the excess wash solution, slides were probed with 450 μl of 0.2 μM glycoprotein sample for 1 h. Following sample incubation, slides were washed 3×5 min with 25 ml of "Complete wash solution", washed once with 25 ml of R.O water and dried at 200 *g* for 10 min in a centrifuge containing a slide carrier.

"Labeled probe" (secondary labeled antibody protocol): arrays were blocked in 25 ml of "complete blocking solution" for 1 h. Slides were washed 3×5 min with 25 ml of "Complete wash solution". After removal of excess wash solution, slides were probed with 450 μl of 0.2 μM glycoprotein sample or control for 1 h. Slides were washed 3×5 min with 25 ml of "Complete wash solution" and probed with 450 μl of 0.06 μM protein-specific unlabeled primary Rabbit IgG for 40 min. Following primary antibody incubation, slides were washed 3×5 min with 25 ml of "Complete wash solution". Slides were probed with labeled Goat anti Rabbit for 30 min, washed 3×5 min with 25 ml of "Complete wash solution" and washed once with 25 ml of R.O water. Slides were dried at 200 *g* for 10 min in a centrifuge containing a slide carrier.

All incubations were performed at room temperature on an orbital shaker at 50 rpm. All steps following incubation with labeled proteins/probes were carried out in the dark.

2.4. Scanning

Slides were scanned using a Perkin Elmer Express HT scanner equipped with a CY3 laser. Scanning parameters were defined so that the maximum signal obtained from lectin spots was $\leq 55{,}000$ RFU or maximal background value was $\leq 10{,}000$ RFU. For "labeled probe" assays sample and control slides were scanned using the same scanning conditions.

2.5. Data analysis

Data analysis was performed automatically using the Qproteome™ GlycoArray Analysis software (Version 1.11).

2.5.1. De-sialylation

0.25 mg/ml PSA (Fitzgerald, Cat. 30-AP15) was incubated for 16 h at 37 °C with 0.1 mU/μl of sialidase (Roche, Cat. 269611) in the presence of 50 mM Tris buffer, pH 7.

2.6. Endo-H cleavage

22.15 mg/ml porcine thyroglobulin (Sigma, Cat. T-1126) was incubated for 60 h at 37 °C with 0.05 mU/μl Endo-H (Calbiochem, Cat. 324717) and Protease Inhibitors (Calbiochem, Cat. 539131) in PBS buffer, pH 7.2.

2.7. PNGase F cleavage

0.8 mg/ml of EPO (Eprex) was incubated for 16 h at 37 °C with 1.8 U/μl of PNGase F (NEB, Cat. P07055) and

Table 1
Glycan epitopes predicted by the Qproteome™ GlycoArray glycoanalysis method

Glycan epitope	Quantification level
N-linked	
High mannose	ND/low/medium/high
Complex	
Bi-antennary	ND/low/medium/high
Tri/tetra-antennary	ND/low/medium/high
Terminal GlcNAc	ND/low/medium/high
Terminal GalNAc	ND/detected
Bisecting GlcNAc	ND/detected
O-linked	ND/detected
Sialic acid	ND/low/medium/high

The levels of the glycan epitopes are calculated as the relative abundance of each epitope. Not detected = up to 10%; Low = 11–30%; Medium = 31–70%; High = 71–100%. N-linked glycans and O-linked glycans are calculated independently.
ND = not detected.

Protease Inhibitors (Calbiochem, Cat. 539131) in PBS buffer, pH 7.2.

3. Results

A set of 24 lectins with defined and overlapping glycan-binding specificities was chosen from over 70 lectins characterized. The selection was based on the level of specificity and activity towards glycans attached to intact glycoproteins, when the lectins are bound to nitrocellulose membranes. The lectins are spotted in duplicate arrays on nitrocellulose membrane-coated glass slides (Whatman) in 6 replicate spots each. The concentrations of the arrayed lectins vary from 1.5 mg/ml to 3.5 mg/ml, depending on their affinity. Overall, the membrane is spotted with two duplicate arrays of 150 spots of 400–450 micron diameter each, including negative and positive control spots. The glycoanalysis process is performed in several steps as detailed in Fig. 1. First, the arrayed slide is blocked for non-specific binding and incubated with 0.2 μM of the intact glycoprotein. Bound glycoproteins can be visualized by labeling of the glycoprotein prior to incubation with the array, either by direct labeling with a fluorescent dye or by biotinylation. Bound biotinylated samples are detected by incubation of the array with fluorescent streptavidin. Alternatively, unlabeled glycoproteins can be detected by an antibody specific for the protein itself, which is either labeled or followed by a secondary labeled antibody. Using an antibody probe excludes the need for sample pretreatment. The processed arrays are scanned using a fluorescence laser microarray scanner, and the resulting images are analyzed using proprietary image analysis software which converts the image into a fingerprint — a histogram of the signals obtained for each lectin. The values for each lectin are calculated as the robust average of the signals obtained from the 6 replicates. The fingerprint is interpreted by proprietary knowledge-based algorithms to produce the glycoanalysis results — a list of epitopes and their relative abundance. The entire procedure requires 4–6 h, depending on the detection method.

A major challenge in the development of the technology was the characterization and understanding of the specificity of lectins on the array, to allow interpretation of the fingerprint into a glycan profile. Although lectins are used as a research tool by glycobiologists already for many years, the level of information that can usually be obtained by lectin blots and lectin columns is limited due to the relatively low affinity and broad specificity for glycans exhibited by most lectins.

In the process of developing the technology, significant efforts were invested into the characterization of the specificity of the lectins by studying their binding to a large collection of carefully chosen, well-characterized glycoproteins, and a set of enzymatically remodeled glycosylation variants of these proteins. The glycoproteins represent a large variability in their source, mass, complexity, number of glycosylation sites, and type of glycans. The list includes human glycoproteins such as lactoferrin, tissue plasminogen activator (tPA), Immunoglobulin G (IgG), prostate specific antigen (PSA), follicle stimulating hormone (FSH), human chorionic gonadotropin (hCG), alpha-1-

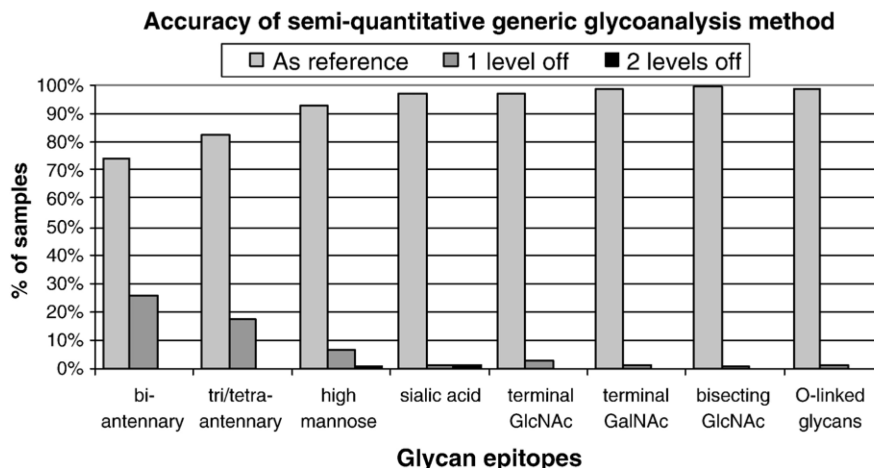

Fig. 2. Statistical summary of the accuracy performance of the generic glycoanalysis method. Interpretation results for 223 fingerprints derived from 15 glycoproteins and their 44 variants were evaluated for accuracy of each epitope prediction by comparison of the prediction with the reference data. Accuracy is measured as the distance of each predicted level (ND, Low, Medium, or High) from the level of the reference.

Table 2
Glycan profile of Cy3-labeled Ribonuclease B (RNase B)

Glycan epitope	RNase B	
	Qproteome™ GlycoArray	Reference
N-linked		
High mannose	High	High (100%)
Complex		
Bi-antennary	ND	ND
Tri/tetra-antennary	ND	ND
Terminal GlcNAc	ND	ND
Terminal GalNAc	ND	ND
Bisecting GlcNAc	ND	ND
O-linked	ND	ND
Sialic acid	ND	ND

Glycan profile produced for RNase B using the Qproteome™ GlycoArray method. The results are calculated from the fingerprint presented in Fig. 3. Results are presented in comparison to reference data produced by traditional methods.
ND = not detected.

acid glycoprotein, and glycophorin, bovine glycoproteins including Fetuin, RNase B, Lactoferrin, and Thyroglobulin, porcine thryroglobulin, recombinant glycoproteins produced in mammalian cells, including erythropoietin (rHuEPO), monoclonal antibodies, heparanase, acetylcholine esterase (AChE), and factor VII. Conalbumin and neoglycoproteins (NGPs) were also utilized. The enzymatic modifications include de-sialylation, de-galactosylation, de-*N*-acetylglucosaminosylation and de-mannosylation at various levels using exoglycosidases, synthesis of Gal(α1–3)Gal epitopes using α-1,3-galactosyltransferase, and removal of N-linked glycans using endo-β-*N*-acetylglucosaminidase Endo-H and amidase PNGase F (peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F). The reference proteins and their variants were fully characterized using chromatographic [13] and mass spectrometric [14] methods, in cases where information from the literature was not available. We then created thousands of fingerprints of several hundreds of glycoform mixtures, derived from the glycoproteins. The analysis of these glycoproteins on the lectin array resulted in detailed specificity definitions for many lectins. This characterization enabled the development of algorithms for glycosylation profile interpretation.

Based on these extensive biochemical, bioinformatics and mathematical analyses, we developed the Qproteome™ GlycoArray method, a rapid, simple to use, kit-based method for the glycoanalysis of mammalian glycoproteins. Several defined detection methods were developed for using both labeled antibody probes and direct labeling of the sample. The epitopes provided by the Qproteome™ GlycoArray method are listed in Table 1. The method provides a 4-level quantification output for most of the epitopes (Not detected = up to 10%; Low = 11–30%; Medium = 31–70%; High = 71–100%) and a qualitative glycan profile for other epitopes (detected/not detected). The algorithm estimates the ratios between the high-mannose-type, the bi-antennary complex-type and the higher order antennary complex-type N-linked glycans. Additionally, the antenna termini of the complex-type N-linked glycans are calculated, and the presence of O-linked glycans detected. The accuracy of the detection of O-linked glycans depends on removal of the N-linked glycans in cases of large and charged N-linked glycans. N-Linked glycans and O-linked glycans are calculated independently, and the ratio between the two types of glycosylation is therefore not provided. Although lectins recognizing the Gal(α1–3)Gal epitope and Fucose are printed on the array, the interpretation does not provide information on them, as they are not reliably predicted by the fingerprints as a stand-alone value. However, when comparing the same protein from different sources (for example different cell lines, or healthy vs. disease specimens), the relative abundance of these epitopes between the two samples can be evaluated by comparing the signals of these lectins between the samples.

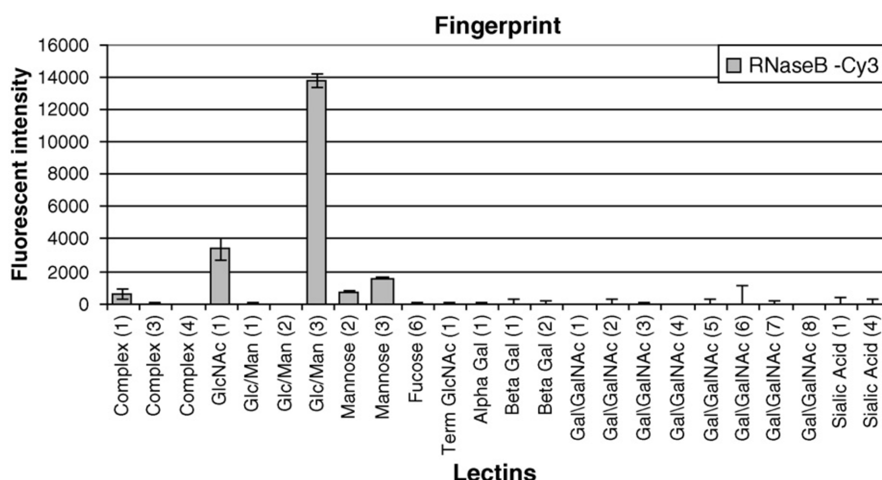

Fig. 3. Fingerprint of Cy3-labeled RNase B. Cy3-labeled RNase B (D/P = 0.31) was diluted with Qproteome™ GlycoArray sample binding buffer to a final concentration of 0.2 μM. 450 μl of the diluted sample was incubated with the lectin array. Sample was processed according to "Labeled samples" protocol (detailed in Materials and methods section). The array was scanned and a fingerprint was produced using the Qproteome™ GlycoArray software. The arrayed lectins (*X* axis) are grouped and labeled according to their specificities. The fingerprint was used by the Qproteome™ GlycoArray software to produce the glycan profile presented in Table 2.

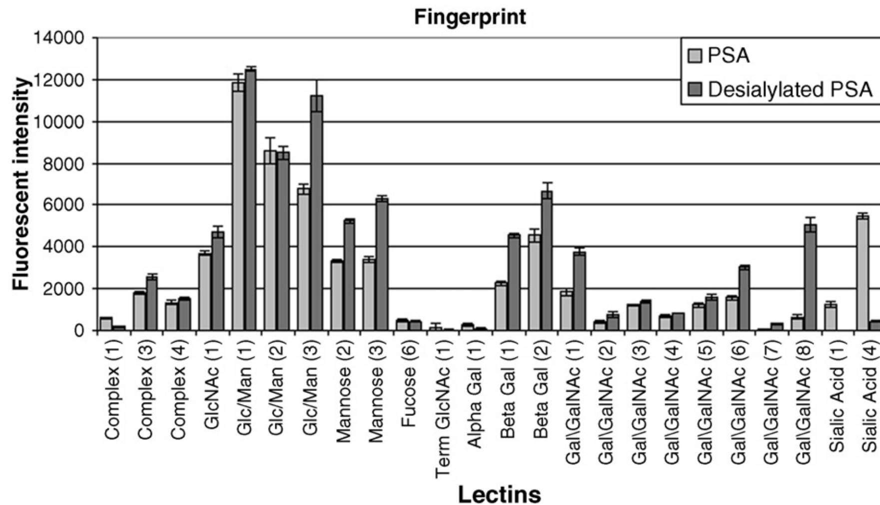

Fig. 4. Fingerprints of native and de-sialylated PSA. Human seminal fluid PSA (light gray bars) and its desialylated variant (dark gray bars) were diluted with Qproteome™ GlycoArray sample binding buffer to a final concentration of 0.2 μM. 450 μl of each diluted sample was incubated with the lectin array. Samples were processed according to "Labeled probe" protocol (detailed in Materials and methods section). Binding of PSA to the lectin arrays was detected using a polyclonal anti-PSA antibody (Dako, cat no. A0562), followed by a secondary FITC-labeled anti-IgG polyclonal antibody (Goat anti Rabbit, Jackson Cat. 111-095-045). The entire process was performed in parallel without sample on a separate control array. Fingerprints were calculated by subtraction of the control array signals from the sample array signals. The arrays were scanned and fingerprints were produced using the Qproteome™ GlycoArray software. The arrayed lectins (*X* axis) are grouped and labeled according to their specificities. The fingerprints were used by the Qproteome™ GlycoArray software to produce the glycan profiles presented in Table 3.

In order to estimate the accuracy of the software tools, a set of 223 fingerprints derived from various glycovariants from 15 different glycoproteins were analyzed. We estimated the accuracy by the distance (calculated as distance between categories) of the interpretation result from the reference data, per each epitope separately. The statistics for the 223 fingerprints are presented in Fig. 2. For most epitopes, the results are within a maximal error of 1 level, although in some cases higher errors may occur. We regard this level of accuracy as acceptable, considering the diversity of the glycoproteins analyzed and the fact that sample and probe concentrations are not optimized for each sample.

In the following we demonstrate the performance of the generic assay using a series of examples of glycoproteins and their glycovariants.

Table 3
Glycan profile of prostate specific antigen (PSA)

Glycan epitope	PSA		Desialylated PSA	
	Qproteome™ GlycoArray	Reference	Qproteome™ GlycoArray	Reference
N-linked				
High mannose	ND	ND	ND	ND
Complex				
Bi-antennary	High	High (100%)	High	High (100%)
Tri/tetra-antennary	ND	ND	ND	ND
Terminal GlcNAc	ND	ND	ND	ND
Terminal GalNAc	ND	ND	ND	ND
Bisecting GlcNAc	ND	ND	ND	ND
O-linked	ND	ND	ND	ND
Sialic acid	Medium	High (76%)	ND	ND

Glycan profiles produced for untreated and desialylated PSA using the Qproteome™ GlycoArray method. The results are calculated from the fingerprints presented in Fig. 4. Results are presented in comparison to reference data produced by traditional methods.
ND = not detected.

3.1. Bovine pancreatic ribonuclease B (RNase B)

RNase B is a well-studied glycoprotein with a molecular mass of approximately 15 kDa. It contains a single N-linked glycosylation site at Asn34 occupied by high-mannose-type N-linked glycans comprising $Man_{5-9}GlcNAc_2$ [15–18].

RNase B glycoanalysis identifies the presence of high levels of high mannose-type N-linked glycans only, in agreement with literature data (Table 2). The fingerprint of RNase B (Fig. 3) reflects a simple profile of a glycoprotein with high-mannose-type glycans. Strong signals are observed for the Glc/Man- and Man-recognizing lectins, while practically no signals are obtained for the complex-type and the antenna-termini-recognizing lectins. Although a small signal is observed on the first complex-type-binding lectin, this signal is not reliable as no antennae termini are detected.

3.2. Prostate-specific antigen (PSA)

PSA is a serine-protease secreted by epithelial prostate cells into the seminal fluid [19]. The presence of PSA in serum serves as a marker for the detection and monitoring of prostate carcinoma [20–22]. PSA is a 34-kDa glycoprotein with a single N-linked glycosylation site at Asn45. The N-linked glycans of PSA have been described as partially sialylated complex-type bi-antennary structures, mostly core (α1–6)-fucosylated; a minor percentage of GalNAc terminated antennae have been

Table 4
Glycan profile of FITC-labeled porcine Thyroglobulin

Glycan epitope	pThyroglobulin		pThyroglobulin-EndoH	
	Qproteome™ GlycoArray	Reference	Qproteome™ GlycoArray	Reference
N-linked				
High mannose	Low	Low (27%)	ND	ND
Complex				
Bi-antennary	Medium	Medium (41%)	Medium	Medium (56%)
Tri/tetra-antennary	Medium	Medium (32%)	Medium	Medium (44%)
Terminal GlcNAc	ND	ND	ND	ND
Terminal GalNAc	ND	ND	ND	ND
Bisecting GlcNAc	ND	ND	ND	ND
O-linked	ND	ND	ND	ND
Sialic acid	Low	Medium (45%)	Low	Medium (62%)

Glycan profiles produced for untreated pThyroglobulin and EndoH-treated pThyroglobulin using the Qproteome™ GlycoArray method. The results are calculated from the fingerprints presented in Fig. 5. Results are presented in comparison to reference data produced by traditional methods.
ND = not detected.

detected [23,24]. There is increasing evidence that the PSA glycosylation is altered in prostate cancer serum in comparison to normal PSA [24,25].

Fig. 4 presents the glycoanalysis results obtained for normal seminal fluid PSA and its de-sialylated variant. The results presented for seminal fluid PSA in Table 3 are highly similar to the information obtained from the literature [24], describing a profile of partially sialylated N-linked bi-antennary glycans. Glycan structures are predicted by the signals observed in the fingerprints presented in Fig. 4. The highest signals are observed from the Man- and Glc/Man-recognizing lectin groups, which are lectins that can bind core mannose of bi-antennary N-linked glycans as well as high-mannose-type N-linked glycans. The relative binding of these lectins varies with respect to the type of N-linked glycan, the number of Man residues, and the type of antennae termini residues, allowing the resolution between high-mannose-type and complex-type bi-antennary glycans. Strong indications for the presence of complex-type bi-antennary glycans and the absence of high-mannose-type glycans are the signal levels observed for the complex-type antenna termini recognizing lectins. The results for untreated PSA indicate medium levels of sialic acid (Table 3). This is concluded mainly by the presence of significant signals from the β-Gal-recognizing lectins, which bind non-sialylated antenna on one hand, and by the presence of significantly stronger signal observed for sialic acid-specific lectin number 4 (Fig. 4). The specificity of the latter is towards (α2–6)-linked sialic acid, while the specificity of sialic acid-specific lectin number 1 is towards (α2–3)-linked sialic acid. De-sialylation of PSA results in the expected non-detectible sialic acid levels, with no affect on N-linked complex antennarity prediction (Table 3). This is concluded by the changes observed in the fingerprint (Fig. 4) for the binding of the antenna-termini-recognizing lectins, i.e. a dramatic decrease in the signals of the sialic acid-specific lectins, and a consistent increase in the signals of the β-Gal- and β-Gal/GalNAc-recognizing lectins.

3.3. Porcine thyroglobulin

Thyroglobulin is a 660-kDa-glycoprotein composed of two subunits; it has 15–20 N-linked glycosylation sites, which comprise 10% of its mass. Porcine thyroglobulin was found to contain both (α1–6)-fucosylated complex-type and high-mannose-type oligosaccharides. Of the complex-type N-linked glycans, there are complex bi-antennary and tri-antennary

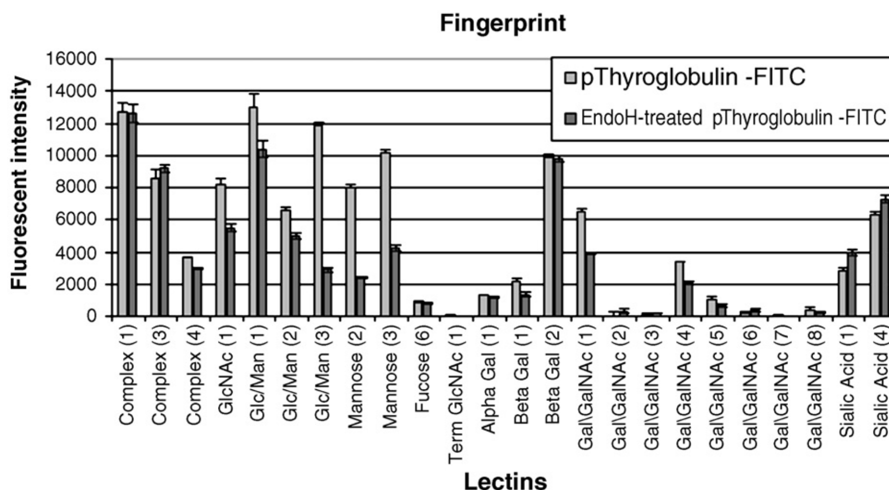

Fig. 5. Fingerprints of FITC-labeled porcine thyroglobulin. FITC-labeled porcine thyroglobulin (light gray bars, D/P = 6.86) and its Endo-H-treated variant (dark gray bars D/P = 6.86) were diluted with Qproteome™ GlycoArray sample binding buffer to a final concentration of 0.2 μM. 450 μl of each diluted sample was incubated with a lectin array. Samples were processed according to "Labeled samples" protocol (detailed in Materials and methods section). The arrays were scanned and fingerprints were produced using the Qproteome™ GlycoArray software. The arrayed lectins (*X* axis) are grouped and labeled according to their specificities. The fingerprints were used by the Qproteome™ GlycoArray software to produce the glycan profiles presented in Table 4.

Table 5
Glycan profile of Cy3-labeled Tamm-Horsfall glycoprotein (THp)

Glycan epitope	THp	
	Qproteome™ GlycoArray	Reference
N-linked		
High mannose	ND	Low
Complex		
Bi-antennary	Low	Low
Tri/tetra-antennary	High	High
Terminal GlcNAc	ND	ND
Terminal GalNAc	ND	ND [a]
Bisecting GlcNAc	ND	ND
O-linked	ND	ND
Sialic acid	High	High

Glycan profile produced for Tamm-Horsfall glycoprotein using the Qproteome™ GlycoArray method. The results are calculated from the fingerprint presented in Fig. 6. Results are presented in comparison to reference data produced by traditional methods.
ND = not detected.
[a] 4-*O*-sulfated GalNAc structures are detected.

structures, partially capped with sialic acid (data obtained by detailed analyses using HPLC and MALDI-TOF analyses; not shown). Sulfation at O3 of terminal Galactose and O6 of antennary GlcNAc were also detected, as well as the Gal(α1–3) Gal element [26].

In order to demonstrate the sensitivity of the glycoanalysis method to changes in the relative levels of the high-mannose- and complex-type N-linked glycans, an enzymatic modification in the glycosylation pattern of thyroglobulin was studied.

The glycan distribution predicted by the lectin array method for thyroglobulin is highly similar to the reference data, as presented in Table 4. The structures are predicted by the fingerprint profile shown in Fig. 5: a relatively large number of lectins bind the non-modified thyroglobulin, corresponding to the complex pattern of its many glycans. These include the lectins specific for (i) complex-type tri-/tetra-antennary glycans, (ii) complex-type bi-antennary glycans, and (iii) high-mannose-type and complex-type bi-antennary glycans (mannose- and Glc/Man-recognizing lectins). The high signals observed for all three mannose-specific lectins point to the presence of high-mannose-type N-linked glycans, while the signals from the complex-type specific lectins as well as the signals from the antenna termini binding lectins are detecting the complex-type glycans. Enzymatic removal of the high-mannose-type glycans by Endo-H treatment results in the expected non-detectible levels of high-mannose-type N-linked glycans (Table 4), predicted by the decrease in binding of the mannose- and Glc/Man-specific lectins. No effect is observed for the complex-type- and antenna termini-specific lectins (Fig. 5). The remaining signals observed for the Glc/Man-specific lectins arise from the complex-type bi-antennary glycans.

Both sialic acid-specific lectins 1 and 4 bind the thyroglobulin sample. Binding of sialic acid-specific lectin 4 indicates the presence of (α2–6) sialic acid structures, as expected. The signals from the (α2–3) sialic acid-specific lectin (Fig. 5, lectin 1) may result from the binding of this lectin to the 3-*O*-sulfated Gal structures reported by Kamerling et al. [26].

3.4. Tamm Horsfall glycoprotein (THp)

Tamm Horsfall glycoprotein (THp) is the most abundant glycoprotein in normal human urine [27]. The glycoprotein consists of a 616 amino acids polypeptide chain with seven occupied N-linked glycosylation sites, a carbohydrate content of 25–30%, and totally over 150 structures [28,29]. The majority of its N-linked glycans are highly sialylated [(α2–3) and (α2–6)-linked] tetra-antennary complex-type chains, with relatively low levels of tri- and bi-antennary glycans; a

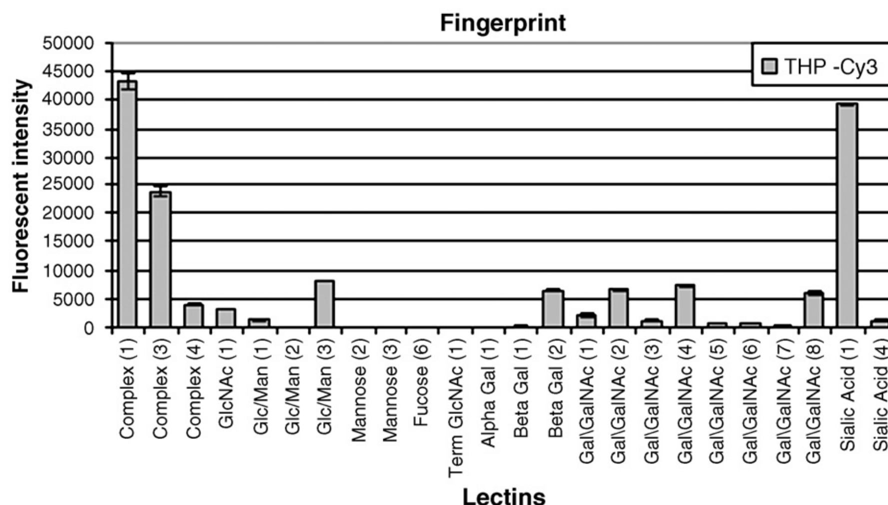

Fig. 6. Fingerprints of Cy3-labeled Tamm-Horsfall glycoprotein (THp). Cy3-labeled Tamm-Horsfall glycoprotein (D/P = 0.43) was diluted with Qproteome™ GlycoArray sample binding buffer to a final concentration of 0.2 μM. 450 μl of the diluted sample was incubated with the lectin array. Sample was processed according to "Labeled samples" protocol (detailed in Materials and methods section). The array was scanned and a fingerprint was produced using the Qproteome™ GlycoArray software. The arrayed lectins (*X* axis) are grouped and labeled according to their specificities. The fingerprint was used by the Qproteome™ GlycoArray software to produce the glycan profile presented in Table 5.

Table 6
Glycan profile of Eprex, a recombinant human erythropoietin (rHuEPO)

Glycan epitope	Eprex		Eprex-PNGase	
	Qproteome™ GlycoArray	Reference	Qproteome™ GlycoArray	Reference
N-linked				
High mannose	ND	ND	ND	ND
Complex				
Bi-antennary	Low	Low (7%)	ND	ND
Tri/tetra-antennary	High	High (93%)	ND	ND
Terminal GlcNAc	ND	ND	ND	ND
Terminal GalNAc	ND	ND	ND	ND
Bisecting GlcNAc	ND	ND	ND	ND
O-linked	ND	Detected	Detected	Detected
Sialic acid	High	High (89%)	Medium	High

Glycan profiles produced for untreated and PNGase-treated Eprex using the Qproteome™ GlycoArray method. The results are calculated from the fingerprints presented in Fig. 7. Results are presented in comparison to reference data produced by traditional methods.
ND = not detected.

relatively high degree of (α1–6)-fucosylation does occur. A wide range of different kinds of terminal epitopes occurs, including minor Gal3*S*(β1–4)GlcNAc, GalNAc4*S*(β1–4), and Neu5Ac(α2–3)[GalNAc(β1–4)]Gal(β1–4)GlcNAc elements [28,30,31]. Depending on the donor, relative to the total carbohydrate content, 1–20% high-mannose-type N-linked glycans can be present. Additionally, the occurrence of O-linked glycans was reported [32], a finding that seems to be donor-specific [33]. The physiological function of the kidney-secreted glycoprotein is still a subject of investigation, with several studies indicating its involvement in modulation of the immune system [34], a role in which its glycosylation was shown to be involved [35,36].

Glycoanalysis of THp yielded the expected results for a highly sialylated glycoprotein with high levels of tri-/tetra-antennary and low levels of bi-antennary complex-type N-linked glycans (Table 5). The results are concluded from the fingerprint presented in Fig. 6. Strong binding is observed for the (α2–3)-linked sialic-acid-specific lectin (Fig. 6, sialic acid lectin 1), consistent with the low signals from the terminal β-Gal-recognizing lectins, indicating high levels of sialylation. No (α2–6)-linked sialylation was detected. The signals of the mannose- and Glc/Man-binding lectins in the fingerprint presented in Fig. 6 are very weak, interpreted as undetectable levels of high-mannose-type glycans. The relatively low levels of high-mannose-type glycans reported in the literature, which hold for the investigated sample, are therefore not detected (Table 5) as they are below the sensitivity of the method, which is 10%. The 4-*O*-sulfated terminal GalNAc structures are also not detected by the lectin array method.

O-linked glycans were not detected in the analyzed THp sample (Table 5), consistent with NMR spectroscopy findings.

3.5. Recombinant human erythropoietin (rHuEPO)

For the production of rHuEPO different host cell lines are in use, with Chinese hamster ovary (CHO) cells being the most

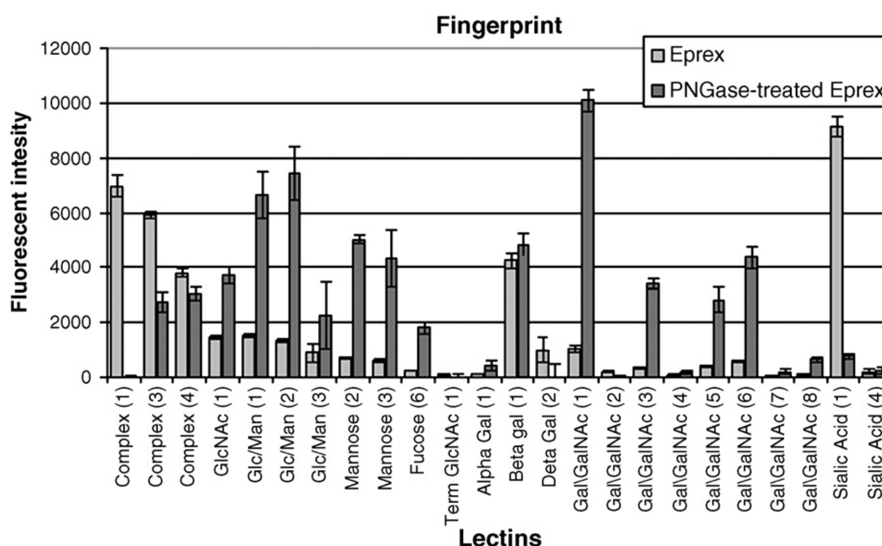

Fig. 7. Fingerprints of native and PNGase-treated Eprex. Recombinant human erythropoietin (Eprex, light-gray bars) and its PNGase-treated variant (dark-gray bars) were diluted with Qproteome™ GlycoArray sample binding buffer to a final concentration of 0.2 μM. 450 μl of each diluted sample was incubated with the lectin array. Samples were processed according to "Labeled probe" protocol (detailed in Materials and methods section). Binding of Eprex to the lectin arrays was detected using a polyclonal anti-human erythropoietin antibody (Rabbit anti rhEPO, homemade), followed by a secondary FITC-labeled anti-IgG polyclonal antibody (Goat anti Rabbit, Jackson Cat. 111-095-045). The entire process was performed in parallel without sample on a separate control array. Fingerprints were calculated by subtraction of the control array signals from the sample array signals. The arrays were scanned and fingerprints were produced using the Qproteome™ GlycoArray software. The arrayed lectins (*X* axis) are grouped and labeled according to their specificities. The fingerprints were used by the Qproteome™ GlycoArray software to produce the glycan profiles presented in Table 6.

popular ones. The glycoprotein hormone is used in replacement therapy for renal failure patients as well as for cancer therapy-induced anemia. EPO has a protein backbone of 166 amino acids, containing three N-linked glycosylation sites (Asn-24, Asn-38, and Asn-83) and one O-glycosylation site (Ser-126). The carbohydrate portion (40% of the total molecule) is important for the *in vivo* half-life of circulating EPO [37]. The 3 N-linked glycosylation sites are occupied by highly sialylated tri- and tetra-antennary complex-type glycans, with minor amounts of bi-antennary glycans; the O-linked glycans comprise the simple ensemble of small sialylated structures [38,39].

In the present study the glycosylation of Eprex, a commercially available epoetin alpha type rHuEPO used for treating anemia, was analyzed. As expected for a highly sialylated glycoprotein produced in CHO cells, the glycoanalysis results obtained for Eprex depict a glycoprotein with highly sialylated tri-/tetra-antennary complex-type glycans; the percentage of bi-antennary glycans is low (Table 6). The high levels of sialylation are predicted by the strong binding observed in the fingerprint for the (α2–3)-linked sialic acid-specific lectin (Fig. 7). The low percentage of bi-antennary complex-type glycans is deduced from the signals that are observed for the mannose- and Glc/Man-recognizing lectins. The various N-linked glycan structures are in accordance with the literature [39]. However, the O-linked glycosylation at Ser-126 is not detected. Our experience shows that O-glycosylation may be masked by highly branched sialylated N-linked glycans; the branched N-linked glycans may significantly reduce the accessibility of the O-linked sugar residues to the lectins. To allow detection of the O-linked glycans, the N-linked glycans of rHuEPO were removed with PNGase-F treatment, and the modified rHuEPO was applied to the lectin array (Fig. 7, Eprex-PNGase). As expected, N-linked glycans are no longer detected by the interpretation algorithm, and the only structures identified are O-linked glycans (Table 6). This results by the significant decrease in the signals of the complex-type glycans-recognizing lectins observed in the fingerprint (Fig. 7), particularly in the signal of lectin 1 which shows the strongest binding to the non-modified rHuEPO. Signals observed for the sialic acid-specific lectins dramatically decrease due to the removal of the sialylated N-linked antennae, leaving only one sialic acid residue, on the O-linked glycans, per protein molecule. On the other hand, there is a dramatic increase in the signals of the Gal/GalNAc-recognizing lectins, detecting the O-linked glycans. Binding is also observed for the Glc/Man, core GlcNAc, and mannose lectins, probably due to non-specific binding of these lectins. Note that the ratios between the mannose- and Glc/Man-recognizing lectins are different when compared with the ratios observed for high-mannose-type glycans (see porcine thyroglobulin and RNase B), enabling the differentiation of these signals from high-mannose-indicative signals. Thus, although the detection of the O-linked glycosylation in rHuEPO is not possible in the presence of its branched complex-type N-linked glycosylation pattern, removal of the N-linked glycans by a simple enzymatic pretreatment for a 16-hour incubation period allows the detection of its O-linked glycans.

4. Discussion

Glycosylation, one of the most important modifications of proteins, has significant impact on the biological properties of proteins. Proteomics is a continuously growing field; glycobiology being an important and integrated portion of it as significant area of research is focused on glycosylated proteins [40]. Glycobiology research is limited by the lack of a technology for rapid analysis of glycan composition of glycoproteins. Our novel protein glycoanalysis method addresses a previously unmet need for the preparation and understanding of proteins in the rapidly emerging field of glycomics. The simple, 4–6 h sample processing protocol, followed by automated data analysis, is a useful tool for life-science researchers of the various disciplines. The level of information obtained by the method is highly informative for the molecular and cell biologist, cancer and development researcher and many others. Unlike the current available HPLC- and mass-spectrometry-based methods, only basic biochemical skills and equipment are required to obtain valuable information about the glycosylation of the protein of interest, information which may be of great added value to the research. The method may be of great value for the glycobiologists researcher, providing the researcher with a tool for obtaining first-line information on its samples, aiding and directing him/her in choosing the appropriate type of analysis for further, more detailed characterization of the protein of interest.

Recently, several reports on lectin arrays have appeared in the literature [41–44]. In all of the approaches, lectins are arrayed onto glass slides, and the binding of either intact glycoproteins [41–43] or whole cells [44] is measured. The major advantages of the method presented herein compared to these reports result from the bioinformatics and algorithmic development, based on data obtained by a benchmark of hundreds of fingerprints, for dozens of glycovariants, enabling the interpretation of the lectin fingerprints, and providing the user with a list of glycan epitopes upon a mouse-click. Moreover, the method is available as easy to use off-the-shelf kits, for the convenience of the user.

As we have demonstrated, our method is also applicable to glycoanalysis of recombinant biopharmaceutical proteins, which are gaining a rapidly increasing share of the pharmaceutical industry [45]. Over 150 biopharmaceuticals have now gained medical approval and several hundred are in the pipeline, most of which are produced in mammalian cell systems [46–49]. Monitoring and characterization of glycosylation at early stages of biopharmaceuticals development is a subject of intense efforts for improving the throughput and efficiency of glycoanalysis methods. The survival of circulating EPO, for example, relies on the presence of terminal sialic acid residues of the N-linked glycans as de-sialylated EPO is rapidly cleared in the liver via galactosyl receptors of the hepatocytes [50]. The method is useful as a screening tool for multiple samples in parallel, making it possible to compare, monitor and control glycosylation throughout the stages of clone screening, clone selection and optimization, process development, and manufacturing. Samples can be analyzed in 4–6 h using non-purified bioreactor supernatant samples, requiring only μM concentrations.

Monitoring glycosylation throughout the drug development process gives scientists in cell culture and process development access to accurate information to guide decision-making. Making informed decisions early in the process will maximize the chance of success in subsequent clinical trials and beyond.

An important aspect of the glycomics research is the analysis of complex biological mixtures such as serum, tissues, and cell extracts. Aberrant glycosylation has been implicated in various disease states such as inflammatory diseases and cancer. A key obstacle limiting such studies is the lack of a technology that can analyze the glycan composition of glycoproteins at high-throughput, directly from in-vivo samples. The lectin array technology, presented here, can be used as a first-line tool for the characterization of global glycosylation patterns of biological samples such as serum, cellular extracts, tissues and whole cells, for identifying changes that accompany biological processes. These applications are currently under development for obtaining comparative analysis tools of complex biological glycosylation profiles, with the aim to enable characterization of glycosylation-related biological effects and to discover novel biomarkers.

5. Simplified description

The Qproteome™ GlycoArray kits, for rapid analysis of glycosylation profiles of glycoproteins is a lectin array-based method in an easy-to-use kit format. Glycoanalysis is performed on intact glycoproteins, requiring only 4–6 h. The method is based on binding of an intact glycoprotein to the arrayed lectins, resulting in a characteristic fingerprint that is highly sensitive to changes in the protein's glycan composition. Proprietary algorithms automatically interpret the fingerprint to provide a comprehensive glycan epitope list. The method is a useful tool for life-science researchers of the various disciplines, providing valuable information on glycosylation for the molecular and cell biologist, cancer and development researcher and many others. It may also be used as a first-line tool for the glycobiologists, directing them to further appropriate, more detailed analyses for characterization of the protein of interest.

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