analytical chemistry

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# Label-Free Glycoprofiling with Multiplex Surface Plasmon Resonance: A Tool To Quantify Sialylation of Erythropoietin

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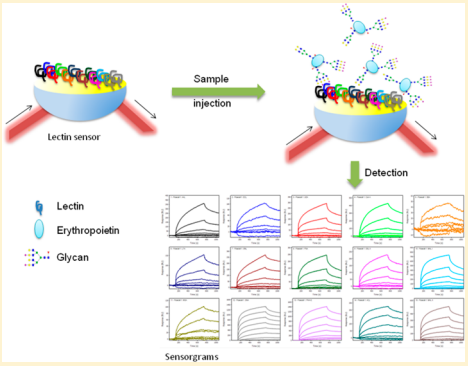
**ABSTRACT:** Protein glycosylation is among the most common and well-defined post-translational modifications due to its vital role in protein function. Monitoring variation in glycosylation is necessary for producing more effective therapeutic proteins. Glycans attached to glycoproteins interact highly specific with lectins, natural carbohydrate-binding proteins, which property is used in the current label-free methodology. We have established a lectin microarray for label-free detection of lectin-carbohydrate interactions allowing us to study protein glycosylation directly on unmodified glycoproteins. The method enables simultaneous measurement of up to 96 lectin-carbohydrate interactions on a multiplex surface plasmon resonance imaging platform within 20 min. Specificity determination of lectins succeeded by analysis of neoglycoproteins and enzymatically remodeled glycoproteins to verify carbohydrate binding. We demonstrated the possibilities for glycosylation fingerprinting by comparing different Erythropoietin sources without the need for any sample pretreatment and we were able to accurately quantify relative sialylation levels of Erythropoietin.




Glycosylation is one of the most important and well-studied post-translational modifications on proteins. Glycans may affect the structure of glycoproteins, can stabilize the conformation of proteins, and may influence the activity of the protein. Furthermore, glycans are involved in protein−protein interactions and protein-cell communication. In biological samples, alterations in glycosylation are typical biomarkers of many diseases such as diabetes,[1,2] rheumatoid arthritis,[3] inflammatory bowel diseases,[4] or metastatic breast cancer.[5]

Additionally, from a therapeutic viewpoint, protein glycosylation is important as it influences the function and efficacy of biopharmaceutical medicines.[6,7] For example, both secretion and efficacy of recombinant Erythropoietin (rhEPO) are largely dependent on glycosylation in general.[8] More specifically, the half-life of circulating Erythropoietin in the blood and in vivo bioactivity are affected by sialylation of the various glycans.[9,10] Erythropoietin is a glycosylated hormone that is produced in the kidneys and liver and regulates red blood cell (erythrocyte) production. Microheterogeneity of rhEPO products mainly originates from glycosylation variants at the three N-linked glycosylation and one O-linked glycosylation sites of the molecule. Glycosylation of rhEPO is one of the critical quality attributes (CQAs) and many different analytical methods exist to characterize the glycans.[11,12]

Current analytical methods mainly study protein glycosylation based on detached glycans, requiring extensive sample preparation for release and labeling of the glycans followed by chromatographic or electrophoretic separation.[13,14] Other methods are based on mass spectrometric measurements and also require several sample preparation steps.[15−17] In the past decade, glycan analysis, or glycoprofiling, has advanced to study intact glycoproteins by affinity-based methods. The majority of these affinity-based methods use lectins as ligands toward carbohydrates. Lectins are naturally occurring carbohydrate-binding proteins that are able to noncovalently bind sugars in a highly specific manner.[18]

**Received:** November 11, 2014
**Accepted:** July 20, 2015
**Published:** July 20, 2015

DOI: 10.1021/acs.analchem.5b00870
*Anal. Chem.* 2015, 87, 8115−8122

Table 1. Lectins Selected for Immobilization, p*I* Value, Immobilization pH, and Their Reported and Determined Specificity

| abbreviation | lectin name | p*I* | immobilization pH | primary specificity[a,b] | other epitopes | binding in our study[c] |
|---|---|---|---|---|---|---|
| AAL | *Aleuria aurantia* lectin | 9.0 | 4.5 | Fuc | – | Fuc |
| LTA | *Lotus tetragonolobus* agglutinin | 7.3–8.2 | 4.5 | Fuc | – | Fuc |
| UEA | *Ulex europaeus* agglutinin | 4.5–5.1 | 4.0 | Fuc | – | Fuc |
| Con A | Concanavalin A | 4.5–5.5 | 4.5 | Man | Glu | Man |
| GNL | *Galanthus nivalis* lectin | 3.5–4.0 | 3.5 | Man | – | Man |
| HHL | *Hippeastrum* hybrid lectin | 4.7–5.1 | 3.0 | Man | – | Man |
| LCA | *Lens culinaris* agglutinin | 7.6–8.4 | 4.5 | Man | Glu, Fuc | Man |
| NPA | *Narcissus pseudonarcissus* agglutinin | 4.2–4.6 | 3.5 | Man | – | Man |
| PSA | *Pisum sativum* agglutinin | 6.0–6.7 | 4.5 | Man | Glu | Man |
| GSL II | *Griffonia* (Bandeiraa) *simplicifolia* lectin II | 5.0–6.0 | 4.5 | GlcNAc | – | GlcNAc, Man |
| WGA | Wheat germ agglutinin | > 9.0 | 4.5 | GlcNAc | SA | GlcNAc (also LacNAc was bound via GlcNAc) |
| SBA | Soybean agglutinin | 5.8–6.0 | 4.5 | GalNAc | Gal | Gal (also LacNAc was bound via Gal) |
| PA-I | *Pseudomonas aeruginosa* lectin | –[d] | -[d] | Gal | – | n.d. |
| RCA I | *Ricinus communis* agglutinin | 7.8 | 4.5 | Gal | – | Gal, LacNAc, Man, Fuc |
| Ricin B | *Ricinus communis* agglutinin B chain | 4.5 | 4.0 | Gal | – | n.d. |
| ECL | *Erythrina cristagalli* lectin | 6.3–6.5 | 4.5 | LacNAc | – | Gal, LacNAc |
| MAL I | *Maackia amurensis* lectin I | 4.7 | 4.0 | LacNAc | – | LacNAc |
| ACL | *Amaranthus caudatus* lectin | 6.7–7.7 | 4.5 | Gal-GalNAc | SA | SA |
| MAL II | *Maackia amurensis* lectin II | 4.7 | 4.0 | SA (−Gal − GalNAc) | – | SA, LacNAc |
| SNA | *Sambucus nigra* agglutinin | 5.4–5.8 | 4.5 | SA (−Gal) | – | All tested glycan moieties |
| PHA-E | *Phaseolus vulgaris* Erythroagglutinin | 6.0–8.0 | 4.5 | LacNac-Man | – | SA, Gal, Man |
| PHA-L | *Phaseolus vulgaris* Leucoagglutinin | 4.2–4.8 | 3.5 | LacNac-Man in triantennary structures | – | no binding with neoglycoproteins |

[a]Primary specificity according to suppliers' information (www.vectorlabs.com/data/brochure/VectorCatalogue2012.pdf) and Sigma-Aldrich Web site, and according to CFG Web site (www.functionalglycomics.org). [b]Fuc, fucose; Man, mannose; Glu, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; LacNAc, *N*-acetyllactosamine; SA, sialic acid. [c]n.d.: not determined. [d]No p*I* of PA-I lectin was specified by the supplier (Sigma-Aldrich); lectin was not immobilized in any of the tested buffers.

Lectin-arrays are able to screen glycosylation profiles and detect differences in these profiles. A recent review by Hirabayashi et al.[19] emphasizes the opportunities for lectin microarrays in glycan analysis. Although current lectin microarrays have eliminated the time-consuming glycan release, fluorescent protein labeling reactions are still required in lectin arrays as described by Hsu et al.,[20] Tao et al.,[21] Wang et al.,[22] Kuno et al.,[23] Pilobello et al.,[24] Chen et al.,[25] and Rosenfeld et al.[26]

Label-free methods such as quartz crystal microbalance (QCM) or surface plasmon resonance (SPR) have been used to study carbohydrate–lectin interactions in real-time. The drawbacks of these methods include monitoring a limited number of lectin-carbohydrate interactions simultaneously[27,28] or using indirect coupling of lectins.[29] Karamanska et al.[30] have established a multiplex carbohydrate assay in order to analyze lectins, but glycan profiling of glycoproteins is not possible on such a microarray because the carbohydrates are immobilized.

We have developed a method that studies lectin–carbohydrate interactions on intact glycoproteins in a rapid, high-throughput, multiplex and label-free manner by surface plasmon resonance imaging. We are able to immobilize unmodified lectins on a sensor in multiplex format while they retain their active carbohydrate binding site. Lectin–glycoprotein interactions are measured without labeling glycoproteins before analysis. We examined the lectins on the array for the specific recognition of glycans and determined affinities/avidities of the selected lectins by means of neoglycoprotein analysis. Furthermore, we used the lectin array to measure glycosylation fingerprints of differentially glycosylated proteins, such as enzymatically remodeled proteins and different sources of recombinant Erythropoietin (EPO).

The method further demonstrated that sialylation of EPO could be accurately quantified. Relative quantitation of sialylation on EPO samples was performed with the lectin microarray, based on the binding to *Erythrina cristagalli* lectin (ECL) and Soybean agglutinin (SBA) lectins.



DOI: 10.1021/acs.analchem.5b00870
*Anal. Chem.* 2015, 87, 8115–8122

## EXPERIMENTAL SECTION

**Surface Plasmon Resonance Method.** Lectin−glycoprotein interactions were measured on an IBIS MX96 surface plasmon resonance (SPR) instrument (IBIS Technologies, Enschede, The Netherlands). Running buffer consisted of HEPES buffered saline (HBS; 20 mM HEPES and 150 mM NaCl) pH 7.2 with 0.05 wt/vol % Tween80 and 1 mM $ZnCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$ added. After a baseline of 2 min, association times of 10 to 20 min and dissociation times of 5 to 20 min were programmed. These were followed by a regeneration of 1 min in 2 steps and a wash step of 1 min. Regeneration was performed with either 3 M $MgCl_2$ or 25 mM phosphoric acid. Analyses were performed at 25 °C and samples were also kept at 25 °C. Samples were analyzed in duplicate or triplicate on sensor surfaces with at least three independent spots of each lectin. All samples were buffer exchanged by 10 kDa spin filters to running buffer or directly diluted in running buffer. All chemicals were of analytical grade and purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) or Merck (Darmstadt, Germany)

**Kinetic Analysis of Neoglycoproteins.** Affinity measurements of lectins were performed with neoglycoproteins. Fucose-BSA, Mannose-BSA, and Galactose-BSA (GlycoDiag, Orleans, France), *N*-acetyllactosamine-BSA (Dextra, Reading, U.K.), *N*-acetylglucosamine-BSA, and Sialic acid-BSA (Vector Laboratories, Burlingame) were the neoglycoproteins of choice. A kinetic titration setup was used, in which 13 dilutions from 0.5 nM to 2 μM (0.45−0.9−1.9−3.9−7.8−15.6−31.3−62.5−125−250−500−1000−2000 nM) of the neoglycoproteins were injected without regeneration between the injections. An association time of 5 min was followed by a dissociation time of 4 min. The sensor was regenerated with 25 mM phosphoric acid after an entire series of one neoglycoprotein for 0.5 min. Running buffer and temperature settings were as mentioned above. BSA was included as a control.

Data analysis was performed in Scrubber software (BioLogic, Campbell, Australia). A 1:1 binding model was used for curve fitting. A selection of the 13 dilutions was made for each lectin−neoglycoprotein pair by selecting the lowest possible concentrations at which an interaction was measured. $K_D$, $k_d$, $k_a$ and Rmax values were determined from 1:1 curve fitting models. At least three independent $K_D$, $k_d$, or $k_a$ and Rmax values were calculated and plotted against each other. A $K_D$, $k_d$, or $k_a$ at Rmax = 100 RU was interpolated or extrapolated from a logarithmic curve for each combination to determine the affinity. No corrections for avidity effects have been made, as the same analyte was used for each lectin and the number of glycan moieties on the neoglycoproteins may vary and is an average.

**Exoglycosidase Treatments.** Approximately 2.5 mg of fetuin was sequentially treated with exoglycosidases α-2-3,6,8,9-neuraminidase (12.5U), β-(1-4,6)-galactosidase (1U), β-*N*-acetylhexosaminidase (2U), α-mannosidase (0.5U), and β-mannosidase (0.5U). All exoglycosidases were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) except (1-4,6)-galactosidase which was purchased from Prozyme (Hayward, CA). Reaction volumes were 150−200 μL, and after each incubation step a fraction of the sample was removed. Remaining sample was buffer exchanged to the recommended buffer for each of the exoglycosidases with 10 kDa cutoff filters. Sample concentrations were checked with Nanodrop after each buffer exchange.

Erythropoietin was desialylated with α-2-3,6,8,9-neuraminidase. Erythropoietin (100 μL of a 0.5 μg/μL solution) was incubated with 10 μL of α-2-3,6,8,9-neuraminidase (5U) at 37 °C for 24 h. Sample was buffer exchanged to running buffer with 10 kDa cutoff filters and concentration was determined with Nanodrop. The five EPO brands were a kind gift of Prof. H. Schellekens from University Utrecht, The Netherlands.

**Quantitation of Sialylation.** Untreated and desialylated EPO (Calbiochem, Merck Millipore (Darmstadt, Germany)) were diluted to 7 μg/mL (200 nM) in running buffer. Sialylation levels of these standards were set to 100% and 0%, respectively. Calibration standards at 50%, 60%, 70%, 80%, 90%, and 100% sialylation were made by mixing the two standards. Binding to four independent spots of both SBA and ECL lectins was measured as the response in RU after 10 min association time and plotted against the theoretical sialylation level. Quadratic curve fitting was applied to the individual calibration curves. Unknown samples were interpolated from the calibration curve to determine sialylation levels.

## RESULTS AND DISCUSSION

**Immobilization and Activity of Lectins.** A panel of lectins displaying recognition toward glycan epitopes on mammalian glycoproteins was selected based on their specificity indicated by the supplier (Table 1). Lectins were immobilized after optimization of the immobilization pH (Table 1) and ligand densities (experimental section in the Supporting Information). A sensor with 18 different lectins in five dilutions including six reference spots (blanks) was successfully applied using two sequential 48 spot prints, enabling monitoring of glycan binding to 18 different lectins on a total of 96 spots simultaneously. The number of lectins to be studied can even be extended further using fewer dilutions or replicates per lectin.

Glycan-binding domains of the lectins could become inaccessible for interaction analysis when lectins are immobilized, especially when the binding site is positioned next to the reactive primary amine with which the lectin will be immobilized with EDC/NHS coupling. Activity of the lectins after covalent coupling to the sensor surface was checked using glycoproteins; Transferrin (Figure S-1), Fetuin, and RNase B (data not shown) were checked for dose-dependent responses. Dose-dependency of glycoproteins was measured on each of the lectins, indicating that lectins can be immobilized using EDC/NHS coupling chemistry on carboxyl sensors without loss of the carbohydrate-binding function.

**Specificity and Apparent Affinity Determination by Neoglycoproteins.** Although the specific binding of certain monosaccharides or glycan epitopes by lectins is known, this specificity is not always consistent between different publications[18,19,23−25,31,32] and the specificity indicated by the supplier. To verify the specificity of the selected lectins, we examined neoglycoproteins as model compounds after immobilization of the lectins. After that, we used the SPR to determine apparent affinities for each lectin−neoglycoprotein pair. Neoglycoproteins are chemically glycosylated bovine serum albumin (BSA) proteins carrying 20−30 homogeneous glycan residues per molecule. Neoglycoproteins modified with sialic acid, galactose, *N*-acetylglucosamine (GlcNAC), *N*-acetyllactosamine (LacNAc), mannose, or fucose residues were chosen to determine apparent affinity and specificity of the immobilized lectins.



DOI: 10.1021/acs.analchem.5b00870
*Anal. Chem.* 2015, 87, 8115−8122

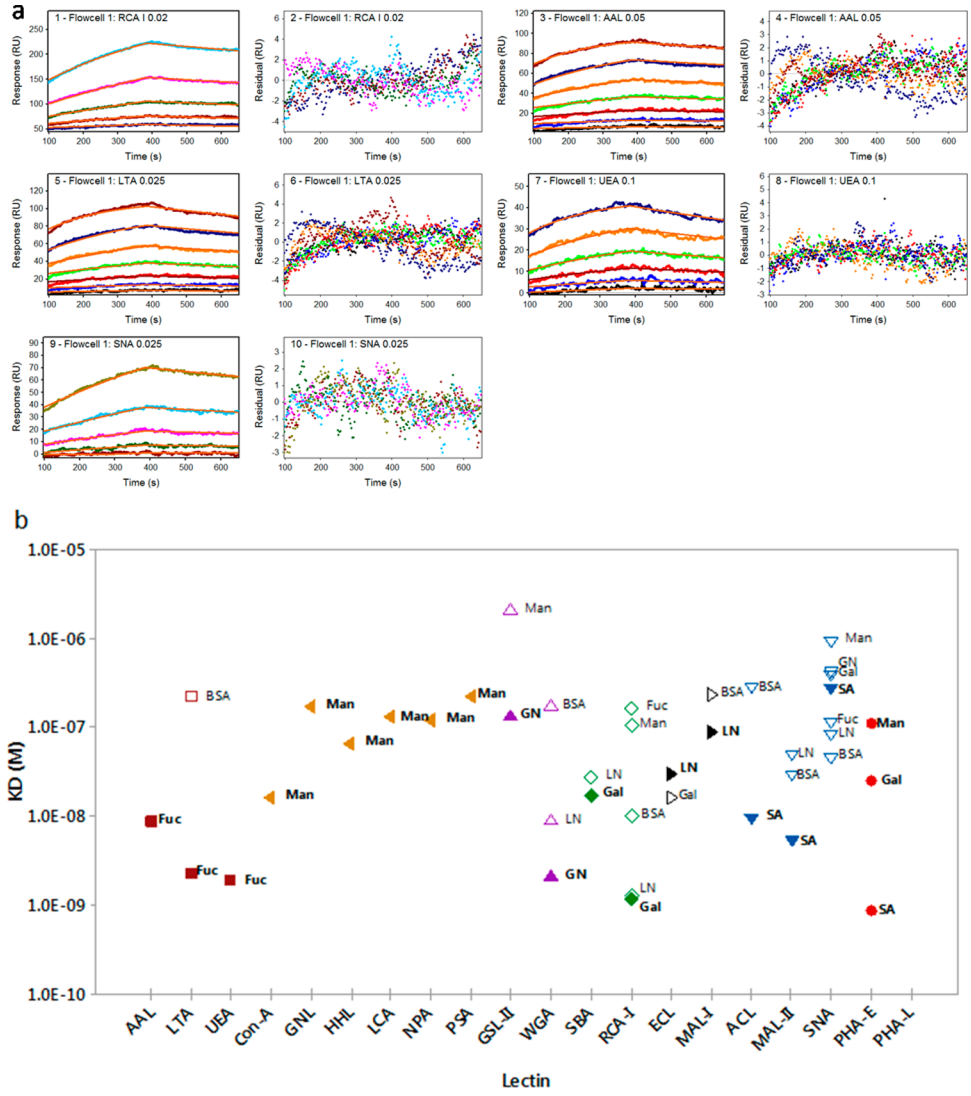



**Figure 1.** Specificity measurements of 20 tested lectins. (a) Sensorgrams from a kinetic titration of fucose−BSA binding to RCA I, AAL, LTA, UEA, and SNA, respectively, including the results of 1:1 Langmuir model global fitting and the corresponding residuals for each global fit. The residuals indicate how closely the modeled curves match with the measured curves and should be randomly distributed over the time axis and over the various concentrations that are analyzed. (b) Apparent affinity of neoglycoproteins at Rmax values of 100 RU after kinetic fitting are plotted for each lectin ($n$ = 4, measurements on two different arrayed sensors). Closed data points refer to specific binding, open data poins refer to cross-reactants. Data labels were added for clarity: Fuc, Fucose-BSA; Man, Mannose-BSA; GN, *N*-acetylglucosamine-BSA; LN, *N*-acetyllactosamine-BSA; Gal, galactose-BSA; SA, sialic acid-BSA; BSA, unmodified BSA.

We used a 20-plex lectin microarray with lectins in two dilutions to measure neoglycoprotein binding and apparent affinity or avidity. Neoglycoproteins were injected between 0.5 nM and 2 $\mu$M in a kinetic titration.[33] Apparent affinity, association rates and dissociation rates of each lectin−neoglycoprotein combination was determined by curve fitting with a 1:1 binding model (Figure 1a, Figure S-2a and Figure S-2b, respectively) and determination of lectin specificity was derived from the apparent affinity values and compared to the indicated specificities.[23,24,31] At least three independent curve fittings were applied to each lectin−neoglycoprotein pair, from which $K_D$ and Rmax values were determined. Residual plots were visually checked for correct distribution of residuals (Figure 1a). Apparent affinities at Rmax = 100 RU were interpolated or extrapolated from plotting the individual $K_D$ and Rmax determinations (Figure 1b). The same interpolation or extrapolation was performed for association and dissociation rates (Figure S-2).

Many lectins have a highly defined primary specificity and our results are in accordance with the supplier's specificity. However, on a number of lectins also cross-reactivity with nontarget neoglycoproteins was measured at lower apparent affinities. Apparent affinities of the specific binders are in general in the low nanomolar range (Figure 1b). However, since the neoglycoproteins carry 20−30 glycan residues per molecule, the reported affinities reflect mostly the avidity of the interaction. The fucose-binding lectins AAL, LTA, and UEA are all very specific and bind to fucose-BSA with high apparent affinity (1−10 nM). Only LTA shows cross-reactivity toward unmodified BSA with an apparent affinity around 200 nM. Also the mannose-binding lectins (Con A, GNL, HHL, LCA, NPA and PSA) are all very specific toward mannose, as no cross-reactivity could be measured. Con A has the highest apparent affinity of these lectins, at approximately 16 nM, while the other lectins bind mannose-BSA at apparent affinities between 66 and 230 nM. Specifity for GlcNAc-binding lectins WGA and GSL II


DOI: 10.1021/acs.analchem.5b00870
*Anal. Chem.* 2015, 87, 8115−8122

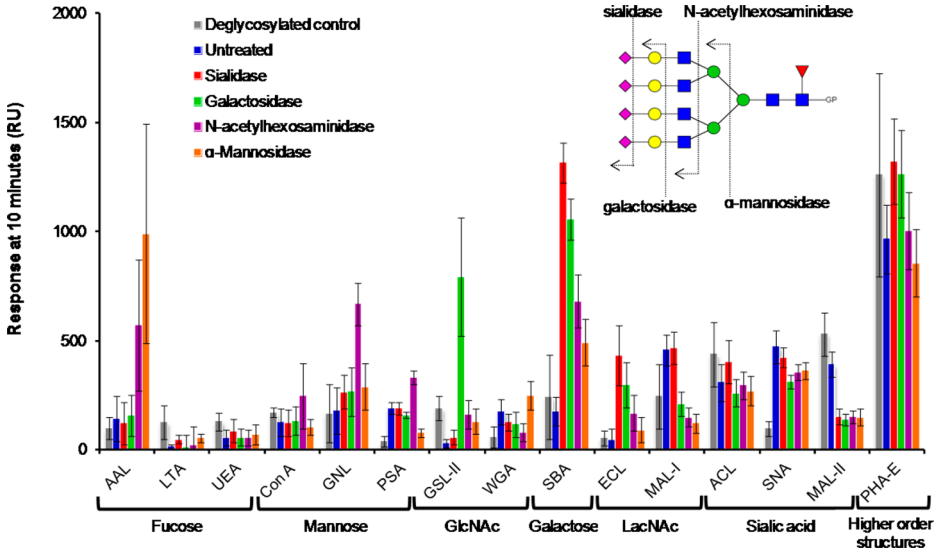



**Figure 2.** Glycosylation fingerprints of remodeled fetuin samples expressed as SPR response units after 10 min association on 15 different lectins. Treated samples ($n$ = 9) were analyzed three times on two different sensors containing triplicate spots of each lectin ($n$ = 6 on sensor 1, $n$ = 3 on sensor 2); the untreated sample ($n$ = 6) was analyzed once on two different sensors containing triplicate spots of each lectin ($n$ = 3 on sensor 1 and on sensor 2). The deglycosylated sample, where N- and O-glycans were removed, was analyzed on one sensor with triplicate spots of each lectin ($n$ = 3). The inset schematically shows the remodeling of a N-glycan.

can also be confirmed with these data, showing that WGA is the stronger binder of the two with an apparent affinity close to 2 nM. WGA also has a strong apparent affinity toward LacNAc-BSA (9 nM), which may be explained by the GlcNAc residue that is part of this LacNAc structure. Cross-reactivity of mannose-BSA was measured on GSL II, indicating that WGA is the favorable lectin for measuring GlcNAc binding based on stronger apparent affinity and no cross-reactivity. In addition to confirmation of lectin specificity (Table 1), many neoglycoproteins bound to RCA I and SNA. RCA I binds galactose-BSA and LacNAc-BSA with highest apparent affinities (1 nM) but cross-reactivity of unmodified BSA (10 nM), mannose-BSA and fucose-BSA (both 100 nM) was found. SNA lectin, selected for its sialic acid binding properties, is the least specific lectin of all lectins tested on our array. All of the analytes, including the BSA control, bind to SNA lectin at apparent affinities between 50 nM and 1 $\mu$M. The strongest binding is measured for nonmodified BSA, while the binding of sialic acid is much weaker compared to unmodified BSA and apparent affinities of fucose-BSA and LacNAc-BSA.

SBA seems to be the lectin of choice for monitoring galactose-binding, with an apparent affinity of approximately 20 nM and only cross-reactivity toward LacNAc which contains a galactose residue in its structure. Lectins that were selected for their specific binding toward LacNAc (ECL and MAL I) are quite specific but have rather low apparent affinities (30−100 nM). ECL binds galactose-BSA at similar apparent affinity compared to LacNAc-BSA, which indicates that ECL does not only recognize the LacNAc moiety but is able to bind a single galactose as well. ACL is the most specific lectin toward sialic acid, of the lectins that were included in this study. Both ACL and MAL II have apparent affinities for sialic acid in the 5−10 nM range. However, MAL II cross reacts with unmodified BSA and LacNAc-BSA. As already discussed previously, SNA is the least specific lectin and is not recommended to use for sialic acid binding.

Furthermore, PHA-E bound all neoglycoproteins, but we were only able to determine apparent affinities for sialic acid-BSA, galactose-BSA, and mannose-BSA, which makes it nonspecific for glycosylated BSA proteins (Figure 1b). On the other hand, PHA-L bound none of the neoglycoproteins which may be explained by its specificity toward triantennary glycans carrying the gal-GlcNAc-man epitope. This epitope is not present at tested neoglycoproteins and therefore no binding could be measured with these models. All of the lectins that show specific binding do so in the nanomolar range, which may be considered strong binding. Nonspecific binding is generally not measured, and if measured it is in the micromolar range or the high nanomolar range, which is relatively weak binding when compared to the specific binding.

**Specificity Determination by Glycan Remodeling.** Specificity of the lectins was further investigated using enzymatically remodeled fetuin and transferrin. Both glycoproteins were sequentially treated with $\alpha$-2-3,6,8,9-neuraminidase, $\beta$-(1-4,6)-galactosidase, $\beta$-$N$-acetylhexosaminidase, and $\alpha$-mannosidase to cleave the N-glycans (Figure 2). Fetuin also carries O-glycans that are cleaved off by exoglycosidases such as neuraminidase and $\beta$-$N$-acetylhexosaminidase. Full cleavage of monosaccharides by exoglycosidases was checked with N-UHPLC analysis after release and 2-AB labeling as a reference method (Figure S3a). As a control, a fully deglycosylated sample was included in the analysis.

Remodeled fetuin (Figure S-3b) and transferrin (data not shown) were analyzed on the lectin microarray to measure differential binding related to the specificity of the lectins based on the exposed glycan moiety. Because of the complexity of the protein glycosylation compared to the neoglycoproteins, it was decided to only evaluate the specificity in a qualitative fashion by comparing binding intensities in the equilibrium state. Overall the specificity measured with remodeled fetuin and transferrin confirmed the results of neoglycoprotein analysis and is in agreement with suppliers' information. Only for a minority of lectins we have found differences in lectin specificity. The deglycosylated control was used to determine background binding on each lectin as no specific glycan binding is expected in the fully deglycosylated samples. Signals of


DOI: 10.1021/acs.analchem.5b00870
Anal. Chem. 2015, 87, 8115−8122

glycosylated samples that were at least three times the response level of the control sample were considered to be true glycan binding. We based this evaluation on the limit of detection qualification in the EMEA guidelines, where a signal-to-noise ratio of 3 is applied.[34]

Clear elevated responses of sialidase-treated fetuin, exposing the galactose, were measured on SBA and ECL lectins (Figure 2). This binding decreased again in samples cleaved with galactosidase and *N*-acetylhexosaminidase, which verifies its binding toward galactose and *N*-acetylgalactosamine. Signals did not decrease to zero because O-glycans of fetuin contain *N*-acetylgalactosamine which can also bind to SBA lectin. The binding measured after galactosidase treatment predominantly originates from *N*- acetylgalactosamine on O-glycans, which are removed after *N*-acetylhexosaminidase treatment resulting in further decrease.

Increasing response on AAL lectin, a fucose-binder, was measured after *N*-acetylhexosaminidase and $\alpha$-mannosidase treatment (Figure 2). Core fucose may be shielded or sterically hindered by sialic acids and galactoses on the N-glycan structure and therefore may not be well recognized by AAL lectin. Upon exoglycosidase treatments, the glycan structure is reduced and the fucose residue may become more accessible for AAL to bind to.

Responses of fetuin after treatment with *N*-acetylhexosaminidase increased on mannose-specific lectins Con A, GNL and PSA lectins confirming mannose recognition as mannose becomes the terminal monosaccharide. A more pronounced increase in signal is measured with GNL and PSA lectins compared to Con A (Figure 2).

An increase in binding of fetuin on GSL II lectin was measured after treatment with galactosidase, which decreased again after treatment with *N*-acetylhexosaminidase. These changes clearly verify the specificity toward GlcNAc. Specificity toward GlcNAc was assigned to WGA with neoglycoproteins (Figure 1b), whereas in the remodeling experiments with fetuin hardly any binding to WGA was measured for the different treated samples (Figure 2). Possibly the cross-reaction of fetuin itself, demonstrated with deglycosylated fetuin (data not shown), prevents binding of exposed GlcNAc residues to WGA lectin.

Sialic acid binders ACL and SNA bound all variants of remodeled fetuin at similar levels compared to untreated, i.e., sialylated, fetuin and were not considered as sialic acid specific (Figure 2). In the neoglycoprotein experiments we already determined that SNA binds to all tested monosaccharides. On the other hand, MAL II lectin bound untreated, i.e., sialylated, fetuin at higher levels than the remodeled samples, although response of deglycosylated fetuin was comparable to sialylated fetuin again. Cross-reactivity of deglycosylated fetuin, where N- and O-glycans were enzymatically removed, was measured on each lectin. Certain lectins (e.g., PHA-E, MAL II) had a higher cross-reactivity toward deglycosylated protein than others. Potentially these lectins specifically bind to exposed glycan moieties but are able to strongly cross-react with non-glycosylated fetuin, in absence of glycans, as well.

Binding of untreated and sialidase-treated fetuin, both of which display the LacNAc epitope, on MAL I lectin was measured whereas further cleaved samples displayed lower levels of binding (Figure 2). Upon cleavage with galactosidase, the LacNAc epitope is broken which caused the reduction in signals. Responses of fetuin samples after galactosidase treatment and further treatments were comparable to the response measured for deglycosylated fetuin and can thus be attributed to cross-reactivity of the protein.

**Erythropoietin (EPO) Analysis with Lectin Microarray.** The developed lectin microarray was validated on Erythropoietin (EPO). Erythropoietin is a highly glycosylated protein. EPO N-glycosylation is mainly present as tri- and tetra-antennary glycans terminating with up to four sialic acids linked to the N-acetyllactosamine chains.[15,35] EPO O-glycosylation is of the mucin-type and carries up to two sialic acids connected to either galactose or *N*-acetylgalactosamine.[15,35,36] The relation between sialylation of EPO and its in vivo activity has been proven by Dubé et al.[8] and can therefore be an important critical quality attribute (CQA) of EPO therapeutics.

We measured five different brands of EPO and tested the lectin microarray to discriminate between the batches based on a glycosylation fingerprint. Three of the batches contained HSA as a stabilizer, which caused a high background signal. The HSA was removed with a HSA depletion kit in less than 30 min (Supporting Information). The glycosylation fingerprints of the five EPO batches were quite distinct from each other, especially on those lectins that bind to LacNAc (MAL I), sialic acid (ACL and MAL II), or higher order structures, i.e., tri- and tetra-antennary glycans (PHA-E) (Figure 3a). None of the tested brands bound to fucose, mannose, and *N*-acetylglucosamine specific lectins (not shown). EPO was desialylated in order to further characterize these differences. Binding of the sialylated

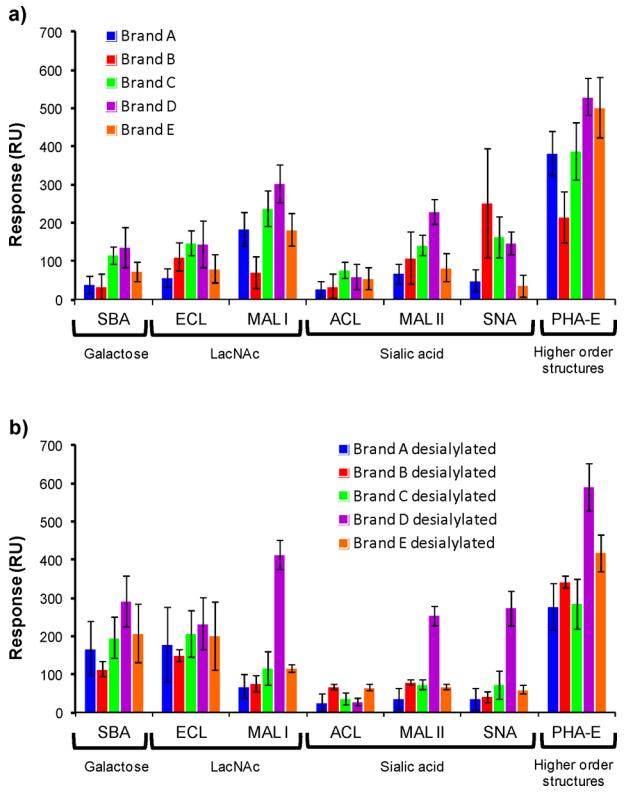



**Figure 3.** Glycosylation fingerprints of five different EPO brands (1000 IU/mL) on a subset of lectins on the lectin microarray of (a) native EPO samples and (b) desialylated EPO samples. Desialylated "Brand B" was analyzed at 733 IU/mL instead of 1000 IU/mL. Samples were analyzed on a sensor with three independent spots of each lectin and repeated on 2 days with independent sample dilutions ($n$ = 6).



DOI: 10.1021/acs.analchem.5b00870
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Table 2. Relative Quantification of EPO Sialylation on SBA and ECL Lectins[a]

| | | SBA | | | ECL | | |
|---|---|---|---|---|---|---|---|
| EPO sample | theoretical % sialylation | avg % sialylation | % CV | deviation (% sialylation) | avg % sialylation | % CV | deviation (% sialylation) |
| **1** | 66.7 | 70.0 | 1.1 | 3.3 | 70.1 | 3.2 | 3.4 |
| **2** | 98.3 | 97.0 | 0.8 | −1.4 | 98.6 | 1.3 | 0.2 |
| **3** | 75.0 | 76.6 | 2.0 | 1.6 | 76.4 | 1.0 | 1.4 |
| **4** | 96.7 | 95.8 | 1.4 | −0.9 | 96.7 | 1.3 | 0.0 |
| **5** | 88.7 | 88.3 | 0.8 | −0.3 | 87.9 | 0.6 | 0.7 |

[a]EPO samples 1 to 5 are mixtures of untreated (100% sialylated) and sialidase-treated (0% sialylated) EPO to known relative sialylation levels.

(Figure 3a) and desialylated (Figure 3b) EPO batches to lectins specific toward terminating galactose and sialic acids indicates that there are differences in terms of glycosylation on these EPO batches. The EPO samples were all diluted to the same concentration in activity (IU/mL), since large differences in responses were measured when all brands were diluted to the same protein concentration in μg/mL.

Brands A and E have highly similar glycosylation fingerprints on the selected lectins. Sialylated samples of both brands only bind to MAL I and PHA-E lectins, whereas desialylated samples bind to SBA and ECL. Brand B has a different glycosylation fingerprint compared to the other four brands, especially with respect to binding to MAL I, SNA, and PHA-E. It hardly binds to MAL I and PHA-E lectins and has the highest response on SNA lectin. However, desialylated brand B is highly comparable to the brands A, C, and E, whereas brand D has a very distinct glycosylation fingerprint after desialylation. Binding to MAL II, SNA, and MAL I remains only for brand D after desialylation. Although no complete identification of glycans on each of the brands can be performed with the current results, we can clearly demonstrate that the lectin sensor is capable of measuring differences between different EPO brands based on a glycan fingerprint. The relevance of these differences should be demonstrated by comparison with an in vivo study. The method was further evaluated for relative quantitation of sialylation. The quantitation was evaluated by comparing binding of desialylated EPO and untreated EPO to galactose-binding lectin SBA and LacNAc-binding lectin ECL, which both increased upon desialylation. Optimization of sample concentrations resulted in analysis of EPO samples down to 1.4 μg/mL (Figure S-4), which corresponds to a total EPO consumption of no more than 200 ng. At this concentration, both SBA and ECL lectin were still capable of measuring clear differences in untreated EPO and desialylated EPO. Higher sensitivity, i.e., larger differences in responses between desialylated EPO and untreated EPO, is obtained at higher sample concentrations and therefore 7 μg/mL was chosen for relative quantitation.

Calibration standards from Calbiochem EPO at different sialylation levels were measured and responses at equilibrium were plotted against the theoretical sialylation level (Figure S-5). We used a relative quantitation method to proof the quantitative capabilities of the lectin sensor. Next to the calibration standards, we prepared five samples by mixing untreated and desialylated EPO from the same brand in different ratios and determined the sialylation by interpolation of the calibration curves. Sialylation of five different EPO samples were accurately quantified (Table 2). Relative quantification on SBA lectin resulted in no more than 3.3% deviation between determined and theoretical level of sialylation, with a % CV of 2.0% or less on an average of four independent lectin spots. Slightly higher deviations between determined and theoretical level of sialylation were detected on ECL lectin, with a maximum deviation of 3.4%. Variation on ECL lectin was 3.2% CV or lower on an average of four independent lectin spots.

## CONCLUSIONS

The broad diversity and general occurrence of glycans as post-translational modification on proteins requires rapid and sensitive methods to profile and monitor glycosylation. We demonstrated that surface plasmon resonance imaging can be employed to study lectin−carbohydrate interactions in a multiplexed manner. Glycan fingerprints are measured in a high-throughput setup as up to 96 lectin−carbohydrate interactions are measured simultaneously generating a glycoprofile which can be used for comparative purposes. Screening of up to 70 samples can be performed within 1 day as each analysis only takes up to 20 min. Intact glycoproteins can be analyzed after diluting into the corresponding system buffer without any laborious sample pretreatment steps. In case of the EPO analyses, we needed to remove HSA which is added as a stabilizer in certain EPO brands. The depletion step that we applied took less than 30 min. Multiple samples can be depleted simultaneously and are directly buffer exchanged to running buffer. Lectins can be directly immobilized on the SPR sensor while maintaining their carbohydrate-recognition properties. Different glycosylation patterns on a panel of 15 lectins were measured for distinctly glycosylated proteins which could be related to the expected glycans. Glycan alterations on the proteins, deliberately applied by exoglycosidase cleavages, were effectively detected by the created lectin microarray. Binding to the lectins was enhanced or decreased after sequential cleavage of the glycan structure and was in accordance with the specificity of the lectins. Specificity of the lectins on the microarray was checked by neoglycoprotein analysis in this study and verified the reported specificities for nearly all studied lectins. Affinities of the lectins binding to defined carbohydrates on neoglycoproteins could easily be determined with the multiplex SPR method. We used these affinities to verify the specificity of the lectins and determine the strong and weak binders. Few lectins bound all neoglycoproteins or remodeled proteins regardless of the exposed glycan epitope and were considered rather nonspecific. In many cases of high cross-reactivity, also binding of nonglycosylated proteins was measured and these lectins were no further included on our array.

The presented method is able to measure lectin−carbohydrate interactions in real-time and label-free. The method has no prerequisites for labeling as SPR measures differences in refractive index at a sensor surface; both ligand and analyte can be successfully analyzed in their native state. Furthermore, it is a true multiplex method since 96 interactions


DOI: 10.1021/acs.analchem.5b00870
Anal. Chem. 2015, 87, 8115−8122

are studied simultaneously on a single sensor with sufficient possibilities to apply negative, positive controls and controls for normalization of the responses, whereas in other SPR-based methods the number of different ligands is limited.

The power of the method was further proven by relative quantitation of EPO sialylation. We optimized measurements on two lectins specific toward galactose to quantify sialylation levels on EPO. Relative EPO sialylation can be accurately determined at levels between 50% and 100% with the developed lectin microarray, consuming only 700 ng of EPO for a single measurement. The deviation between actual and measured sialylation levels is no more than 3.4%. Furthermore, a % CV of 3.2 or less was measured based on four independent lectin spots. Different brands of EPO were analyzed on the lectin microarray and distinct glycosylation fingerprints were obtained. Especially large differences in both N- and O-sialylation were measured for the different batches. The relevance of these differences should be proven with in vivo data, which is currently ongoing.

The combination of label-free, multiplex and real-time measurements opens up new prospects for rapid glycosylation fingerprinting. The lectin microarray has many advantages over existing glycoprofiling methods, such as the ability to measure intact glycoproteins, the low sample consumption, the high accuracy, sensitivity, and the possibility to quantitatively determine specific glycan moieties.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b00870.

Additional figures and detailed experimental section (PDF)

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All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank EFRO Province of Gelderland and Overijssel, The Netherlands, for giving us the opportunity by financially supporting the research project. We would like to thank J. Brouwer for most of the experiments with Erythropoietin.

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DOI: 10.1021/acs.analchem.5b00870
*Anal. Chem.* 2015, 87, 8115−8122