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Modulation of glycan detection on specific glycoproteins by lectin multimerization

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Abstract

Improved methods for studying glycans could spur significant advances in the understanding and application of glycobiology. The use of affinity reagents such as lectins and glycan-binding antibodies is a valuable complement to methods involving mass spectrometry and chromatography. Many lectins, however, are not useful as analytic tools due to low affinity *in vitro*. As an approach to increasing lectin avidity to targeted glycans, we tested the use of lectin multimerization. Several biotinylated lectins were linked together through streptavidin interactions. The binding of certain lectins for purified glycoproteins and glycoproteins captured directly out of biological solutions was increased using multimerization, resulting in the detection of lower concentrations of glycoprotein than possible using monomeric detection. The analysis of glycoproteins in plasma samples showed that the level of binding enhancement through multimerization was not equivalent across patient samples. Wheat germ agglutinin (WGA) reactive glycans on fibronectin and thrombospondin-5 were preferentially bound by multimers in pancreatic cancer patient samples relative to control samples, suggesting a cancer-associated change in glycan density that could be detected only through lectin multimerization. This strategy could lead to the more sensitive and informative detection of glycans in biological samples and a broader spectrum of lectins that are useful as analytical reagents.

Introduction

Lectins function in every organism to recognize and interact with specific glycan structures. This activity is important in cell-cell communication, immune system activities, protein structure and interactions, cellular metabolism, and other areas of biology¹. The glycan-binding ability of lectins makes them potentially useful as analytical reagents to detect specific glycan structures in biological samples², and many lectins have been isolated and used for that purpose. In contrast to other methods for analyzing glycans, such as chromatography, mass spectrometry, and enzymatic digestion, lectins can provide precise measurements of specific structures from minute quantities of biological sample. This capability is important for distinguishing changes in glycan structures between samples or conditions, which is fundamental to understanding the involvement of particular glycans in disease³. Lectins have been used successfully in a wide variety of formats^{1,4} such as histochemistry⁵⁻⁶, affinity electrophoresis⁷, immunofluorescence cell staining⁸, lectin

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arrays^{9–11}, and antibody^{12–13} and protein arrays^{14–15}, to characterize both normal and pathological glycosylation.

A valuable use of lectin-based assays is to detect target glycans on specific protein carriers^{3, 16–17}. For example, an immobilized antibody can capture a protein of interest, and the glycans on that protein may be probed using a variety of lectins (Figure 1). This information is useful because the glycosylation state of a protein may be critically important to its function or may be associated with a particular condition. The glycosylation state of many different proteins can be probed in parallel using antibody-lectin sandwich arrays (ALSA)¹². By spotting many different antibodies in a small array, a single incubation of a lectin can be used to examine the glycans on many different proteins. We have previously shown the value of this method for biomarker research: for example, the abundances of certain proteins may not change much between healthy and diseased populations, yet their glycosylation state does^{12–13}. Thus, measuring the glycans on specific proteins provides improved biomarker performance. We currently are applying this method to problems in the diagnosis and management of pancreatic and breast cancers. This approach could be for other cancers and diseases in which alterations to glycosylation is observed.

Although lectins and glycan-binding antibodies have experimental advantages for measuring glycans, not many are used routinely in biological research. Some glycan-binding proteins have a weak affinity for their target glycan, typically in the μM to mM range¹⁸, at least when used as a reagent outside the biological context. Weak affinity leads to poor sensitivity in an analytical assay; this is a serious limitation, because rarely is any glycan found at a high abundance in a biological sample.

In biology, lectins frequently rely upon multivalent interactions to effect their functions¹⁸. For example, the glycan ligands of a lectin may be tethered to a cell surface and clustered together in multiple interactions when bound by lectins. The galectin family of lectins uses a well-described system of forming lattices in order to induce downstream effects¹⁹. Bacterial and viral infections typically rely on multivalent interactions to achieve necessary binding strengths²⁰. Plant lectins also have multivalent forms that have increased binding to cell wall glycans relative to their monovalent forms. In such systems, the strength of any one protein-glycan interaction is not great, but linking several such interactions together increases the overall interaction strength.

Given these aspects of lectin-glycan interactions *in vivo*, it follows that the *in vitro* binding of such lectins to their target glycans may be enhanced by inducing multivalent interactions. Multivalent ligands have been extensively investigated for their effect on lectin binding and as potentially more potent inhibitors of lectin-glycan interactions. Synthetic versions of glycans with precisely controlled densities have revealed strong preferences of certain lectins for particular glycan presentations^{21–22}. Another approach to achieving multivalency is linking lectins together, as would be found on cell surfaces or in multimeric galectin structures¹⁹. Alterations to the valency and orientation of linked lectins could be used to achieve increased binding of properly-spaced glycan targets. This strategy was used to improve the *in vitro* binding of the influenza hemagglutinin (HA) protein to its sialic acid ligand in a glycan array assay²³. The HA proteins were recombinantly produced with a His-6 tag, and the spontaneously formed trimers of the His-labeled HA proteins were linked together with a goat antibody against the His tag. Two of the antibody-bound clusters were further joined through an anti-goat IgG antibody to form a final cluster of 12 HA proteins. Another approach used the physical admixture of separate lectins bound to either agarose or pressure resistant supports to facilitate the clustering effect and demonstrated up to a 10-fold enhancement of binding affinity by ellipsometry measurements in glycoproteins with glycan motifs complimentary to the lectin specificity²⁴. This approach was used to develop the

multiple lectin affinity chromatography approach (M-LAC) and used in studies of plasma glycoproteins^{25–26}.

The same type of strategy could be pursued other types of lectins. One way to cluster lectins would use the multiple binding sites of streptavidin, which has four subunits that each contains a binding site for biotin (Figure 1A). Therefore, by mixing biotinylated lectins with streptavidin, clusters of one to four lectins should be formed. This strategy was previously used to detect weak interactions between the human protein VIP36 and membrane sugar chains^{27–28}. Here we investigated whether this strategy results in increased glycan binding for certain lectins in in-vitro assays such as antibody-lectin sandwich arrays¹² (Figure 1B). We demonstrate the enhanced binding of particular lectins to purified glycoproteins and glycoproteins captured directly out of biological samples. Applied to the analysis of glycoprotein glycosylation in plasma samples, multimeric detection revealed differences between the cancer and control subjects that were not observable using monomeric detection.

Materials and Methods

Plasma Samples

Plasma samples from pancreatic cancer and healthy subjects were collected at the University of Pittsburgh Medical Center. All stage (I – IV) pancreatic cancer samples were collected. The pancreatitis patients were a mixture of chronic and acute and the healthy subjects had no evidence of pancreatic, biliary or liver disease. Both of pancreatitis and healthy subjects were classified as control in this study. Five serum samples from patients with breast cancer stage II and five from healthy individuals were provided by Dr. Samir Hanash at the Fred Hutchinson Cancer Center. All the samples at each site were processed in BD Vacutainer® K2 EDTA Plus Blood Collection Tubes (Becton Dickinson, Franklin Lakes, NJ) using a standard operating procedure based on the serum and plasma protocols from the Early Detection Research Network (available upon request). All samples were stored at –80°C and sent frozen on dry ice. Each aliquot had been thawed no more than three times before use. The study was conducted in strict compliance with the guidelines of local Institutional Review Boards.

Antibodies and Lectins

The antibodies and lectins were purchased from various sources (Table S-1). The antibodies to be printed onto microarray slides were purified by dialysis (Slide-A-lyzer, Pierce Biotechnology, Rockford, IL) to phosphate buffered saline (PBS) and ultracentrifuged, and their concentrations were adjusted to 250 µg/ml. Biotinylation was performed using the EZ-Link-sulfo-NHS-LC-Biotin kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. The Cy3 labeling of the Anti-biotin antibody was performed according to kit instructions (Cy3 NHS Ester, #11020, Lumiprobe, Hallandale Beach, FL).

Microarray Fabrication

The antibodies and proteins (Table S-1) were spotted onto Nexterion® Slide H (Schott North America Inc., Louisville, KY) or PATH® microscope slides (Grace Bio-Labs, OR) using a robotic microarrayer (2470, Aushon Biosystems, Billerica, MA), at a concentration of 250 µg/mL in 1X phosphate-buffered saline with 0.05% Tween-20 (PBST0.05). The serial dilutions of the proteins were performed with 250 µg/ml BSA PBST0.05 in order to keep the total protein concentration constant. Each antibody or protein was printed with six replicate spots within each array, and each microscope slide contained 48 identical arrays arranged in a 4×12 grid with a 4.5 mm spacing between arrays. A wax-based hydrophobic boarder was imprinted on the slide to define boundaries between the arrays (SlideImprinter, The Gel

Company, San Francisco, CA). The printed slides were stored at 4 °C in a desiccated, vacuum-sealed slide box until use.

Microarray Assays

The antibody microarray assays with Slide H was adapted and modified from the protocol described previously^{12, 29–30}. Briefly, plasma samples were diluted two-fold into a sample dilution buffer with the final concentrations of 1X PBS, 0.1% Tween-20, 0.1% Brij-35 (Thermo Scientific, Rockford, IL), 1X IgG blocking cocktail (100 µg/mL each for mouse, sheep, and goat IgG, 200 µg/mL rabbit IgG in 1X PBS, antibodies from Jackson ImmunoResearch), and 1X protease inhibitor (Complete Tablet, Roche Applied Science, Indianapolis, IN) and stored at 4 °C overnight with gentle agitation. The overnight incubation allows the blocking antibodies in the sample buffering to be bound by any species-specific immunoglobulin in the plasma samples (for example, anti-mouse IgG antibodies in human plasma that bind the mouse antibodies in the blocking buffer). Unless otherwise stated, all the following steps were conducted at room temperature. The next day, the Slide H microarray slides were deactivated in Slide H deactivation buffer (25 mM ethanolamine in sodium borate buffer, pH 9.0) in a Coplin jar for 1 hour, washed in three changes of PBST0.05 for 3 min each, and dried by brief centrifugation at 160 × g. The PATH microarray slides (used in experiments with spotted proteins) were blocked in 1% Bovine Serum Albumin (BSA, Fisher Scientific, Fair Lawn, NJ) in PBST0.5 for 1 hour. For slides to be detected with AAL, chemical derivatization of the antibodies was performed as previously described¹² to prevent lectin binding to the capture antibodies.

Six µL of each plasma sample was applied to an array and incubated for 1 hour. (The experiments involving lectin detection of spotted glycoproteins had no plasma incubation step but were otherwise equivalent.) The primary lectin detection and secondary streptavidin-phycoerythrin (SA-PE) detection reagents were prepared in PBST0.05 separately (for the non-multimer protocol) or mixed together and incubated on ice for at least 1 hour (for the multimer protocol). For the sugar competition experiments, the primary lectins (either in the multimer or the non-multimer format) were pre-incubated for one hour on ice with 0, 100-fold, or 200-fold molar excess of N,N'-Diacetylchitobiose (Carbosynth 35061-50-8, Compton, UK) or D-mannose (Acros Organics 3458-28-4, Fair Lawn, NJ). After the plasma sample incubation, the slides were washed three times in PBST0.05 and spin-dried. In the non-multimer protocol, the slides were incubated with the primary and secondary detection reagents sequentially for 1 hour each, with three washes between steps, and in the multimer protocol, the slides were incubated with the pre-mixed primary and secondary reagents for 1 hour, followed by the wash and dry steps. Lastly, all the slides were scanned for fluorescence at 532 nm at 10 µm resolution using a microarray scanner (LS Reloaded, TECAN, NC), and the resulting images were saved in TIFF format.

Analysis

The images were quantified and analyzed with the software GenePix Pro 5.0 (Molecular Devices, Sunnyvale, CA), using both automatic and manual spot finding features. The local background was subtracted from the median intensity of each spot. Outlier spots among the six replicates for each antibody were identified using the Grubb's test and removed, and the geometric mean was calculated from the remaining replicate spots. In the dilution curves of pooled pancreatic cancer plasma samples, the limit of detection (LOD) was determined by the equation: $Y_{LOD} = Y_{blank} + (3 * \text{standard deviation of } Y_{blank})$, where Y_{LOD} is the fluorescent signal at the LOD, and Y_{blank} refers to the signal measured in a negative control. Here, the negative control was an array that was incubated with PBS instead of sample. The LOD in sample dilution folds can be extrapolated using the best fitted four-parameter curves.

The Student's *t* test was calculated using Microsoft Excel. The area under the curves (AUCs) were calculated from the receiver operating characteristic (ROC) analysis using SigmaPlot 10 (Systat Software, San Jose, CA). The Box Plots were generated with OriginPro 8 (OriginLab, Northampton, MA), and the figures were prepared in Canvas XII (ACD Systems, Saanichton, Canada).

Results

Formation of Lectin Multimers

We used streptavidin-biotin interactions to investigate the effects on glycan binding of linking together multiple lectins (Figure 1). Streptavidin (SA) has four identical biotin binding sites and thus in principle could link up to four biotinylated lectins. To establish that such multimers are formed and to determine the optimal molar ratio between the molecules, we measured the binding of SA to a biotinylated protein after pre-incubating the SA with varying amounts of biotinylated lectin (Figure 2A). SA conjugated to phycoerythrin (referred to as SA-PE) was used to enable detection of bound SA-PE by fluorescence scanning. SA-PE binding to the spotted protein was significantly reduced at a lectin:SA-PE ratio of 4:1 for each lectin, with nearly complete loss at 8:1 (Figure 2B), confirming occupancy of the SA binding sites by the biotinylated lectin. No loss in SA-PE binding was observed using the non-multimer method of sequential incubation (Figure 2B).

An alternative way to detect biotinylated lectins is to use a fluorophore labeled anti-biotin antibody, which has two biotin binding sites and could be used to link two lectins. The pre-incubation of Cy3-labeled anti-biotin with biotinylated WGA partially blocked binding to the spotted, biotinylated protein at a lectin:anti-biotin ratio of 1:1 and almost completely blocked binding at a ratio of 2:1. No change in binding was observed using the standard, sequential protocol (Figure 2B), confirming the formation of lectin:anti-biotin complexes.

Lectin-Glycoprotein Binding using Multimers

Next we determined whether lectins that were pre-conjugated to SA or anti-biotin antibody had enhanced glycan binding activity for particular glycoproteins. The binding was investigated using microarrays of spotted glycoproteins (fibronectin, laminin, and haptoglobin) that were probed with various lectins in multimeric or non-multimeric (sequential) form. We confirmed multimer formation for each lectin through the loss of streptavidin binding to a biotinylated control protein (not shown). The eight lectins tested (WGA, AAL, RCA-1 PHAE, PHAL, GSL-1, EEL and PTL-1) showed divergent behaviors using multimerization (Figure 3 and Figure S-1). WGA showed significantly increased signal and sensitivity for all proteins; RCA and PHAE had moderately enhanced detection; PTL-1 and EEL has similar behavior between the two methods; and AAL, GSL-1, and PHAL showed lower signals using the multimer method. For example, WGA detected the spotted proteins down to 3.9 $\mu\text{g/mL}$ using the multimer method, compared to 8–31 $\mu\text{g/mL}$ using the non-multimer method, representing. This result shows the potential for enhanced binding using multimerization but also indicates that the method is not compatible with certain lectins, such as AAL. The use of anti-biotin antibodies for linking biotinylated lectins (Figure 2) gave similar results to those obtained using streptavidin-based complexes among the same eight lectins tested above (not shown), suggesting no fundamental difference between those methods of forming multimers.

The multimer-based binding with WGA and RCA-1 was further tested by diluting the detection reagents, rather than the target glycoproteins³¹. The multimer-based WGA detection of fibronectin, laminin and haptoglobin showed higher fluorescence signals at each dilution (Figure S-2), and multimer-based RCA-1 showed similar signals at each dilution.

(We were not able to calculate dissociation constants because binding was not saturated at the maximum lectin concentrations.) These results indicate that the multivalent binding interactions were well maintained and stable at decreased multimer concentrations.

Next we tested whether multimerization provides an advantage for detecting proteins captured out of biological solutions. Serial dilutions of plasma samples were incubated on antibody arrays, and the captured proteins were probed by WGA using both the multimer and non-multimer methods. Highly increased signals were observed on anti-tenascin-X, anti-fibronectin and anti-thrombospondin-5 using the multimer protocol (Figure 4), and increased signals were observed on all the remaining capture antibodies except for anti-MUC5AC (data not shown). A signal increase from 4-fold to 40-fold was observed at the highest plasma concentration, and the limit of detection was reduced. For example, the LOD for fibronectin was at the 512-fold plasma dilution using the multimeric WGA detection, compared to 8-fold plasma dilution using the non-multimer method. Given an average plasma concentration of 500 $\mu\text{g/mL}$ for fibronectin, the limit of detection is reduced from ~ 40 to ~ 1 $\mu\text{g/mL}$. Thrombospondin-5 and tenascin-X are minimally detectable at the 2048-fold dilution using the multimer method. Based on typical plasma concentrations of 1 $\mu\text{g/mL}$ for thrombospondin-5³² and 0.5 $\mu\text{g/mL}$ for tenascin-X³³, the detection limits are approximately 0.5 and 0.25 ng/mL , respectively. No such benefit from multimer formation was observed using AAL (not shown), indicating consistency between detection of spotted glycoproteins and detection of proteins captured by antibodies.

We sought to determine whether the specificity of WGA for its primary carbohydrate ligand, N-acetylglucosamine (GlcNAc), remained intact using the multimer method. Competition assays were performed in which WGA was pre-incubated with a 0-fold, 100-fold, or 200-fold molar excess of a competing sugar, chitobiose (the dimer form of GlcNAc), or a non-specific sugar, mannose. Mannose pre-incubation failed to block WGA binding to fibronectin in either the multimer or non-multimer method, but chitobiose specifically reduced WGA binding in both methods (Figure S-3). This result confirmed the maintenance of WGA specificity for GlcNAc after multimer formation with SA-PE.

To investigate the generality of improvement in WGA binding using multimers, lectin Western blots were performed with serially diluted plasma samples from pancreatic cancer patients, which are rich with secretory glycoproteins. Substantially increased signal was observed using multimeric detection (Figure S-4), indicating a benefit for other assay types and for denatured proteins in addition to native proteins.

Detection of Disease-Associated Glycosylation Using Multimers

We tested the possibility that multimerization could not only provide increased signals but also reveal differences in glycosylation between patients that were not apparent using the non-multimer method. Plasma samples from pancreatic cancer patients ($n = 10$) and healthy control subjects ($n = 10$) were incubated on antibody arrays and probed by WGA and AAL using both methods. The arrays were spotted with 11 different capture antibodies, targeting seven unique proteins and one glycan structure (the CA 19-9 antigen), as well as positive and negative control antibodies (Tables S-1 and S-2). Consistent with the above observations, WGA binding to fibronectin, thrombospondin-5, and tenascin-X were significantly increased for all samples using the multimer method (Figure 5A and Table S-2). It was interesting that multimer signals were significantly lower than that of non-multimer at the antibodies targeting the mucin protein MUC16 and CA 19-9 (a glycan antigen found on multiple proteins) and equivalent or slightly lower at the antibodies targeting the mucins MUC1 and MUC5AC. This finding indicates that mucin proteins, which are long, heavily O-glycosylated proteins, may present their glycans in a way that is not amenable to detection by WGA multimers. WGA binding to the spotted capture

antibodies targeting CEA also was significantly higher with the non-multimer method (Table S-2), perhaps indicating the same relationship for the glycans on that protein. Detection with AAL also was consistent with the above results, giving lower signals using the multimer method. The discrimination of cancer from control specimens was unchanged using AAL multimers (not shown).

In addition to increased signals, WGA binding to fibronectin showed increased differences between the cancer subjects and the controls using the multimer method. Certain cancer samples had clearly elevated levels, relative to the controls, that were observed only using the multimer method (Figure 5A), resulting in a significant difference between the groups and increased area-under-the-curve in receiver-operator-characteristic analysis (Figure 5B and Table S-2). The elevations were not simply due to increased fibronectin protein concentrations in particular samples, as the detection of fibronectin protein levels using an antibody sandwich assay showed statistically equivalent protein levels between the cancer patients and the control subjects (Figure S-5). (Previous studies also have never found increased plasma fibronectin levels in pancreatic cancer patients.) In addition, the fibronectin protein levels did not correlate with the glycan levels as detected by WGA (Figure S-5), indicating that fibronectin glycosylation varies independently of protein concentration.

WGA binding to thrombospondin-5 also revealed disease-associated glycosylation using only the multimer method (Figure 5A), with a corresponding increase in AUC (Figure 5B). WGA binding to tenascin-X showed increased signals but no uncovering of disease-associated glycosylation (Figure 5A) and equivalent AUCs (Figure 5B). An additional comparison of plasma samples from breast cancer patients ($n = 4$) to control subjects ($n = 5$) also showed higher WGA binding to tenascin-X in all samples using the multimer method (Figure S-6). In this case, a higher binding of WGA to tenascin-X in the control subjects, relative to the cancer subjects, was more clearly observed using the multimer method.

These results show that particular patterns of glycosylation that occur in selected patients may be optimally detectable using a multimer method, potentially enhancing the detection of disease-associated glycosylation. The possibility exists that the significant differences between the cancer and control samples observed with fibronectin and thrombospondin-5 are due to chance rather than a cancer-derived alteration. The false discovery rate among 11 independent observations (11 unique antibodies were tested) would be 0.55 observations (11×0.05 , using $\alpha = 0.05$ for rejection of the null hypothesis), which is less than our two observations. Also, when we randomly permuted the samples into “case” and “control” groups 10 separate times, no markers showed significance in any of the groupings (data not shown). These analyses support a real difference between the cancer and control samples in fibronectin and thrombospondin-5 glycosylation as detected by WGA multimers.

Discussion

Lectins are powerful tools for probing the abundance and localization of specific glycans, but certain lectins have weak affinity when used *in vitro*, resulting in limited usefulness as analytical tools. Here we investigated the potential for increased binding strength through lectin multimerization, based on the concept that increased valency can be achieved by linking the interactions of multiple glycans with multiple lectins. We used a practical system for linking lectins, taking advantage of the strong interactions of up to four biotinylated lectins with a single streptavidin molecule. We showed that certain lectins, particularly WGA, have greatly increased binding using multimerization for most glycoproteins tested, while others have equivalent or weaker interactions. Furthermore, we showed that the increase in binding was not equivalent between patient samples, but that a proportionally

higher increase was observed in cancer patients for the detection of WGA-reactive glycans on fibronectin and thrombospondin-5. Statistically-significant differences in protein glycosylation between the cancer and control subjects were revealed only with the multimer method.

The presumed mechanism for increased binding using multimers is an increased number of glycan-lectin interactions leading to higher valency. This mechanism depends upon both the glycans and lectins being in the right orientation to allow simultaneous binding. The glycans need to appear in a repeating fashion either along the same protein or on closely spaced proteins, and the lectins need to direct their binding sites towards the presented glycans. It is reasonable to assume that such a relationship would not exist for every glycan-lectin relationship, especially in an *in-vitro* assay, which accords with our observation that the effect of multimerization was variable between lectins and glycoproteins. The development of a theoretical understanding of our current observations would help predict the behavior of other lectins.

Some clues about the factors influencing our observations could be gained by comparing many of the properties of the lectins (Table S-3). The greatest increase occurred with WGA. The WGA target glycan, N-acetylglucosamine, especially as found in lactosamine (galactose β 1,4 N-acetylglucosamine), is a common motif that is found on nearly all extended N-glycans and O-glycans, which is consistent with the availability of multiple target glycans in the proper orientation for multivalent binding. The proteins MUC16 and CEA showed decreased WGA binding (Table S-2), perhaps indicating a fundamental difference between the glycans of these protein and the other glycoproteins. The proteins showing improvement are matrix or matricellular proteins which may tend to aggregate more than MUC16 and CEA, which are cell surface proteins. MUC16 is heavily O-glycosylated and may have shorter glycans than typical glycoproteins with heavily N-glycosylation, perhaps resulting in less accessibility to WGA when multimerized. Availability of the glycan does not solely account for our observations, though, since AAL, which showed decreased binding upon multimerization, binds a glycan (fucose) that is typically easily accessible on the outer arm of glycans.

The biophysical and structural properties of the lectins (basic information summarized in Table S-3) likely account for much of their *in vitro* behavior. WGA is the smallest protein among those tested, in solution typically existing as a 36 kDa heterodimer, although tetramers also can form. The smaller size of WGA might allow effective organization around the 60 kDa streptavidin molecule or proper insertion between neighboring glycans. The larger RCA-1 and PHAE lectins showed potentially improved binding using multimers, indicating that small size is not required. WGA also has the fewest biotinylation sites, with 8 lysine residues per subunit, compared to 9–13 lysines per subunit for the other lectins, which may reduce the likelihood of interference of glycan binding by the streptavidin interaction. Another favorable feature of WGA is the multiple glycan binding sites per subunit. Because biotinylation sites are normally located at various sites on molecular surfaces, the attachment of biotinylated molecules to streptavidin results in diverse orientations. Some of these orientations may hide certain glycan binding sites, but if multiple glycan binding sites exists, some will be available regardless of the orientation. The PHAE tetramer has four glycan binding sites, two each on opposite sides of back-to-back dimers of legume type, “jelly roll” folds, which may facilitate glycan binding in any orientation. PHAL has this structure but showed decreased binding upon multimerization. The difference may be due to the nature of its glycan targets, since both PHAL and PHAE showed contrasting effects of multimerization among the protein targets (Figs. 3 and S-1). RCA-1 likewise has four well-separated glycan-binding domains in its tetrameric structure, facilitating access in any orientation. AAL has a six-fold beta-propellor structure, in the shape of a ring, with five

fucose binding sites on the edges of the ring^{34–35}. Such a structure may not be conducive to streptavidin-based linking. Thus the effects of multimerization need to account for the structural characteristics of the lectin, the mode of multimerization, and the characteristics of the bound glycans.

A study by Dam and Brewer provides insights into the energetics involved in matching lectin orientation to glycan clustering³⁶. In their model, the best improvement of lectin affinity to glycoproteins occurs face-to-face interactions, in which multiple binding sites of a lectin multimer are aligned in a common direction and bind to glycoproteins with clustered epitopes. Under a contrasting scenario, a tetramer-shaped lectin, which resembles the streptavidin-lectin multimer complex in our study, displays a much lower affinity to linear glycoproteins. The tetrameric lectin with binding sites in four directions would have lower binding efficiency to linear glycan epitopes. In our study, some of the glycoproteins captured on the 2-dimensional planar surface may have given an unfavorable interaction face relative to the SA-tetramerized lectins with binding sites in three dimensions. In this case, a non-multimerized lectin might have an equal or better chance to engage the glycans.

Considering the above factors, it will be valuable to test other multimerization methods for potentially better lectin orientation in certain cases. In this work we demonstrated the potential for multimerization using anti-biotin (Figure 2), which may be better than SA in certain situations due to the high flexibility of the globular arms of IgG molecules. It may be possible to achieve improved effects using a polymer with linkage sites for a protein at some periodic interval along the polymer backbone^{37–39}. Proteins could be attached to the backbone using a C-terminal or N-terminal fusion tag, which may provide more consistent orientation than the variable biotinylation sites used here. Given a flexible system, various spacings could be tested to determine the best spacing for each lectin. The level of improvement would depend on the organization of the target glycans and the structural ability of the lectin to properly bind adjacent glycans. Therefore, no protocol will be optimal for every lectin; rather, each lectin will require individual testing and optimization using a variety of multimer and non-multimer protocols.

Additional experimental information about the conditions that result in increased binding using multimers could be pursued using glycan arrays^{40–44} or glycopeptide arrays⁴⁵, especially arrays which contain each glycan at different densities²¹. In previous work, various amounts of glycan structures were covalently attached to the lysine residues of BSA to achieve a range of glycan densities, from which the preferential binding of certain lectins and antibodies to higher specific glycan densities could be observed²¹. Another approach to varying glycan density is to mix various concentrations of a non-glycosylated protein with a fixed amount of glycosylated protein²². These types of arrays may reveal unexpected effects. For example, the lectin *bauhinia purpurea* was found to switch carbohydrate ligands as a function of the density of glycan-modified, self-assembled monolayers⁴⁶. We plan to determine if similar effects can occur when lectins are linked together.

An unexpected finding was the increased differentiation of cancer from control subjects using WGA multimer detection of fibronectin. This effect may be due to altered density of the WGA ligands along the fibronectin backbone. For example, the total number of ligands might be similar between the cancer and control subjects, but in the cancer subjects they might be more closely spaced. Thus instead of observing equivalent signal increases across all patients, an extra increase would be observed in the cancer samples using multimer detection. Another possibility is changed heterogeneity in the glycans, which has been shown to affect lectin specificity⁴⁷ and may particularly affect multimeric lectins. In any case, the increased differentiation of cancer from control subjects points to a change in glycan presentation, rather than abundance, which particularly favors detection by

multimers. Previous research found increased poly(lactosamine) on fetal fibronectin relative to plasma fibronectin⁴⁸. Increased poly(lactosamine) could be responsible for our increased multimer binding and could result from the reactivation of developmental pathways in cancer⁴⁹. The nature of the glycan alterations leading to decreased WGA binding to tenascin-X likewise is not known, but these observations are consistent with previous studies showing differential binding of tenascin-X to a multiple lectin affinity column (M-LAC) as well as a pI shift in subsequent fractionation of the M-LAC fractions⁵⁰. Further studies could include characterizing the glycans on fibronectin and tenascin-X collected from cancer and control samples and to map the densities of glycosylation along the polypeptide backbone.

Conclusions

This work shows that multimerization can enhance the binding of particular lectins to their targeted glycans. The use of this method could result in an increased ability to observe and measure specific glycan structures in biological samples, particularly those at low abundance. Currently only a handful of lectins are widely used as analytical reagents due to the weak affinity of most lectins, which limits the repertoire of glycans that can be probed. The increased affinity of certain lectins could broaden the range of glycan structures that may be routinely probed by lectins. In addition, multimerization may provide fundamentally different information than monomeric detection, as it may reveal changes in glycan presentation or density—features that are extremely difficult to probe using conventional glycobiology methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Lectin multimers for the detection of glycoproteins captured on antibody arrays
 (A) The pre-incubation of biotinylated lectins with streptavidin leads to multimer complex formation. (B) Biological samples are incubated on antibody arrays to allow protein capture by the immobilized antibodies. The glycans on the captured proteins are then probed using lectins. In the conventional non-multimer method, the primary and secondary detection reagents are added sequentially, but in the multimer method, the pre-formed multimer complex is added in a single step, potentially achieving enhanced binding through multivalent interactions.

Figure 2. Optimizing lectin:streptavidin/anti-biotin molar ratios

(A) The binding of streptavidin-phycoerythrin (SA-PE) or anti-biotin to a biotinylated control protein (here depicted as an antibody) is expected to be prevented by the pre-incubation of biotinylated lectins with the SA-PE or anti-biotin. (B) The signal at the biotinylated control protein (non-specific sheep IgG) was quantified after detection at various SA-PE:lectin or anti-biotin:lectin molar ratios, both using sequential (non-multimer) and multimer detection. The images show representative raw data from the control protein spots. The quantified data represent averages from six replicate spots for AAL and SA-PE detection (left), WGA and SA-PE detection (middle), and WGA and anti-biotin detection (right). The SA-PE and anti-biotin concentrations were fixed at 3 $\mu\text{g}/\text{mL}$, and the concentrations of biotinylated AAL and WGA were varied to give the indicated molar ratios.

Figure 3. Detection of purified glycoproteins with the multimer and non-multimer protocols
 Four different purified glycoproteins were serially diluted into 250 µg/mL BSA (keeping total protein concentration at 250 µg/mL), printed in microarrays, and detected with four different lectins using either the multimer or non-multimer protocol. The fluorescence signal is the mean of five replicate arrays, each containing six replicate spots per protein.

Figure 4. Detection of proteins captured from plasma samples using the multimer and non-multimer protocols

Plasma samples from pancreatic cancer patients (n=10) were pooled, serially diluted with 2X sample buffer, and detected by WGA:SA-PE at a 4: 1 molar ratio (SA-PE at 3 μ g/ml) using either the multimer or non-multimer protocol. The fluorescence values are the means of triplicate arrays, with each array containing six replicate spots per antibody. The solid lines represent the best four-parameter fits. The insets show representative raw data for each of the spotted antibodies.

Figure 5. Enhanced discrimination of patient groups using multimer detection

Individual plasma samples from pancreatic cancer patients ($n = 10$) and control subjects ($n = 10$) were incubated on antibody arrays and detected by WGA using either the multimer or non-multimer protocol. (A) The signals at each of the three indicated capture antibodies were quantified for each sample. Each data point is the average signal from three replicate array for an individual sample. The box gives the upper and lower quartiles, the vertical lines define the signal range, and the horizontal lines mark the median values. (B) The receiver-operator characteristic curves are plotted for the signals at each of the three capture antibodies. AUC, area under the curve.