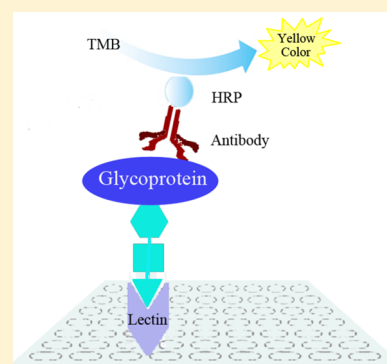


# Analysis of Glycan Variation on Glycoproteins from Serum by the Reverse Lectin-Based ELISA Assay

Jing Wu,<sup>†</sup> Jianhui Zhu,<sup>†</sup> Haidi Yin,<sup>†</sup> Ronald J. Buckanovich,<sup>‡</sup> and David M. Lubman<sup>\*,†</sup><sup>†</sup>University of Michigan, Department of Surgery, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109, United States<sup>‡</sup>University of Michigan, Department of Internal Medicine, 1500 East Medical Center Drive, Ann Arbor, Michigan 48109, United States**S** Supporting Information

**ABSTRACT:** Altered glycosylation in glycoproteins is associated with carcinogenesis, and certain glycan structures and glycoproteins are well-known markers for tumor progression. To identify potential diagnostic candidate markers, we have developed a novel method for analysis of glycosylation changes of glycoproteins from crude serum samples using lectin-based glycoprotein capture followed by detection with biotin/HRP-conjugated antibodies. The amount of lectin coated on the microplate well was optimized to achieve low background and improved S/N compared with current lectin ELISA methods. In the presence of competing sugars of lectin AAL or with sialic acid removed from the glycoproteins, we confirmed that this method specifically detects glycosylation changes of proteins rather than protein abundance variation. Using our reverse lectin-based ELISA assay, increased fucosylated haptoglobin was observed in sera of patients with ovarian cancer, while the protein level of haptoglobin remained the same between cancers and noncases. The combination of fucosylated haptoglobin and CA125 (AUC = 0.88) showed improved performance for distinguishing stage-III ovarian cancer from noncases compared with CA125 alone (AUC = 0.86). In differentiating early-stage ovarian cancer from noncases, fucosylated haptoglobin showed comparable performance to CA125. The combination of CA125 and fucosylated haptoglobin resulted in an AUC of 0.855, which outperforms CA125 to distinguish early-stage cancer from noncases. Our study provides an alternative method to quantify glycosylation changes of proteins from serum samples, which will be essential for biomarker discovery and validation studies.

**KEYWORDS:** reverse lectin-based ELISA, glycosylation, biomarkers, ovarian cancer

**INTRODUCTION**

Glycosylation is a posttranslational modification that has significantly contributed to protein–protein interactions, cellular recognition, and, in particular, cancer development and progression.<sup>1,2</sup> Increasing evidence has indicated that abnormal posttranslational modifications are associated with cancer progression and that potential biomarkers may be identified based on their changes in protein modifications.<sup>3,4</sup> Glycosylation changes in serum proteins have been reported to contribute to the progression of various cancers, including pancreatic,<sup>5</sup> ovarian,<sup>6</sup> hepatocellular,<sup>7</sup> and breast cancer.<sup>8</sup> Therefore, analysis of glycosylation changes of serum glycoproteins may provide a promising strategy to identify new diagnostic biomarkers.

Methods to identify and quantify variations in glycosylation in complex biological samples have been widely established, which are mainly based on the removal of glycans by enzymatic digestion, followed by chromatographic separation and mass spectrometry analysis.<sup>9,10</sup> These methods can provide detailed information about glycan structures but are not suitable for analyzing a large number of biological samples. Because of the lack of glycopeptide standards, SRM/MRM assays, which have been widely used to quantify protein level changes, are not optimal for quantifying glycosylation changes of target proteins.

Because a number of glycosylated proteins in serum have been identified as promising candidate biomarkers, an improved method for quantification of glycosylation changes of proteins from original sera is needed.

Recently, lectin-based antibody microarrays and lectin-ELISA assays have been developed to analyze glycosylation changes of proteins from serum samples.<sup>11–13</sup> The lectin-based antibody microarrays and the lectin-ELISA assay are both based on coating antibodies to the slides or 96-well plates, where the glycans on the antibodies need to be oxidized by sodium periodate ( $\text{NaIO}_4$ ), followed by derivatization with MPBH and dipeptide solution (Cys-Gly). Unfortunately, periodate oxidation can diminish or inactivate antibody immunoreactivities,<sup>14</sup> which may increase nonspecific binding and lead to inaccurate quantitative results. Furthermore, precipitates are formed during the process of oxidation with  $\text{NaIO}_4$ , which can increase background if not completely removed.<sup>15</sup>

Herein, we have developed an alternative method, a reverse lectin-based ELISA assay to quantify glycosylated proteins from crude serum samples without the oxidization and derivatization of glycans on the antibodies. Because fucosylation/sialylation

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Table 1. Characteristics of the Patients

	normal ( <i>n</i> = 15)	benign ( <i>n</i> = 12)	stage I/II ( <i>n</i> = 21)	stage III ( <i>n</i> = 29)
median age (range, <i>y</i> )	59 (43–74)	61 (16–87)	53 (24–71)	61 (41–78)
histology				
serous	n/a <sup>a</sup>	9	21	25
endometrioid	n/a	0	0	0
other <sup>b</sup>	n/a	3	0	4
grade <sup>c</sup>				
1/2	n/a	n/a	18	5
3	n/a	n/a	2	17

<sup>a</sup>Abbreviation: n/a, not applicable. <sup>b</sup>Fibroids and ovarian thecoma for benign diseases; poorly differentiated adenocarcinoma and fallopian tube carcinoma for ovarian cancer. <sup>c</sup>For some ovarian cancer patients, the grade information was not available.

changes have been reported in a number of cancers and are regarded as promising targets of cancer diagnosis and therapy,<sup>16,17</sup> in this study, we mainly focused on optimizing the reverse lectin-based ELISA assay to quantify fucosylation/sialylation changes of target proteins in ovarian cancer. The reverse lectin-based ELISA assay is based on coating lectin on the microtiter plate followed by detection of captured glycoproteins with biotin/HRP-conjugated primary antibodies. The concentration of lectins coated on the 96-well plate was optimized to achieve a low background compared with the current lectin-ELISA format. With this method, we confirmed the increased expression of fucosylated haptoglobin in ovarian cancer, especially in early-stage ovarian cancer. Our findings suggest that fucosylated haptoglobin could be a promising candidate biomarker, which can supplement the clinically used biomarker CA125 (cancer antigen 125) to detect ovarian cancer with improved sensitivity and specificity.

## MATERIALS AND METHODS

### Serum Samples

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The set of serum samples used in this study comprised 15 healthy controls, 12 benign diseases, 21 early-stage ovarian cancers, and 29 late-stage ovarian cancers. All of the healthy controls were provided by the Great Lakes-New England CVC (EDRNLNE). Serum samples from 12 benign diseases and 17 late stage ovarian cancers were collected preoperatively at the University of Michigan as part of an IRB-approved tumor banking protocol, while the other samples were obtained from ProteoGenex (Manhattan Beach, CA). All specimens were processed using the same protocol. A summary of clinical data is given in Table 1.

### Developing Reverse Lectin-Based ELISA Assay

A reverse lectin-based ELISA assay was developed to analyze glycosylation changes of proteins from crude serum samples based on the differential binding of glycoproteins to their specific lectins. One hundred microliters of lectin (1.25, 2.5, 5, 10, 50, 100  $\mu\text{g}/\text{mL}$  AAL or 1, 5, 10, 50  $\mu\text{g}/\text{mL}$  SNA) was added to each well of a 96-well ELISA plate (Thermo Scientific, IL) and incubated at 37 °C for 2 h. After the lectin solution was removed, the plate was washed five times with PBST (0.1% Tween-20 in PBS). The plate was then blocked with 3% BSA in PBST for 1 h. One hundred microliters of each 200-fold diluted serum sample or 2  $\mu\text{g}$  of purified protein was applied to each well of a 96-well ELISA plate. After 1 h of incubation, the plate was washed with PBST five times to remove unbound proteins. One hundred microliters of biotinylated/HRP-conjugated

antibodies (1:1000 for antihaptoglobin, and 1:50 000 for anti-IgG) purchased from Abcam (Cambridge, MA) was added to bind with their corresponding antigens. TMB working solution was added to each well, followed by stop solution. The absorbance values were read on a microplate reader (BioTek, Synergy HT) at a wavelength of 450 nm.

### Lectin-Blot for Haptoglobin and IgG

Human full-length proteins of haptoglobin and IgG were purchased from Abcam (Cambridge, MA). Five micrograms of the proteins was separated by SDS-PAGE. The resolved proteins were then transferred onto a PVDF membrane (Bio-Rad). The membrane was blocked by 3% nonfat milk in PBST (0.1% Tween-20 in PBS) for 1 h. The membrane was probed with 0.5  $\mu\text{g}/\text{mL}$  biotinylated AAL or biotinylated SNA to bind with their preferred oligosaccharides. HRP-conjugated streptavidin (1  $\mu\text{g}/\text{mL}$ ) was then added, and the blot was detected by DAB detection kits.

### ELISA Assay

The protein abundances of HAP, IgG, and CA125 in sera of ovarian cancer and control groups were measured by ELISA assay. ELISA kits for HAP, IgG, and CA125 were all purchased from Genway (San Diego, CA).

### Statistical Analysis

All statistical analyses were performed using SPSS 11.5. Statistical differences were determined using Wilcoxon rank-sum test. For all statistical comparisons,  $p < 0.05$  was taken as statistically significant. Receiver operating characteristic (ROC) curves were produced in terms of the sensitivity and specificity of markers at their specific cutoff values. Multivariate analysis was also done by logistic regression to find the best-fitting multivariate model for each comparison group.

## RESULTS AND DISCUSSION

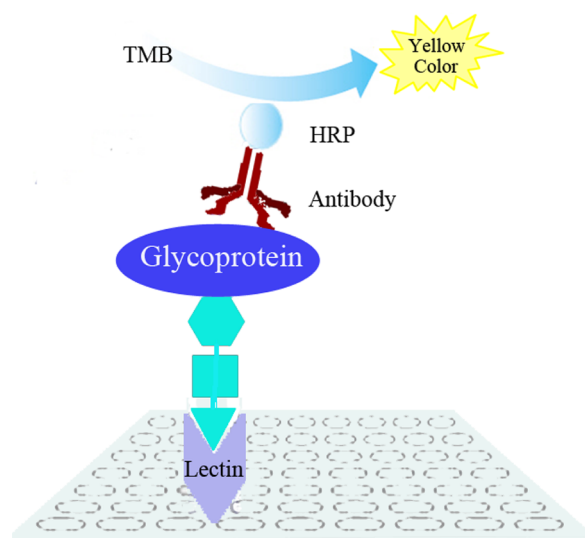
### Determining the Presence of Fucosylation/Sialylation of Haptoglobin and IgG by Lectin-Blot

Using the reverse lectin-based ELISA assay, we found high responses of haptoglobin and IgG to AAL or SNA. To exclude false-positives, we used lectin-blot to verify the presence of fucosylated/sialylated glycans on haptoglobin and IgG. AAL is a lectin that responds to fucose linked ( $\alpha$ -1,6) to *N*-acetylglucosamine or to fucose linked ( $\alpha$ -1,3) to *N*-acetylglucosamine related structures, while SNA responds to sialic acid attached to terminal galactose in  $\alpha$ -2,6 or  $\alpha$ -2,3 linkage. As shown in Supplemental Figure S1 in the Supporting Information, the fucosylated/sialylated haptoglobin and IgG were detected by the AAL-blot and SNA-blot. The results confirmed the

presence of fucosylated/sialylated glycans on the haptoglobin and IgG, which had responses to AAL/SNA detection.

### Optimizing Reverse Lectin-Based ELISA Assay Conditions

The workflow of the reverse lectin-based ELISA assay is shown in Figure 1. In this experiment, a number of control



**Figure 1.** Diagram of reverse lectin-based ELISA assay for the analysis of glycosylation of target glycoproteins.

determinations with the removal of serum or lectin from the mixture of reverse lectin-based ELISA assay were tested to avoid potential interfering factors. To reduce the background caused by nonspecific binding of serum proteins or biotinylated/HRP-conjugated antibodies to the 96-well plate, a buffer containing 1% BSA is used for the dilution of the serum and antibodies. In addition, we also measured the absorbance values when lectin, serum, or antibody was omitted from the reverse lectin-based ELISA assay. To measure sialylated haptoglobin by reverse SNA-based ELISA assay, low absorbance values were obtained when lectin or serum was removed from the assay. The results indicate that there was no nonspecific binding of serum proteins to the plate, and the glycans on the antibodies had no effect on detection of the sialylated haptoglobin. To measure fucosylated haptoglobin by reverse AAL-based ELISA assay, low absorbance values were obtained when lectin was omitted from the assay, while a slightly higher absorbance value was obtained when serum was left out of the assay, indicating the presence of fucosylated glycans on the antibody. The S/N ratio of the reverse AAL-based ELISA assay for detecting fucosylated haptoglobin is still higher than 3, as shown in Supplemental Table S1 in the Supporting Information. Therefore, the low binding of AAL to the glycans on the antibodies did not influence the quantification of fucosylated haptoglobin from the serum samples.

Because of the high abundance of IgG in the serum, we observed absorbance values when lectin was left out of the reverse SNA/AAL-based ELISA assay. However, compared with the high absorbance values for the sample group, this low background did not influence the accuracy of quantifying fucosylated or sialylated IgG from crude serum samples.

For detection of fucosylated IgG by reverse AAL-based ELISA assay, high background was observed when serum was

omitted from the assay, indicating high binding of AAL to the fucosylated glycans on the anti-IgG (Fc). Attempts to reduce this background by oxidizing the glycans on the anti-IgG (Fc) were unsuccessful. Finally, we found that using anti-IgG (Fab) in place of anti-IgG (Fc) could dramatically reduce the background caused by the binding of AAL to fucosylated glycans on the antibodies. The high background obtained by anti-IgG (Fc) is due to the presence of fucosylated oligosaccharide in its Fc portion (Supplemental Table S1 in the Supporting Information). Antibodies with the F(ab) portion have been used in several studies to eliminate the binding of lectin to glycans on the antibodies.<sup>18,19</sup>

### Optimizing Concentration of Lectins for Well-Coating

To reduce the background and increase the S/N ratio of the assay, we further optimized the amount of lectin for well coating. The data on optimization of AAL concentration used for well coating are shown in Figure 2. When the amount of AAL for well coating increased, higher intensities for both test and control (no serum) group were obtained (Figure 2a). The highest S/N ratio (>5) was obtained when 5  $\mu\text{g}/\text{mL}$  AAL was used for coating the plate (Figure 2b). Therefore, 5  $\mu\text{g}/\text{mL}$  AAL was chosen for the well coating for the reverse AAL-based ELISA assay.

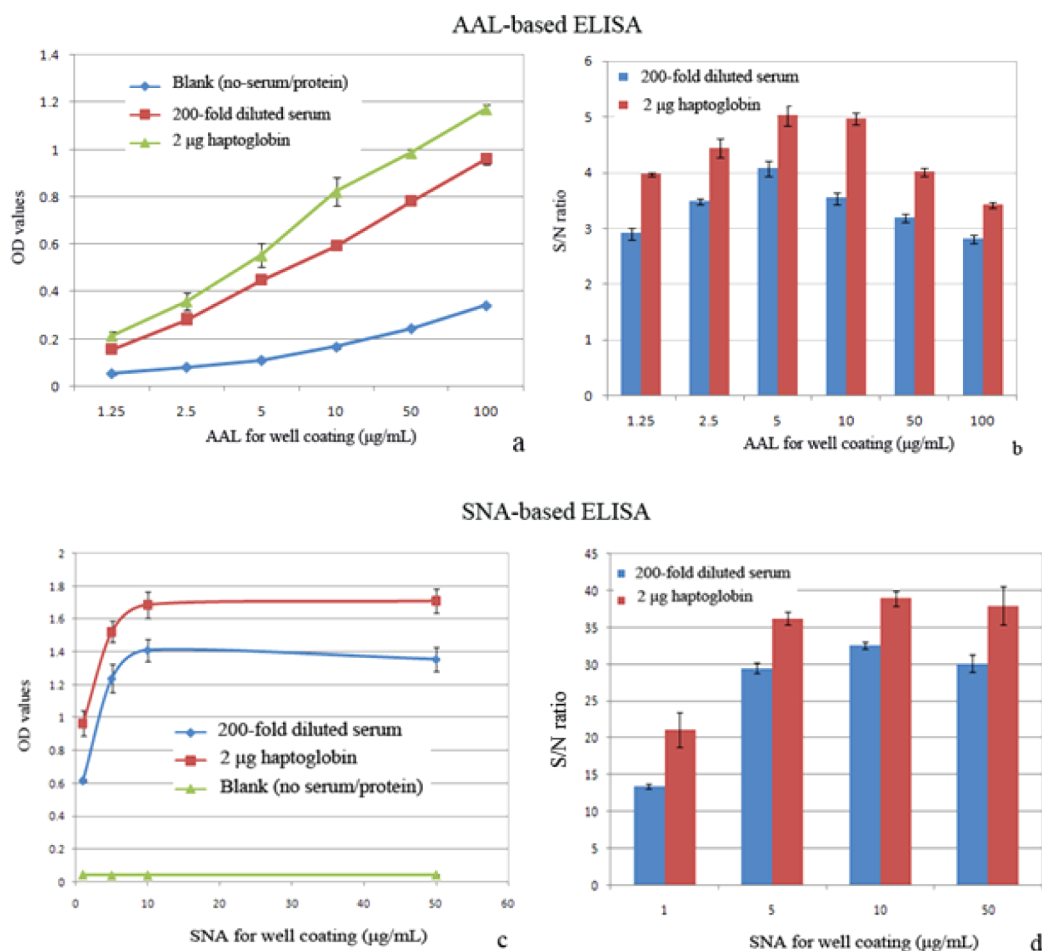
Compared with reverse AAL-based ELISA assay, lower background was obtained for the reverse SNA-based ELISA assay (Figure 2c), which may be due to the lower sialylation level of the tested antibodies. The S/N ratio reached the maximum when 10  $\mu\text{g}/\text{mL}$  SNA was applied for well coating, and the higher concentration of SNA (50  $\mu\text{g}/\text{mL}$ ) did not increase the amount of bound protein. Therefore, 10  $\mu\text{g}/\text{mL}$  SNA was used for well coating for reverse SNA-based ELISA assay, as shown in Figure 2d.

### Evaluating the Specificity of the Reverse Lectin-Based ELISA Assay

To verify that AAL specifically bound to fucosylated glycans on the glycoproteins rather than nonspecifically interacted with other glycan structures, we measured AAL binding performance in the presence of competing sugars, L-fucose. As shown in Figure 3a, preincubation of AAL with competing sugar, L-fucose, resulted in a dramatic reduction in AAL binding to fucosylated haptoglobin after serum/protein incubation. A parallel experiment using lactose showed no effect on AAL binding performance. The reduced binding of AAL to fucosylated proteins using its competing sugar indicates the specific binding of AAL to fucosylated glycans on the captured proteins.

To study the specificity of the reverse SNA-based ELISA assay, we tested the lectin binding to the captured proteins by treating samples with neuraminidase to remove the sialic acid of the glycoproteins. After samples were treated with neuraminidase, no proteins were found to bind to SNA when detected by antihaptoglobin (HRP), while serum/protein without sialic acid removal showed the high binding to SNA, as shown in Figure 3b. The results confirmed that lectins specifically bind to glycans rather than nonspecifically interact with proteins. The specificity of reverse AAL/SNA-based ELISA assays were also confirmed by testing protein IgG (Supplemental Figure S2 in the Supporting Information). Our results indicated that the reverse lectin-based ELISA assay showed the specific glycosylation changes of proteins.

To further address the specificities of the reverse lectin-based ELISA assay, we tested the specificity of the antibodies of



**Figure 2.** Optimizing the amount of lectin for well coating. (a) To obtain the highest S/N ratios for the reverse AAL-based ELISA assay, we measured the OD values for the sample (with serum samples/purified haptoglobin incubation) and control (no serum/haptoglobin incubation) groups after different amounts of AAL were coated on the microtiter plates. (b) S/N ratios for reverse AAL-based ELISA assay with different amounts of AAL coated on the microtiter plates. (c) OD values for sample and control groups were determined after different amounts of SNA were coated on the microtiter plates. (d) S/N ratios for the reverse SNA-based ELISA assay.

haptoglobin and IgG used in this study. Serum samples with haptoglobin- and IgG-depleted were used for the reverse lectin-based ELISA assay to check whether these two proteins can still be detected with their antibodies. As shown in Supplemental Figure S3 in the Supporting Information, a significant reduction in antibody bindings to their antigens was observed, which indicated the specificity of the antibodies. The results further confirmed the specificity of the reverse lectin-based ELISA assay to quantify the fucosylation/sialylation changes of haptoglobin and IgG from crude serum samples.

#### Reproducibility and Precision of the Reverse Lectin-Based ELISA Assay

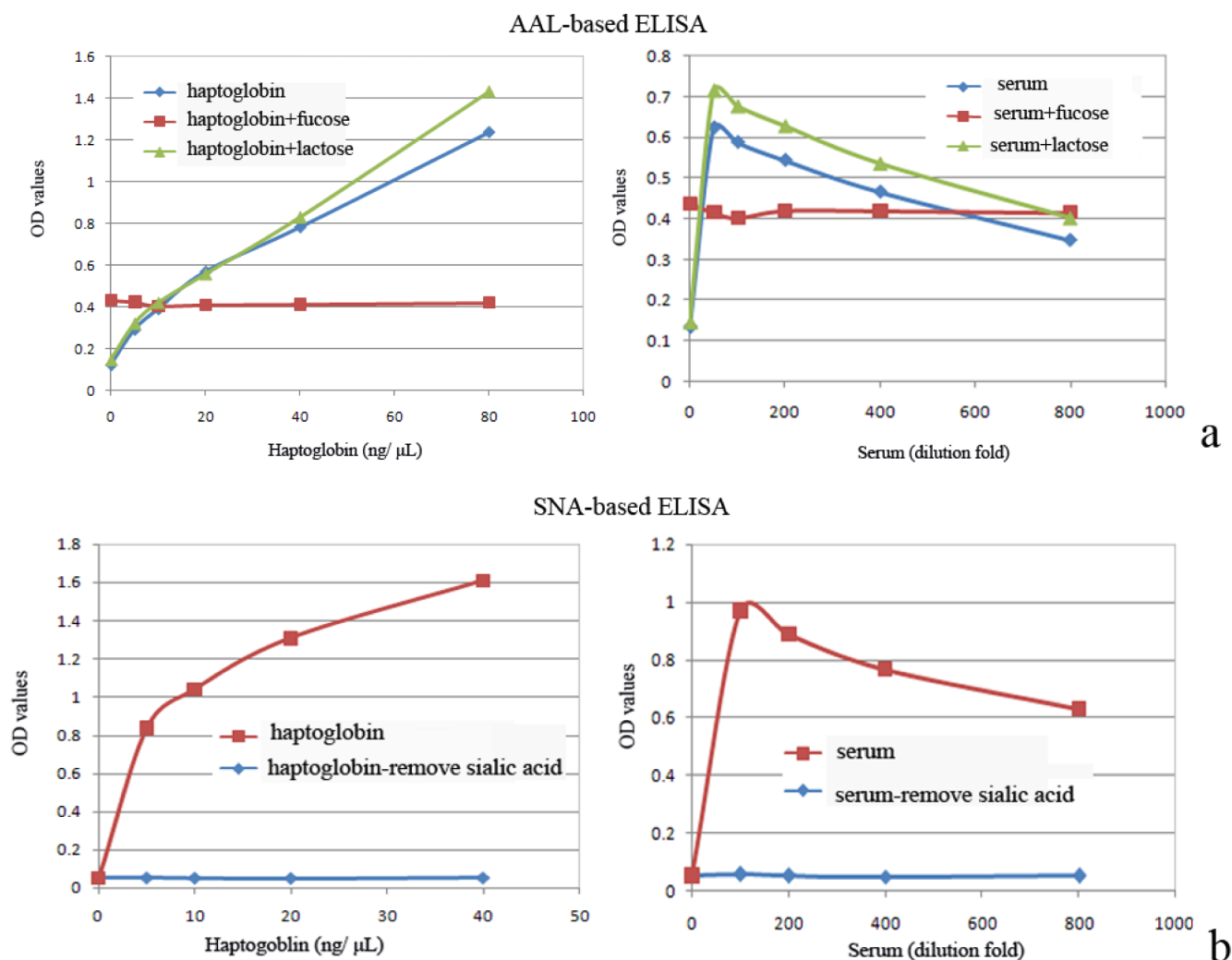
To compare samples analyzed on different microtiter plates or in different laboratories, standard samples need to be used to normalize the results. Because most of standard samples of differentially fucosylated/sialylated proteins are not commercially available, purified proteins were used to establish the standard curves in this study. As shown in Figure 3, haptoglobin showed a linear response in SNA/AAL binding between 5 and 80 ng/mL ( $R^2 = 0.991$ ). Although this type of standard curve is not ideal to calculate the absolute concentrations of the glycosylated proteins, it could be successfully used to enable comparisons of samples analyzed on different microtiter plates or in different laboratories.

The intra- and interassay precision of the reverse lectin-based ELISA assay was determined by repeated analysis of the serum samples on the same (in triplicate) and different microtiter plates (in duplicate). The coefficient of variation (CV) for intra- and interassay precision was 3.6 and 5.7%, respectively.

#### Determining Glycosylation Changes on Serum Proteins in Cancer Patients

One of the most important applications of the developed reverse lectin-based ELISA assays is to measure glycosylation changes on serum proteins from a large cohort of patients. The fucosylation/sialylation changes of haptoglobin and IgG in sera of patients with ovarian cancer were measured in duplicate by reverse lectin-based ELISA assay. We found the increased fucosylation levels of haptoglobin in ovarian cancer, especially in early-stage cancer compared with healthy controls or benign diseases (Figure 4), while the sialylated haptoglobin and IgG as well as fucosylated IgG showed no significant changes (Supplemental Figure S4 in the Supporting Information).

ROC curves were constructed for the changes in fucosylated glycoproteins to distinguish cases (late stage and early stage cancers) from noncases (healthy controls and benign diseases). The clinically used marker CA125 obtained the highest AUC (0.86) to differentiate cancer from noncases. The AUC for fucosylated haptoglobin was 0.739 (Figure 5). The combination



**Figure 3.** Determining the specificity of the reverse lectin-based ELISA assay. (a) For reverse AAL-based ELISA assay, OD values were measured after the lectin coated on the microtiter plates had or had not been preincubated with 100-fold molar excess of a competing sugar. (b) For reverse SNA-based ELISA assay, OD values were measured with removal of the sialic acid on the glycoproteins.

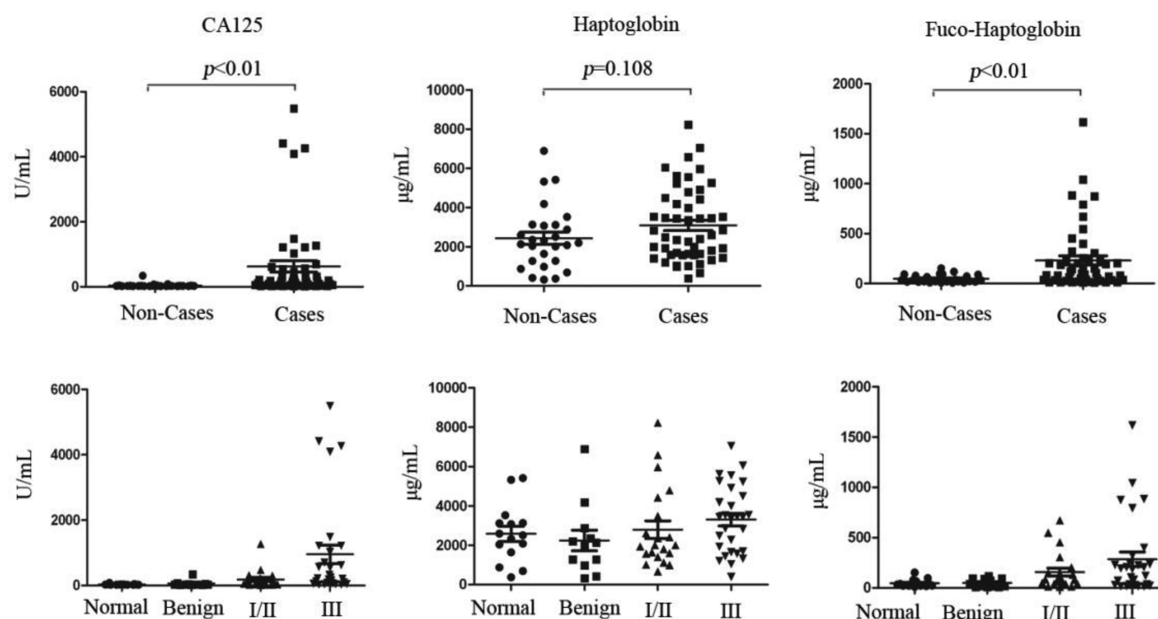
of CA125 and fucosylated haptoglobin had an AUC of 0.88 with specificity of 96.3% at a sensitivity of 78%, which improved both sensitivity and specificity when compared with CA125 alone (Figure 5). It should be noted that the fucosylated haptoglobin had an AUC of 0.741 to distinguish early stage from noncases, which was comparable to CA125 (0.795). The combination of CA125 and fucosylated haptoglobin resulted in an AUC of 0.855, which outperforms CA125 to distinguish early-stage cancer from noncases (Figure 5).

We performed a power analysis to determine the power of our experiments. At the given sample size, the variance of expression values, and the difference we want to detect (two-tailed, 0.05), the power of the experiment was calculated. The powers at the calculated differences of the means (delta mean) of comparison groups of differentially expressed fucosylated haptoglobin and CA125 are higher than 99%, which provides the statistical support for the number of samples included in our study.

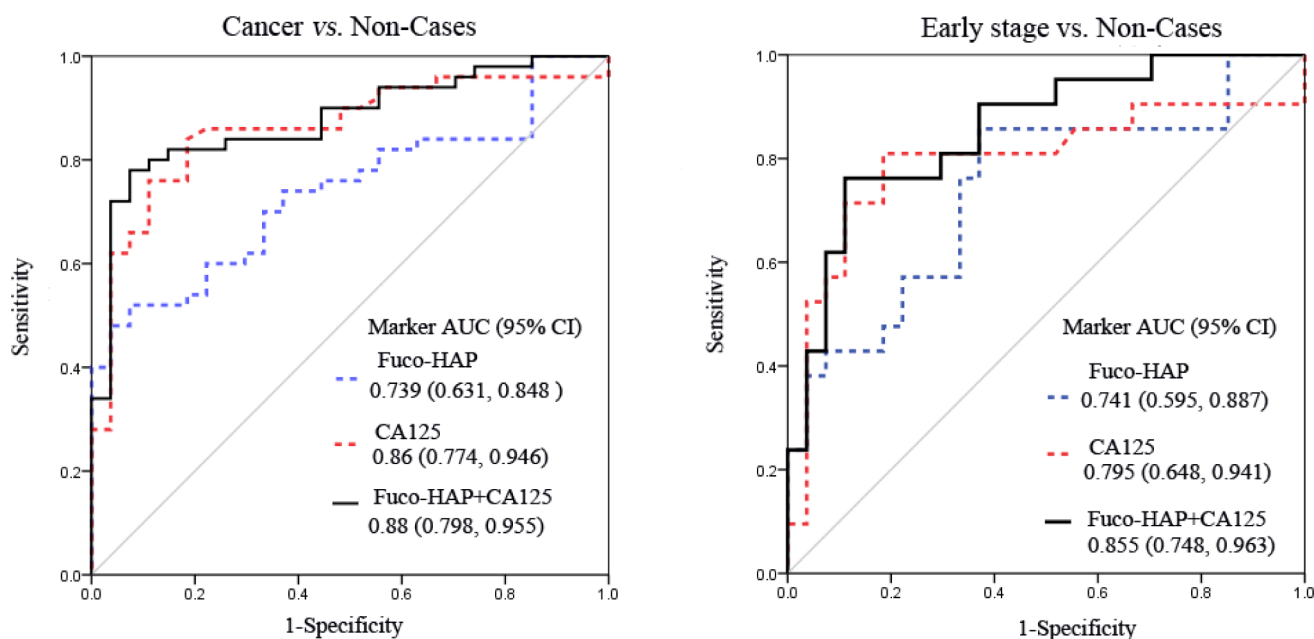
Haptoglobin, a glycosylated protein, is mainly produced in the liver and composed of two  $\alpha$  and two  $\beta$  subunits. Four N-linked glycans are attached to each  $\beta$  subunit.<sup>20</sup> Increased fucosylated haptoglobin has also been observed in various types of cancers, such as pancreatic cancer,<sup>21</sup> hepatoma,<sup>22</sup> prostate

cancer,<sup>23</sup> lung cancer,<sup>24</sup> and ovarian cancer.<sup>25</sup> There are several key advantages of the reverse lectin-based ELISA method compared with other methods. In these previous studies, to quantify fucosylated haptoglobin, several high-abundance proteins such as IgG were depleted, or haptoglobin needed to be purified from serum samples before mass spectrometry or lectin blotting analysis. Also, a large quantity of purified glycoprotein (micrograms to milligrams) is required for glycan analysis using mass spectrometry, which needs at least 10  $\mu$ L of sera,<sup>21,26</sup> while for the reverse lectin-based ELISA assay, nanogram levels of protein or <0.5  $\mu$ L serum is sufficient to analyze the glycosylation changes of protein. Because glycans need to be released from glycoprotein purified from depleted serum samples before MS analysis, contaminants from other glycoproteins may interfere with glycan quantification of target proteins. In contrast, our results showed high specificity of reverse lectin-based ELISA assay for analyzing the glycosylation changes of target proteins (Figure 3 and Supplemental Figure S3 in the Supporting Information).

Recently, using glycopeptides CID MS/MS and glycan database search, Chandler et al.<sup>27</sup> have studied site-specific N-glycosylation microheterogeneity of haptoglobin, which provided detailed glycosylation patterns of haptoglobin. By site-



**Figure 4.** Levels of CA125, haptoglobin, and fucosylated haptoglobin in sera of patients with ovarian cancer were determined by ELISA and reverse lectin-based ELISA assays. CA125 and fucosylated haptoglobin showed significant changes between noncases (normal and benign) and cases (early-stage and late-stage ovarian cancer) ( $p < 0.01$ ).



**Figure 5.** ROC analyses for CA125 and fucosylated haptoglobin to differentiate ovarian cancer from noncases.

specific glycan analysis with LC-ESI-MS, Nakano et al.<sup>28</sup> have shown that fucosylated glycans are markedly increased at N211 in pancreatic cancer. However, these studies analyzed haptoglobin glycans in a qualitative instead of a quantitative manner, which are not applicable to quantify glycosylation changes of haptoglobin from individual samples. Quantification methods such as MS and lectin blotting lack sensitivity, accuracy and high sample throughput,<sup>29</sup> which may impede their application in clinical examination. A system that is suitable for analyzing a large number of specimens is required. In this study, the developed reverse lectin-based ELISA assay provides a reproducible method to quantify glycosylation changes of proteins from crude serum samples. Because

biotinylated/HRP-conjugated antibodies are required for the reverse lectin-based ELISA assay, the glycoproteins that lack commercial biotinylated/HRP-conjugated antibodies could not be analyzed by the reverse lectin-based ELISA assay.

Because the abundance changes of the underlying protein could account for the detected glycosylation changes, an ELISA assay was used to measure the underlying protein concentrations of haptoglobin from the original serum samples. As shown in Figure 4, fucosylated haptoglobin showed significant changes between cancer and noncases, while its protein levels showed no significant changes between cancer and control groups. The results support previous findings that potential

biomarkers may be identified based on their changes in protein modifications rather than changes in protein abundance.<sup>4,13,15</sup>

## CONCLUSIONS

Our study shows an effective method for the analysis of glycosylation changes on glycoproteins from serum samples. This method can be used not only to analyze the high-abundance serum proteins, but also to quantify moderate-abundance proteins that are potential markers, as described in our previous study.<sup>15</sup> Its effectiveness for low abundance glycoproteins has yet to be determined. Nevertheless, using this novel reverse lectin-based ELISA assay, we found that fucosylated haptoglobin could be a potential candidate biomarker, which can be used to supplement CA125 for detecting ovarian cancer with improved sensitivity and specificity. The method has distinct advantages over current lectin-ELISAs that require oxidation of glycans on the IgGs to minimize interaction with the lectins. However, even with this procedure, there is often significant background in these lectin-ELISA experiments compared with our current platform.

In future work, there are various ways in which the reverse lectin-based ELISA method could be expanded and improved. It could be expanded to a 384 well format to increase the throughput for a larger number of samples. Also, there has been recent work to improve the response of lectin arrays based on oriented lectins using an immobilization method.<sup>30–32</sup> These oriented lectin arrays have been shown to markedly increase the response of some lectins to glycoproteins. AAL and SNA though were not tested in this work. Nevertheless, this strategy has worked for many lectins and could help in expanding this method to lower abundance glycoproteins.

## ASSOCIATED CONTENT

### Supporting Information

Determining the presence of fucosylation/sialylation of haptoglobin and IgG by Lectin-blot. Determining the specificity of the reverse lectin-based ELISA assay by detecting IgG. Evaluating the specificity of anti-haptoglobin and anti-IgG for the reverse lectin-based ELISA assay with depleted/original serum samples incubation. Levels of sialylated haptoglobin, sialylated IgG, and fucosylated IgG in sera of patients with ovarian cancer determined by reverse lectin-based ELISA assays. Detection of glycosylation on proteins after omitting various components from the mixture of reverse lectin-based ELISA assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## AUTHOR INFORMATION

### Corresponding Author

\*E-mail: [dmlubman@umich.edu](mailto:dmlubman@umich.edu). Tel: (734) 615-5081. Fax: (734) 615-2088.

### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS:

AAL, aleuria aurantia lectin; SNA, *Sambucus nigra* lectin; ELISA, enzyme-linked immunosorbent assay; CA125, cancer antigen 125; HRP, horseradish peroxidase; TMB, 3,3',5,5'-tetramethylbenzidine; ROC curve, receiver operating characteristic curve; AUC, area under an ROC curve

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