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Label-Free Impedimetric Detection of Glycan−Lectin Interactions

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A compact biosensor for a label-free, rapid (<80 s) detection of glycan−lectin interactions using ac impedance measurements was developed for the first time. A galactose-binding peanut agglutinin (PNA) and sialic acid-binding *Sambucus nigra* agglutinin (SNA) were covalently surface-immobilized on the layered Cu/Ni/Au printed circuit board (PCB) electrodes. Samples of artificial and natural glycoconjugates consisting of (1) gold glyconanoparticles encapsulated with ~90−100 copies of TF-antigen disaccharide Galα1-3GalNAc (TF-AuNP), (2) asialofetuin (ASF) containing both LacNAc (Galβ1-4GlcNAc) and TF-antigen, and (3) fetuin (FET), the sialylated glycoform of ASF. The samples were run separately on PNA- and SNA-immobilized PCB electrodes. Our results indicate that TF-AuNP could be rapidly and reliably detected up to 1 pg/mL (13 fM) concentration on PNA electrode but, as expected, yielded no response on the SNA electrode. ASF and FET glycocoproteins were unambiguously detectable up to 10 pg/mL (150 fM) on PNA and SNA electrodes, respectively. Moreover, the technique allowed us to observe glyco-microheterogeneity of FET as well as establish the presence of two isoforms of SNA lectin, SNA-I and SNA-II, in one of the vendor's formulations. Further elaboration of the described technology into novel electrochemically driven lectin arrays may find applications in diagnosis of cancer and other diseases with multiple glycobiomarkers or as a rapid low-cost bioanalytical tool for glycoproteome analyses.

Carbohydrates are of central importance in the development and maintenance of all biological systems as they confer structural and functional diversity onto roughly 80% of all secreted and cell surface proteins[1] and other biomolecules. Changes in glycosylation are often associated with a variety of diseases[2] including cancer.[3] The field of glycomics has recently emerged to systematically address the structural and functional roles of carbohydrates at a level of complexity previously unseen in other areas of applied biology.

The principal limitations in the field of glycomics are technological in nature. Identification of glycans traditionally has been achieved by the use of lectins or monoclonal antibodies, high-performance liquid chromatography, 2-D gel and capillary electrophoresis, mass spectrometry, and, most recently, optically labeled lectin arrays.[4−6] Although these approaches have been partially successful, the field would greatly benefit from additional bioanalytical tools with the capacity to scrutinize glycosignatures rapidly and, preferably, in a label-free fashion. This would accelerate the development of novel high-throughput (HTP) technologies for studying the unprecedented diversity of carbohydrate-mediated biological interactions. Among label-free methods, surface plasmon resonance (SPR),[7] quartz crystal microbalance (QCM),[8] and electrochemical impedance spectroscopy (EIS)[9] have received insufficient attention in HTP glycan profiling despite their extensive and often successful use in the genomics and proteomics areas.[10,11] Similar to SPR and QCM, EIS enables rapid label-free assays with the added advantages of simplicity, amenability to miniaturization, and mass producibility, which may translate into low-cost online or on-site detection devices.[12] These advantages make electrochemical devices especially attractive for point-of-care applications, such as clinical analysis.[13]

EIS detection is generally based on the differences in the electrochemical behavior (impedance, resistance, and capacitance) of a medium consisting of ligands bound to the surface of electrodes functionalized with appropriate receptors.[9] EIS is extensively used in immunosensors to study the strong monovalent antigen−antibody molecular recognition events by measur-

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ing the electrochemical changes induced by binding reactions.[14] Recognition of individual carbohydrates by proteins, on the other hand, is usually weak, but it can be strongly amplified by multiple presentations of carbohydrates.[15] Glycosensor designs employing carbohydrate−lectin bimolecular recognition have been reviewed;[16] it appears that thus far the label-free electrochemical approach has been rarely[17] if ever applied for the detection of glycans.

We recently reported the electrochemical detection of carbohydrate−lectin interactions based on the competition between nanocrystal-tagged sugars and the target sugars for the binding sites of surface-confined lectins monitored by highly sensitive amperometric stripping voltammetry.[18] Although this method, in addition to excellent sensitivity, offers unique multiplexing abilities by using nanocrystals of different compositions, the necessity for labeling increases complexity and length of the assay procedure. Here we present the first report on label-free detection of artificial and natural multivalent glycoconjugates using ac frequency-based EIS on low-cost, gold-electroplated, lectin-immobilized printed circuit board (PCB) electrodes.

MATERIALS AND METHODS

Electrode Fabrication. All electrodes are a standard three-electrode impedance spectroscopy design with Ag/AgCl reference electrode and a gold counter and a working electrode. These electrodes were designed using CAD software and printed onto 6 in. × 6 in. presensitized PCB boards. The process is as follows: a UV light box exposes the photoresist on the presensitized boards that have the CAD designed mask on top of them, blocking the light on the desired portions, and exposing the uncovered surfaces. This surface of photoresist is removed in a 0.25 M, pH 14 sodium hydroxide wash for 2 min. Next, a 1.1 M, pH 1 solution of ammonium persulfate at 40 °C etches the exposed copper under agitation for 8−10 min. The individual chips are then cut, the photoresist is removed with acetone, and sample wells are added. The copper is cleaned prior to electroplating, 90 s nickel, 90 s gold, 2 s silver (onto reference only), and finally, the silver is chlorinated to make the Ag/AgCl reference electrode. The electrodes were immediately stored in pH 7.4, 1× phosphate buffer saline (PBS) at room temperature to reduce surface contaminants. PBS stocks, pH 7.4, were used for all solutions (unless otherwise noted).

Lectin Immobilization. A 100-μL solution of 25 mM mercaptohexadecanoic acid (MHDA; Aldrich) in reagent grade ethanol was added to each electrode and incubated for 12 h at room temperature. The electrodes were then rinsed with reagent grade ethanol (Sigma-Aldrich). A 100=μL droplet of a 40 mM *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (Pierce Biotechnology) and 10 mM *N*-hydroxysulfosuccinimide (VWR international) solution in PBS was then placed on each electrode. After 1-h incubation at room temperature, the electrodes were rinsed in PBS buffer. Next, a 100-μL droplet of a 50 μg/mL solution of lectin, from *Arachis hypogaea* (peanut agglutinin, PNA) or lectin from *Sambucus nigra* (SNA) as appropriate, in PBS buffer was placed on the electrode and incubated at room temperature for 1 h. The electrodes were finally incubated with 1 mM ethanolamine in deionized water for 0.5 h at room temperature to block unreacted carboxyl groups of MHDA, washed with PBS, and stored at 4 °C in PBS until future use.

Verification of Electrode Functionalization. Verification of electrode functionalization was performed using a secondary antibody. The PNA functionalized electrode was incubated with an anti-PNA MAb and then a secondary antibody labeled with Alexa680 IR dye (LI-COR) at 2.5 μg/mL for 1 h at room temperature, washed, and immediately imaged using a LI-COR Odyssey imaging system.

Impedance Spectroscopy Measurements. Electrochemical workstation from CH Instruments (CHI660C) was used to record the changes in the impedance, which arise due to the binding events occurring at the surface of the electrodes. Each PCB electrode was connected to the impedance spectrometer using leads and checking for continuity. Fresh redox probe solution was prepared using PBS buffer (pH 7.4) and 5 mM potassium ferrocyanide/ferricyanide. The target sample solutions were diluted in the redox probe from 0 pg/mL to 100 ng/mL. These samples were stored at 4 °C until used. Before running impedance measurements, each electrode was rinsed with the redox probe solution. The sample was placed onto the well of the PCB chip, and the ac (alternating current) impedance setting were changed to an amplitude of 5 mV, a full frequency of 1−100 000 Hz, and an initial potential of 250 mV. The impedance of each PCB chip was recorded for different concentrations.

Optimal Frequency Determination. In order to understand the frequency at which maximum/optimal changes in impedance are seen, a plot was made for frequency and maximal percentage change in impedance for each of the concentrations of samples tested. By doing so, an estimate was made for an average frequency that could be used for comparison between different PCB electrodes, controls, and samples. This frequency also allows the freedom of using impedance analysis on these PCB electrodes over longer periods of time. Plots of frequency versus impedance at various concentrations of target are used to develop the frequency of maximal response for data interpretation.

Desialylation of Bovine Fetuin. A 500-μg sample of commercially available bovine calf serum fetuin (FET) (Sigma-Adrich, Lot No. 094K7695) was reconstituted in 240 μL of 25 mM sodium phosphate buffer, pH 5.0. Slurry containing neuraminidase conjugated to agarose (Calbiochem, Lot No. B54217) was washed twice and suspended in 25 mM sodium phosphate buffer, pH 5.0, just prior to use. A 20-μL sample of the *Clostridium perfringens* neuraminidase−agarose suspension, which cleaves α2−3,6,8-linked terminal sialic acids, was added to the FET solution. Activity of the added neuraminidase slurry was calculated to equal 20 mU. The reaction mixture was placed into a 37 °C incubator for 18 h with gentle shaking. Upon completion of incubation, the neuraminidase−agarose conjugate was separated from the mixture by centrifugation in a 100-kDa MWCO spin device (Pall) for 15 min at 3000*g*. To eliminate free sialic acid, the flow-through containing the desialylated fetuin (asialofetuin, ASF) was placed into a 10-kDa MWCO spin device and centrifuged for 15 min at 4000*g*. Protein retained on the spin device membrane was removed by

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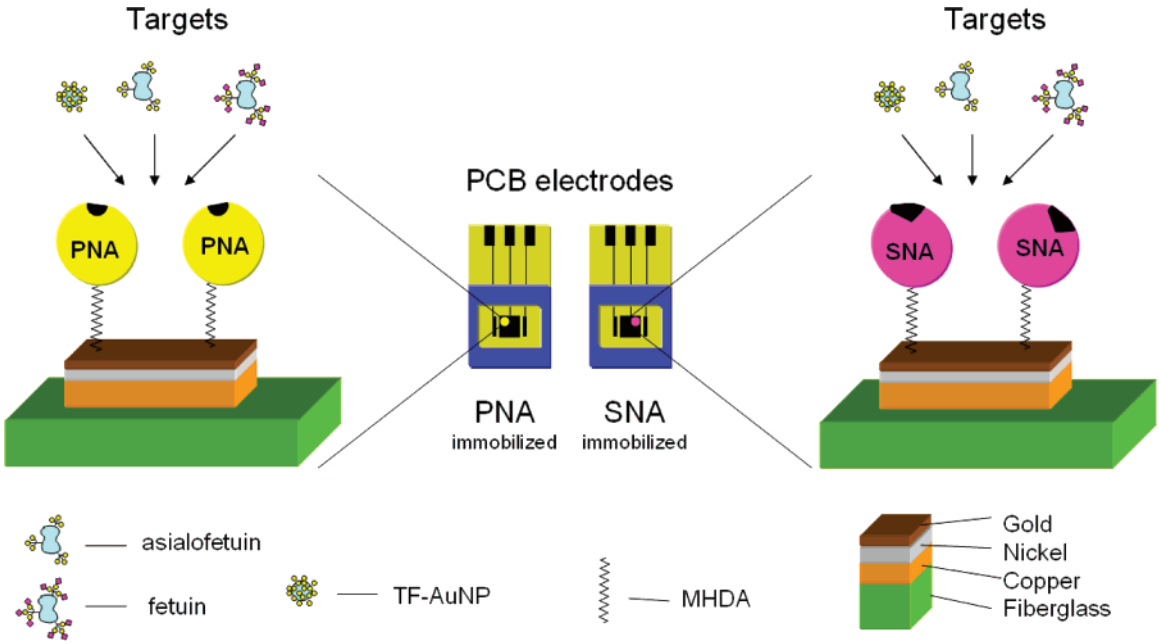

Figure 1. Schematic representation of TF-AuNP, ASF, and FET binding experiments performed in this work.

addition of 100 μL of PBS, briefly vortexed, and then pipetted off. Final yield was found to be ~200 μg (40%) by Bradford analysis using BSA as a standard (Pierce Biotechnology). Desialylation was confirmed by SDS-PAGE comparison of an aliquot of the desialylated protein with natural FET run on 4−12% Bis-Tris polyacrylamide gel (Invitrogen). The ASF displayed a mass shift of 5 kDa (data not shown).

RESULTS AND DISCUSSION

As a proof of concept, for this study we have chosen an established system based on the affinity of PNA and SNA toward terminal galactose and α2,6-linked sialic acid, respectively. PNA lectin is a 110-kDa homotetrameric lectin that preferentially recognizes the tumor-associated TF-antigenic determinant (Galβ1−3GalNAc, where Gal is galactose, GalNAc is *N*-acetylgalactosamine), and, less specifically, other galactose-terminated oligosaccharides, such as lactosamine (LacNAc, Galβ1−4GlcNAc, where GlcNAc is *N*-acetylglucosamine). The extensive analysis of the carbohydrate specificity of PNA has revealed that it is unable to recognize sialylated derivatives of galactose, which is largely attributed to its usefulness as a tool in cancer diagnostics (vide infra). On the other hand, SNA is a 150-kDa homotetrameric lectin that is known to preferentially bind terminal α2,6-sialylated glycoproteins such as FET, orosomucoid, and bovine submaxillary mucin.[19]

Experimental Setup. The overall experimental setup is shown in Figure 1. Briefly, PCB electrodes covalently functionalized on the working electrode with either PNA or SNA were probed with serially diluted PBS solutions of (1) TF-antigen encapsulated gold nanoparticles (TF-AuNP), (2) ASF, and (3) FET. Each electrode was used once and then discarded.

Detection of TF-AuNP Glyconanoparticles. In order to eliminate uncertainties associated with glycoprotein heterogeneities and to benchmark the assay using a well-defined glycoconjugate, we first tested our EIS platform with the gold nanoparticles (TF-AuNP) encapsulated with ~90−100 PEG-tethered TF-antigens (Galβ1-3GalNAc) (Figure 2), prepared as previously described.[20]

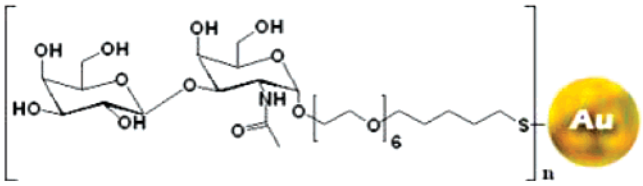

Figure 2. Structure of TF-AuNP with $n = 90-100$. Reproduced with permission from ref 23.

This "artificial glycoprotein" construct, weighing on average 75 kDa, was previously shown to be fully biofunctional as it very specifically and reversibly bound to a PNA affinity column and agglutinated fluorescently labeled PNA but not mannose-specific lectin PSA (*Pisum sativum* agglutinin).[20] Such sugar-encapsulated nanoparticles have proved to be useful instruments for studying carbohydrate−protein and carbohydrate−carbohydrate interactions due to their ability to mimic a multivalent arrangement of glycans in biological systems.[21]

The initial impedance values have been acquired with a three-electrode setup using a CH Instruments 660C electrochemical workstation as previously described.[22] To determine the effects of concentration, the concentration of TF-AuNP was varied from 100 ng/mL to 1 pg/mL via a series of dilutions, with 0 pg/mL being the working buffer (1× PBS, pH 7.4). These frequency sweep resulting impedances $Z(f)$ were then normalized in a differential impedance approach, namely, by normalization to the initial point by $Z(f)-Z(f)_{min}$. Figure 3 shows the results of ac sweep

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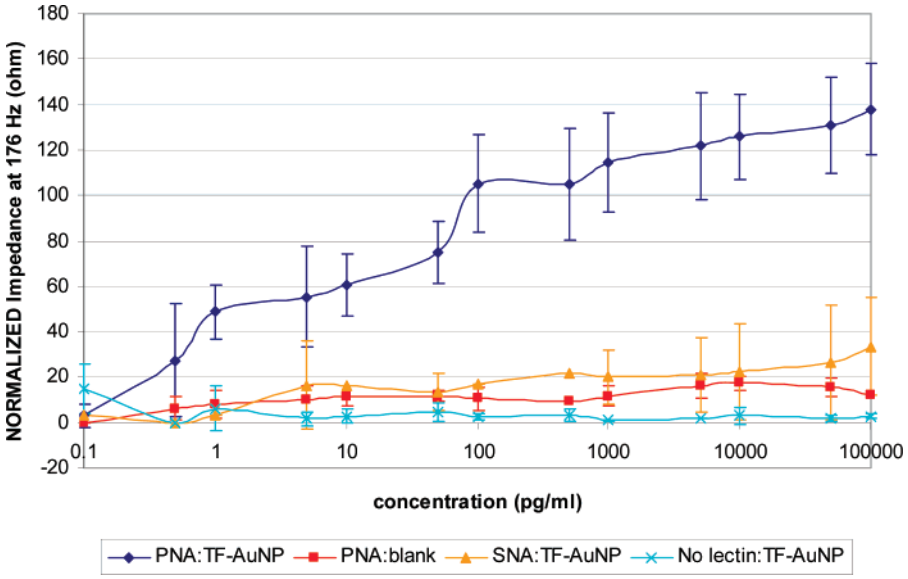

Figure 3. Normalized (response at conc = 0 set to zero impedance) impedance at ~176 Hz for immobilized PNA with TF-AuNP (diamonds), PNA control in blank redox probe (squares), SNA I on TF-AuNP (triangles), and no immobilized lectin control on TF-AuNP (crosses) all $n = 4$. Typical molarity of the TF-AuNP target (at 1 pg/mL) is 13 fM.

spectroscopy with SNA- and PNA-immobilized electrodes. SNA lectin that binds to α2,6-sialic acid residues is inactive toward TF-antigen.[19] Indeed, in Figure 3, we see very little, if any, binding of TF-AuNP to the SNA-immobilized electrode (triangles) while the PNA-immobilized electrode (diamonds) shows a discernibly strong signal up to 1 pg/mL (~13 fM in TF-AuNP, MW_{av} 75 kDa; or 1.3 nM in TF-antigen, based on 100 TF-antigens/AuNP) concentration of TF-AuNP. As expected, the PNA immobilized electrode (diamonds) showed dramatically higher response to the presence of the TF-AuNP than did the SNA electrode (triangles). The 5% variation seen in the PNA control (squares) run against working buffer indicates a strong stability of the electrodes, traditionally an Achilles heel of impedimetric spectroscopy.[9] Minimal binding is seen in the SNA immobilized electrode over the 6 orders of magnitude in concentration runs in these experiments. Importantly, the additional control experiment with no lectin present on the PCB electrode (crosses) showed virtually no response to the presence of TF-AuNP, indicating that there is no unspecific binding of TF-AuNP to the unfunctionalized electrode surface.

Detection of Glycoproteins. Inspired by the exquisitely low detection levels of the TF-AuNP, we further proceeded to determine sensitivity and specificity of our assay in binding of natural glycoproteins, FET and ASF to the SNA and PNA lectins, respectively. FET isolated from fetal calf serum has been studied extensively. It contains three O-linked either linear or branched carbohydrate units, which are attached to serine and threonine (S/T) residues, and three N-linked branched heteropolysaccharides attached to asparagine (N) residues[23,24] as shown in Figure 4. In FET, the majority of the terminal galactose residues are masked by α2,3- or α2,6-linked *N*-acetylneuraminic (sialic) acid. ASF is produced by enzymatic or chemical desialylation of FET,[27] thus exposing the penultimate galactose residues.

PNA is often used as a tool for recognition of TF-antigen on the surfaces of malignant cells.[25] PNA also recognizes, albeit less specifically, the penultimate *N*-acetyllactosamine (LacNAc, Galβ1−4GlcNAc), present on bovine fetal glycoprotein ASF, which carries nine LacNAc residues (Figure 4). SNA preferentially binds α2,6-sialylated glycoproteins such as FET and not their asialo derivatives, such as ASF.[19] It should be noted, however, that elderberry bark (a source of SNA) has been previously shown to contain at least two isoforms of this lectin.[26] While a single isoform is well characterized as a receptor for glycans with terminal sialic acid residues (SNA-I), another isoform (SNA-II) has been shown to also bind nonsialylated residues including terminal galactose (Gal) and *N*-acetylgalactosamine (GalNAc).[27]

We obtained SNA lectins from two different sources during the course of this study. Some binding was observed during addition of ASF to electrodes modified with SNA from Sigma-Aldrich, Inc, but later experiments with SNA lectin sold as the purified isoform SNA-I by EY Labs, Inc. showed little tendency to act as a receptor for ASF (data not shown). We attribute this differential binding to the presence or absence of the SNA-II isoform in the SNA lectin obtained from two different vendors.

PNA and SNA-I immobilized electrodes were chosen for screening the ASF and FET samples as schematically shown in Figure 1. Figure 5 shows the impedance with maximum change at a frequency of ~550 Hz. As anticipated, SNA-I has a significant binding affinity to FET (diamonds) but nearly zero affinity to ASF (crosses). PNA, however, can detect the glycosylation state of ASF (triangles) but also is able to bind FET (squares), although with a substantially weaker affinity at concentrations above 10 pg/mL. This effect is probably due to microheterogeneities involving variable and incomplete sialylation of the glycan chains of sialoproteins. The microheterogeneity of FET glycoforms is well-

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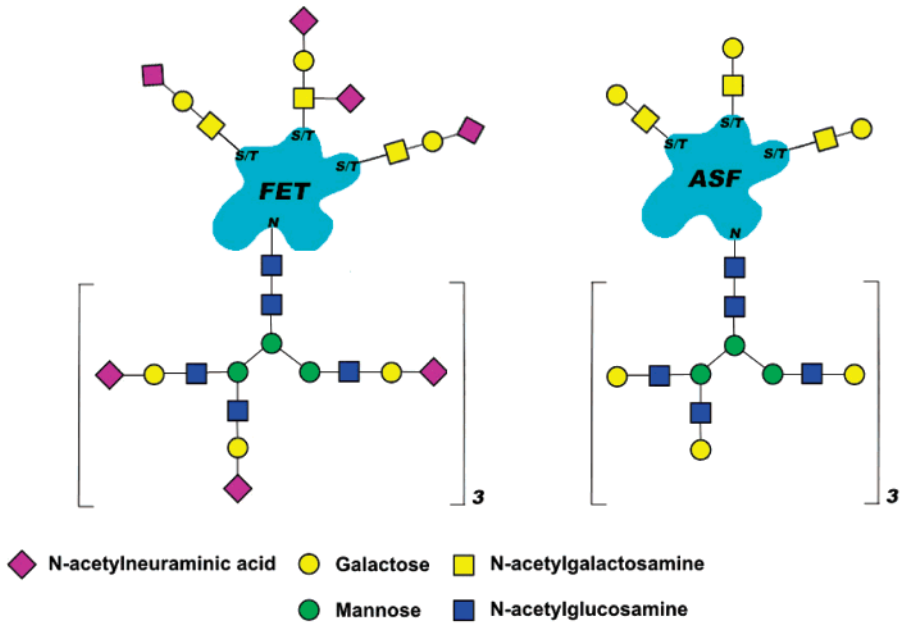

Figure 4. Carbohydrate composition of FET and ASF glycoproteins. Carbohydrate units are represented by geometric shapes according to nomenclature established by the Consortium for Functional Glycomics (CFG).

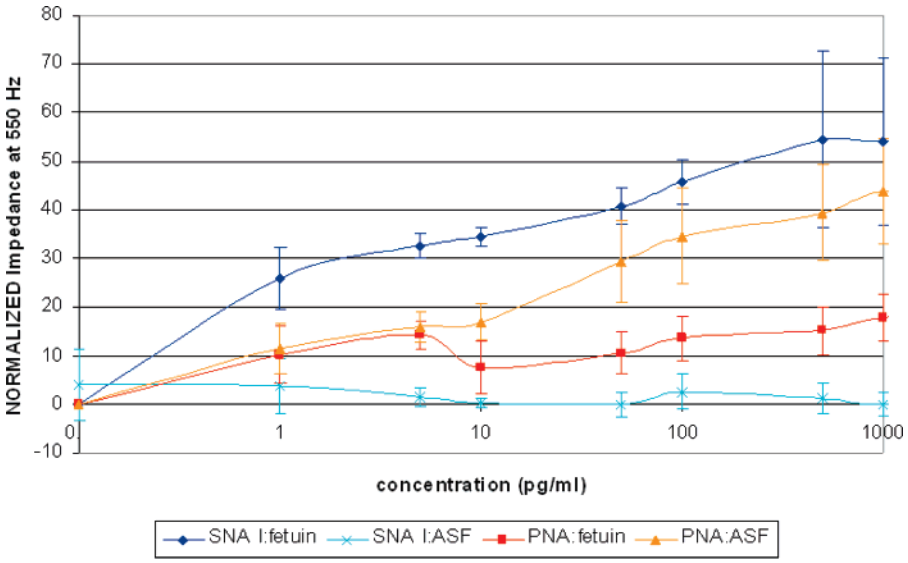

Figure 5. Normalized (response at conc = 0 set to zero impedance) impedance at ~550 Hz for immobilized SNA I with FET (diamonds), PNA control with FET (squares), PNA with ASF (triangles), and SNA I control with ASF (crosses) all $n = 4$. Typical molarities of the ASF and FET targets (at 1 pg/mL) are as follows: 20.4 and 14.9 fM, respectively (assuming a 67 kDa FET and 49 kDa ASF).[29,30]

documented.[28] The variations are due to incomplete sialylation or postsynthetic degradation within the cell or during isolation of the protein. Since *N*-acetylneuraminic acid is the terminal residue, the microheterogeneity due to this sugar is most common. Therefore, it comes as no surprise that we observe some residual binding of PNA to FET. The absence of such binding in the SNA I-ASF system can be explained by efficient enzymatic desialylation of FET to ASF.

Although not as sensitive as detection of gold glyconanoparticles, these results demonstrate that EIS can also be used for rapid and exceptionally sensitive detection of native protein glycoforms as well as for the determination of lectin specificities as was shown for FET and the two isoforms of SNA lectin, SNA-I and SNA-II. With the increasing understanding of the importance of post-translational modifications on the biological activity of proteins, this technique may offer an interesting low-cost alternative to the conventional glycoproteomic approaches.

Implications in Cancer Diagnostics. TF-antigen is now one of the most firmly established cancer glycobiomarkers.[31] The only serum tests approved for use in breast cancer (BC) detection are immunoassay (ELISA) tests for MUC1 glycoprotein and carcinoembryonic antigen (CEA). MUC1 is a major cell surface mucin (MW > 1 MDa) on breast epithelial cells; currently it is most

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widely used marker for BC detection.[32] Mucins found on the surface of cancer cells are not only overexpressed but also aberrantly glycosylated. For example, in normal breast epithelia, MUC1 is heavily glycosylated with the core-2 glycans masking underlying core-1 structures. At the onset of BC, the shorter, core-1 glycans become unmasked exposing several tumor-associated carbohydrate antigens (TACAs), which include the TF-antigens and its precursor, Tn-antigens.[2] These TACAs have been proposed as useful BC glycobiomarkers because of their specific occurrence in BC but rarely in normal tissues.[33]

Recently, an ELISA was developed to measure levels of both TF- and Tn-antigens expressed on MUC1 from nipple aspirate fluid (NAF).[31] It was shown that nearly 90% of breast cancer NAF samples contained measurable quantities of TF- and Tn-antigens in contrast to the vast majority of noncancerous samples that did not contain detectable levels of these carbohydrates. The usefulness of these immunochemical tests is however limited by their marginal sensitivity and overall time-consuming (>6 h/assay) laboratory-based procedure.

The ultrasensitive and simple gold nanoparticle-based EIS assay for the TF-antigen developed here may become an attractive point-of-care testing alternative to the currently used enzyme-linked immunochemical assays. Nanoparticles have received wide attention in the past few years in electrochemical sensing applications mainly due to their extraordinary optical, electronic, and electrocatalytic properties that can be used as additional means for further signal amplification in biosensors.[34] The recent availablity of efficient glycoblotting[35] technology to transfer glycans from the surface of cells and biomolecules onto the surface of nanoparticles can further facilitate the nanoparticle-based approach.

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CONCLUSIONS

In this report, we demonstrate that EIS can be used to reliably detect glycan−lectin interactions in a label-free fashion. The technique was shown to be fast and sensitive. The obtained quantitative and qualitative information was essential for the understanding of affinity and specificity of interactions between glycans and their cognate protein receptors, lectins. Such novel methods for label-free glycan detection may be of value for the point-of-care early cancer detection since many established tumor biomarkers including CEA, CA125, PSA, and MUC1 (CA15−3) are heavily glycosylated. The ability to rapidly differentiate their glycosylation states may provide additional information on the status of the disease. In the past few years, it has become increasingly clear that no single biomarker can be reliably used for cancer diagnosis. Further improvements using more specific carbohydrate-binding receptors and their integration into multiplexed electrode arrays with computer-assisted pattern analysis are needed before this impedimetric approach can be advanced into clinical studies.

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