Label-Free Protein Glycosylation Analysis Using NanoMonitor—An Ultrasensitive Electrochemical Biosensor

Antra Ganguly,[1] Kai-Chun Lin,[1] Sriram Muthukumar,[2] Vinay J. Nagaraj,[1] and Shalini Prasad[1,3]

[1]Biomedical Microdevices and Nanotechnology Laboratory, Department of Bioengineering, University of Texas at Dallas, Richardson, Texas
[2]Enlisense LLC, Allen, Texas
[3]Corresponding author: *Shalini.Prasad@utdallas.edu*

Glycans (oligosaccharide chains attached to glycoproteins) are a promising class of biomarkers, found in body fluids such as serum, saliva, urine, etc., that can be used for the diagnosis of disease conditions. Subtle changes in glycans resulting from altered glycosylation machinery have been reported during various diseases, including carcinogenesis. In this article, we detail protocols for the rapid, label-free analysis of glycans using a previously developed highly sensitive and selective electrochemical impedance spectroscopy−based biosensing diagnostic platform called "NanoMonitor." The glycosensor operation is based on the specific affinity capture of the target glycans on the sensor surface by glycan-binding proteins known as lectins. This glycan-lectin binding activity modulates the impedance of the electrical double layer at the buffer-electrode interface. Protocols for the preparation of glycoprotein samples and glycosylation analysis using NanoMonitor and lectin-based ELISA are described here. The data obtained using these protocols show that NanoMonitor is capable of distinguishing between glycoform variants of the glycoprotein fetuin and glycoproteins derived from cultured human pancreatic cancer cells with high sensitivity (orders of magnitude higher than lectin-based ELISA) and selectivity. The results obtained indicate that NanoMonitor protocols can be further developed to enable use of NanoMonitor as a handheld electronic biosensor device for routine multiplexed detection of glycan biomarkers from clinical samples.

Basic Protocol 1: Preparing the NanoMonitor surface for glycan biosensing
Support Protocol: Synthesis of glycoform variants of fetuin
Basic Protocol 2: Performing Electrochemical Impedance Spectroscopy (EIS) for analyzing glycoprotein structures

Keywords: cancer glycoprofiling • electrochemical impedance spectroscopy • glycoprotein analysis • glycosensor • label-free glycosylation analysis • lectin

How to cite this article:
Ganguly, A., Lin, K.-C., Muthukumar, S., Nagaraj, V. J., & Prasad, S. (2021). Label-free protein glycosylation analysis using nanomonitor—an ultrasensitive electrochemical biosensor. *Current Protocols*, *1,* e150. doi: 10.1002/cpz1.150

INTRODUCTION

Glycosylation is a major class of post-translational protein modification, and influences several biological processes including cell-cell interactions, cell differentiation, cell

CURRENT PROTOCOLS
A Wiley Brand
Current Protocols e150, Volume 1
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpz1.150

development, host–pathogen recognition, and immune response (Haab & Klamer, 2020; Lin, Qing, Liao, & Zhuo, 2020; Reily, Stewart, Renfrow, & Novak, 2019). Disease-induced changes of protein glycosylation are promising biomarkers for the diagnosis of various diseases, including cancer. In addition to the detection of unique glycans, global changes to an individual's glycome are also being investigated for the assessment of disease progression (Reily et al., 2019). Traditional laboratory techniques for glycosylation analysis, such as mass spectrometry, may not be practical for routine and rapid screening of a large number of clinical samples, since they are expensive and time-consuming, and require handling by skilled personnel. Other methods of glycan detection include western blots, lectin-based ELISAs, and microarrays, which make use of lectins, glycan-specific antibodies, aptamers, peptides, etc., and offer simpler screening tools (Haab & Klamer, 2020; Nagaraj et al., 2010; Tommasone et al., 2019). However, these techniques require labeling of molecules and tend to be low-throughput. Among the "label-free" biomarker detection techniques being developed, electrochemical biosensors have excellent potential for glycan analysis. Relative simplicity, high sensitivity, ease of miniaturization, short assay time, and low cost make them appealing clinical diagnostic devices for glycan profiling (Nagaraj et al., 2010). Among these devices, the microelectronic biosensor, NanoMonitor, which utilizes electrochemical impedance spectroscopy (EIS) as the detection modality, has previously been reported as a highly sensitive glycan detection platform (Nagaraj et al., 2010).

Protocols for the use of NanoMonitor for glycosylation analysis are described. Basic Protocol 1 describes the preparation of the NanoMonitor surface for glycan biosensing, including functionalization of the electrode surface and immobilization of lectins as capture probe on the sensor surface. The Support Protocol provides a method to synthesize glycoform variants of fetuin as test glycoproteins. Basic Protocol 2 outlines the electrochemical impedance spectroscopy (EIS) technique for glycan detection. This protocol also includes a comparison of NanoMonitor performance with lectin-based ELISA for analysis of (i) glycoform variants of fetuin and (ii) cancer glycoproteins. The rationale

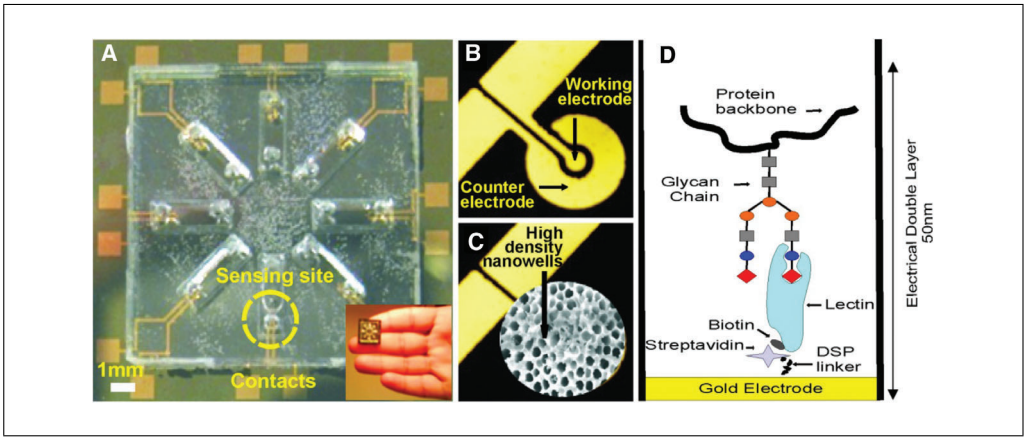

Figure 1 (**A**) The NanoMonitor consists of a base silicon microplatform with eight gold metal sensing sites to form a hand-held diagnostics device (insert). Individual sensing sites are overlaid with the nanoporous alumina membrane, and the microplatform is encapsulated by an acrylic microfluidic chamber to ensure physical isolation between individual sites. (**B**) Each sensing site is composed of a gold working electrode (25 µm diameter) and a counter electrode (125 µm). (**C**) The nanoporous alumina membrane (not to scale) is used to create a high-density array of nanowells on the sensing site. (**D**) Schematic representation of the interaction of biomolecules (not to scale) at the electrical double layer. Within each nanowell, biotinylated lectin molecules are linked to the gold electrode surface through streptavidin that is conjugated to the gold surface via the DSP linker. The binding of glycan structures of proteins from a test sample to lectins within the nanowells results in perturbations of the electrical double layer that is measured as a change in impedance. DSP: dithiobis succinimidyl propionate.

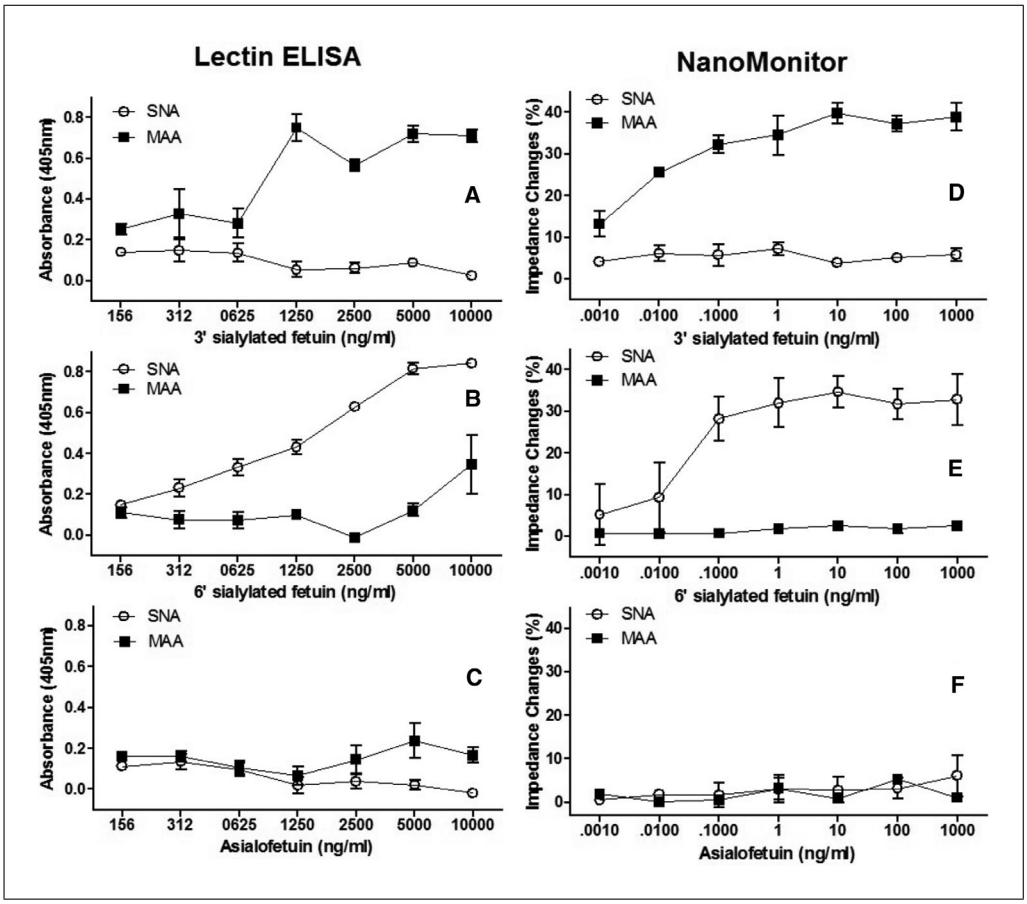

Figure 2 Comparison of the performance of NanoMonitor with that of lectin-based ELISA for the detection of protein glycosylation. Asialofetuin and sialylated glycoforms of fetuin exclusively containing α (2–3)-linked or α (2–6)-linked terminal sialic acids were analyzed for binding to SNA lectin (recognizes terminal sialic acid on glycan with an α(2–6) linkage to galactose) and MAA lectin (recognizes terminal sialic acid on glycan with an α(2–3) linkage to galactose). Graphs on the left panel show lectin-based ELISA results for the interactions of the various concentrations of (**A**) 3′SF, (**B**) 6′SF, and (**C**) ASF with SNA and MAA. Graphs on the right panel show NanoMonitor results for the interactions of the various concentrations of 3′SF (**D**), 6′SF (**E**), and ASF (**F**) with SNA and MAA. Error bars represent standard error of the mean ($n = 3$). 3′SF: 3´ sialylated fetuin; 6′SF: 6´ sialylated fetuin; ASF: asialofetuin; MAA: *Maackia amurensis* agglutinin; SNA: *Sambucus nigra* agglutinin.

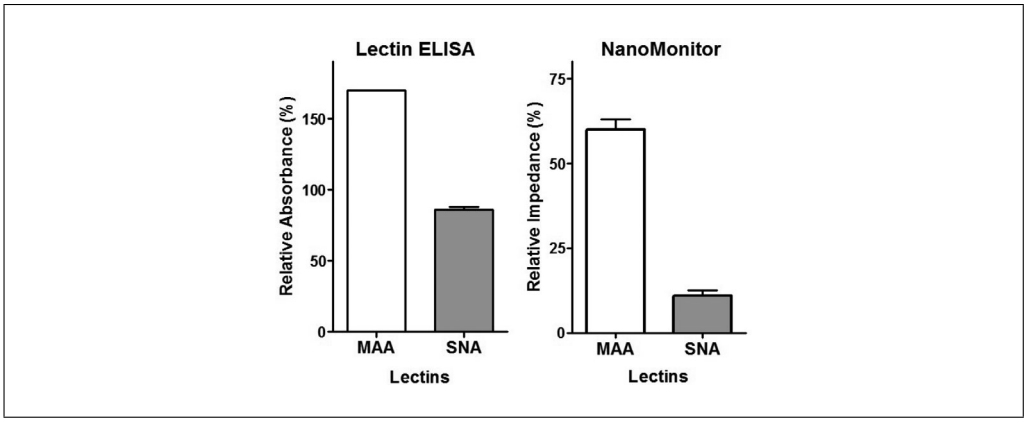

Figure 3 Analysis of global sialylation pattern of proteins from cultured human pancreatic cancer cells (BxPC-3) using lectin-based ELISA and NanoMonitor. The same extract of total proteins from BxPC-3 cells (10 µg/ml) was analyzed for binding to MAA and SNA lectins using lectin-based ELISA and NanoMonitor. Error bars represent standard error of the mean ($n = 3$). MAA: *Maackia amurensis* agglutinin; SNA: *Sambucus nigra* agglutinin.

and methodology for performing EIS experiments using NanoMonitor are discussed in detail in the Commentary section. Figure 1 depicts the NanoMonitor design, assay details, and biosensor operation. Figure 2 shows data comparing the performance of NanoMonitor with that of lectin-based ELISA. Figure 3 illustrates the analysis of global sialylation patterns of proteins from cultured human pancreatic cancer cells (BxPC-3) using lectin-based ELISA and NanoMonitor.

BASIC PROTOCOL 1

PREPARING THE NanoMonitor SURFACE FOR GLYCAN BIOSENSING

This protocol is adapted from a previous work by our group (Nagaraj et al., 2010). The NanoMonitor consists of a microfabricated silicon platform with eight microscale gold sensing sites (Fig. 1A). Each sensing site is composed of two concentric gold circles, which act as the working and counter-electrodes (Fig. 1B). The inner circle, which is the working electrode, has a 25-μm diameter, while the outer circle is the counter-electrode with a 125-μm diameter. The electrodes are overlaid with nanoporous alumina membrane (pore diameter of 20 nm). The nanoporous alumina membrane (50% porosity with 10^6 pores/mm^2) results in the formation of a high density of nanowells on the sensor surface. The device consists of eight fluid channels encapsulated in an acrylic microfluidic chamber (Fig. 1A). The channels are physically isolated from each other, and each channel has an inlet and an outlet port at opposite ends. The volume per channel is 10 μl. The chamber is designed so that the channel is flush over the sensing site. To ensure physical immobilization of the alumina membrane at the sensing site, the dimensions of the chamber are such that its perimeter is flush with the perimeter of the nanoporous alumina membrane on each sensing site (Fig. 1A). The biosensor device utilizes impedimetric transduction of lectin-glycan binding, and the change in electrical impedance (measured across the working and counter electrodes) is reported as a function of the amount of glycoprotein bound to the surface.

The NanoMonitor device for glycan biosensing can be prepared into two steps: (1) functionalizing the gold electrode surface with a cross linker; and (2) immobilizing lectins on the sensor surface. The first step requires chemisorption of a thiol crosslinker on the gold electrode for subsequent immobilization of lectins in the second step. Functionalization of the gold electrode has been achieved by coating the surface with the cross-linking agent, dithiobis succinimidyl propionate (DSP). DSP contains an amine-reactive *N*-hydroxysuccinimide ester at each end of two eight-carbon spacer arms that are linked together with a disulfide bond. The disulfide linkage of DSP chemisorbs rapidly to the gold surface, forming monolayers of the DSP molecules on the gold surfaces while the exposed *N*-hydroxysuccinimide groups are available for binding to the primary amine groups of proteins. Immobilization of lectins to the functionalized gold surface was achieved using biotin-streptavidin linker chemistry. This protocol is adapted from a previous work by our group, and the concentrations and volumes of the reagents and chemicals have been reported based on the electrode dimensions mentioned above (Nagaraj et al., 2010). All volumes used are 10 μl, unless otherwise noted. The samples (both doses and washes) are pipetted into the channel inlet and allowed to incubate on the sensor surface, and almost all the fluid volume is aspirated out and removed from the channel outlet post incubation. It is important to ensure that the membrane surface is not in direct contact with the pipette tip during fluid addition and removal.

NOTE: DSP is non-water-soluble and needs to be first dissolved in an organic solvent (such as DMSO) and added to the aqueous reaction mixture. DMSO has a high freezing point of 18.5°C (65.3 °F); hence, the prepared DSP solution (dissolved in DMSO) should not be stored in the refrigerator.

NOTE: DSP is light sensitive and should be stored in the dark.

NOTE: DSP is moisture-sensitive and should be stored desiccated at 4°-8°C. To avoid moisture condensation, the vial must be equilibrated to room temperature before opening (equilibration may require 30 min).

NOTE: DSP must be reconstituted immediately before use. The NHS-ester moiety readily hydrolyzes and becomes nonreactive; therefore, stock solutions should not be prepared for storage. Any unused reconstituted crosslinker must be discarded.

Materials

Dithiobis succinimidyl propionate (DSP; Thermo Fisher Scientific Inc., cat. no. 22585)

Alternatively, DTSSP [dithiobis(sulfosuccinimidyl propionate)] can be used for crosslinking. DTSSP is water soluble (unlike DSP, it is sulfonated) and can be used directly with aqueous buffers.

Dimethylsulfoxide (DMSO; Thermo Fisher Scientific Inc.; CAS: 67-68-5)

1× phosphate-buffered saline (PBS; Thermo Fisher Scientific Inc., cat. no. 10010023, or equivalent)

Streptavidin (Thermo Fisher Scientific Inc., cat. no. 434301)

Biotin-conjugated lectin: *Sambucus nigra* agglutinin (SNA; EY Laboratories, cat. no. SNA-I BA-6802-1)

Biotin-conjugated lectin: *Maackia amurensis* agglutinin (MAA; EY Laboratories, cat. no. MAA BA-7801-1)

SuperBlock blocking buffer (Thermo Fisher Scientific Inc., cat. no. 37515)

Nanoporous alumina membrane (Whatman Scientific; pore diameter = 20, 50% porosity, 10^6 pores/mm^2)

Single-channel pipettors (1000, 200, and 20 µl)

Functionalize the electrode surface

1. Allow DSP to equilibrate to room temperature prior to mixing with DMSO. Prepare a 4 mg/ml solution of DSP in DMSO.

2. Incubate the gold surface of the working electrode with DSP solution for 30 min at room temperature.

   To clarify, the working electrode is made of gold and is covered with nanoporous alumina membrane. The thiol linkage happens between the DSP cross linker and the underlying gold electrode electrode.

3. Perform two 1× PBS washes to remove excess unbound DSP.

   As in all all other steps, 10 µl of PBS is used for washing. As mentioned in the introductory portion of Basic Protocol 1, the samples (both doses and washes) are pipetted into the channel inlet, allowed to incubate on the sensor surface, and almost all the fluid volume iss aspirated out and removed from the channel outlet post incubation. It is important to ensure that the membrane surface is not in direct contact with the pipette tip during fluid addition and removal. There is no particular incubation time between washes.

4. Next, prepare 1 mg/ml streptavidin solution in 1× PBS.

5. Add 1 mg/ml streptavidin solution to the electrode surface containing the DSP crosslinker and incubate for 2 hr.

6. Wash off excess unbound streptavidin two times with 1× PBS (see step 3).

   DSP linkage to the gold surface and saturation concentrations are previously determined using fluorescently labeled streptavidin (Bothara et al., 2008).

Immobilize lectins on the sensor surface

7. Add biotinylated lectins—*Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA) at 1 mg/ml in PBS —to the streptavidin-coated gold surface and incubate for 15 min at room temperature.
8. Wash off unbound lectins three times with 1× PBS.
9. Add Super Block buffer to block nonspecific binding sites on the sensor surface for 10 min.

Various concentrations of biotinylated lectins were tested to determine the saturation concentration (1 mg/ml) in previous work (Nagaraj et al., 2010).

SUPPORT PROTOCOL

SYNTHESIS OF GLYCOFORM VARIANTS OF FETUIN

Asialofetuin (ASF) is used as a substrate for sialyltransferase reactions to create fetuin with exclusively 3´-linked terminal sialic acid (3´SF) or with exclusively 6´-linked terminal sialic acid (6´SF) on the glycan chain of the protein. ASF is used as a substrate to create 3´SF and 6´SF. No significant binding is expected between ASF and either SNA or MAA. ASF is used as a negative control, and the interfacial modulation (and the resulting capacitive response) from this non-significant binding in the case of ASF is the negative signal.

Materials

Sodium cacodylate (Sigma Aldrich, cat. no. C0250, or equivalent)
Magnesium chloride (Sigma Aldrich, cat. no. M8266, or equivalent)
Calcium chloride (Sigma Aldrich, cat. no. C4901 or equivalent)
Recombinant rat α (2–3) and α (2–6) sialyltransferase (Calbiochem or equivalent)
Asialofetuin (Sigma Aldrich, cat. no. A4781, or equivalent)
100 mM CMP-sialic acid (Calbiochem, cat. no. 233264 or equivalent)
Test glycoproteins: 1 mg/ml stocks of three glycoforms (ASF, 3′SF and 6′SF) of the glycoprotein fetuin

Amicon Ultra-0.5 centrifugal filter (cat. no. C82301)
Single-channel pipettes (1000, 200, and 20 µl)

1. Perform sialyltransferase reaction to create fetuin in a buffer consisting of 100 mM sodium cacodylate, 10 mM $MgCl_2$, and 5 mM $CaCl_2$ (buffer pH 6.0) for a total reaction volume of 100 µl. 10 mM CMP-sialic acid (10 µl of a 100 mM stock) acts as the sialic acid donor and 1 mg ASF (4 µl of a 25 mg/ml stock) acts as the sialic acid acceptor glycoprotein. Use 5 U of recombinant rat α (2–3) or α (2–6) sialyltransferase enzymes to catalyze the reaction.
2. Incubate the sialyltransferase reaction at 37°C overnight.
3. Purify the modified glycoprotein using a size-exclusion spin column (Amicon® Ultra-0.5) according to the manufacturer's instructions.
4. For analysis using the NanoMonitor (Basic Protocol 2), serially dilute the three glycoforms (ASF, 3′SF, and 6′SF) of the glycoprotein fetuin from a 1 mg/ml stock solution down to the required concentrations (10 to 0.156 µg/ml) in 1× PBS.

The sialylation products can be characterized by lectin-based ELISA (see below), by lectin blots, or by microarray analysis (Nagaraj, Eaton, Thirstrup, & Wiktor, 2008).

The test glycoproteins are created to optimize and understand the sensor performance. It may not be necessary to utilize the test glycoproteins prior to the analysis of each and every sample. However, when specificity and sensitivity issues are noted with the sensor performance, the test glycoproteins can help with troubleshooting.

PERFORMING ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY (EIS) FOR ANALYZING GLYCOPROTEIN STRUCTURES

This protocol is adapted from a previous work of our group (Nagaraj et al., 2010). Detection of protein glycosylation using the NanoMonitor (Fig. 1D) is achieved through EIS, which is based on the principle of double-layer capacitive measurement (Bothara et al., 2008; Reddy, Bothara, Barrett, Carruthers, & Prasad, 2008). An electrical double layer, approximately 50 nm in thickness, is formed at the solid/liquid interface at the base of each nanowell (Fig. 1D). Binding of the glycans to lectins at this interface produces specific and measurable change to impedance measured across the sensing site. As the binding of the biomolecules occurs directly on the NanoMonitor surface and is not mediated through a redox probe, the impedance changes are non-inductive (i.e., capacitive and resistive) in nature. A low AC voltage (typically less than 10 mV) is applied across the sensing site comprising multiple nanowells at a frequency of 1 kHz. The impedance is then measured across the working electrode and counter-electrode on each of the sensing sites, by means of an impedance analyzer.

NOTE: Previous studies have shown that maximum signal-to-noise ratio (change to the impedance from baseline dose) as a result of the interaction of glycans with lectins is obtained at a frequency of 1 kHz.

NOTE: Body fluids such as serum, saliva, urine, cerebrospinal fluid, tears, sweat, semen, vaginal fluid, interstitial fluid, biopsy fluids, etc., can all serve as potential sources of glycoproteins for NanoMonitor analysis.

NOTE: Any standard protocol to isolate plasma or serum from whole blood can be used for sample preparation. The prepared samples should be devoid of red blood cells, white blood cells, platelets, and any kind of cell debris. Such samples can be directly analyzed on the NanoMonitor. Similarly, body fluids without any particulate matter can be directly used for analysis. For glycosylation analysis of specific serum glycoproteins, appropriate protein isolation protocols will have to be followed prior to the use of NanoMonitor.

Materials

- 1× phosphate-buffered saline (PBS; Thermo Fisher Scientific Inc., cat. no. 10010023, or equivalent)
- Glycoproteins for analysis
- Glycoform variants of fetuin (Support Protocol)
- Sodium bicarbonate buffer: 0.1 M $NaHCO_3$ in water (Thermo Fisher Scientific Inc., MFCD00003528)
- 100 mM Tris-buffered saline containing 0.05% Tween (TBST; Cepham Life Sciences, cat. no. 10420, or equivalent)
- SuperBlock blocking buffer (Thermo Fisher Scientific Inc., cat. no. 37515)
- Biotin-conjugated lectin: *Sambucus nigra* agglutinin (SNA; EY Laboratories, cat. no. SNA-I BA-6802-1)
- Biotin-conjugated lectin: *Maackia amurensis* agglutinin (MAA; EY Laboratories, cat. no. MAA BA-7801-1)
- Extravidin−alkaline phosphatase (Sigma-Aldrich, Product Number: E2636)
- *p*-nitrophenyl phosphate (SigmaFast PNPP tablets, Sigma-Aldrich, cat. no. N1891)
- Human pancreatic carcinoma BxPC-3 (American Type Culture Collection)
- RPMI 1640 (Invitrogen)
- 10% FBS (Invitrogen)
- Cell stripper (Mediatech)
- M-PER mammalian protein extraction reagent (Pierce Chemicals)
- Protease inhibitor cocktail tablets (Hoffmann-La Roche AG)

Gamry 600 Potentiostat (Gamry Instruments, PA) and computer running Gamry software
NanoMonitor (Basic Protocol 1)
96-well Nunc MaxiSorp plates (Thermo Fisher Scientific, cat. no. 44-2404-21)
Single-channel pipettors (1000, 200, and 20 µl) and multichannel pipettors (200 and 20 µl)
Microplate spectrophotometer (Spectramax M5, Molecular Devices, CA, USA)
Centrifuge
Qubit Protein Quantitation system (Invitrogen Corporation)

Analyze protein glycosylation using NanoMonitor

1. For each of the NanoMonitor's eight sensing sites, connect the working and working sense electrode probes of the Gamry potentiostat to the working electrode of the sensor, and the reference and counter electrode probes of the Gamry potentiostat to the counter electrode of the sensor (Fig. 1).

2. Turn on the Gamry potentiostat. Open Gamry software and ensure that the device is identified by the computer. Select a destination folder and appropriate file name to store EIS data.

   Refer to Internet Resources for instructions for using Gamry Potentiostat.

3. In the Gamry software, open Sequence Wizard and set up sequence to take EIS measurement

   a. Add "Manual Potentiostat Select" to GUI.
   b. Add "Loop" and "Delay" to GUI (double click in GUI to bring up options).
   c. Add "Single Frequency EIS" Module to GUI

      Ensure that the EIS module is embedded under the Loop command.

   d. Assign appropriate filename.
   e. Set frequency to 1 kHz.
   f. Create Gamry sequence by setting desired delays (based on incubation time of assay step) and loops/iterations for each dose.

4. Dispense 10 µl of 1× PBS (0 pg/ml glycoprotein) and execute the Gamry sequence to determine baseline impedance.

5. Next dispense the lowest glycoprotein concentration (1 pg/ml of fetuin) to be analyzed followed by a 15-min incubation to allow sufficient time for the lectin-glycan binding to occur as well as for the signals to stabilize. Obtain EIS measurement.

   Here fetuin can mean 3'SF or 6'SF depending on the target of interest (which may be 3'SF or 6'SF depending on which is being studied) or can also mean ASF when conducting negative control experiments.

6. Next dispense increasing concentrations of glycoproteins/fetuin under study starting from 1 pg/ml, and obtain measurements.

   Steps 5-7 are valid for all kinds of glycoproteins that may be tested. As mentioned in the Critical Parameters section, this protocol is applicable not only for the two test glycoproteins (i.e., 3'SF and 6'SF) and their substrate (ASF which may be for negative control experiments), but is also applicable for any other glycoprotein obtained from clinical samples. To generalize this, the term "glycoproteins/fetuin" has been used.

7. Before addition of the next higher concentration of glycoprotein/fetuin under study, wash the sensing sites two times with 1× PBS.

   A concentration range of 1 pg/ml to 1 µg/ml can be tested on the NanoMonitor.

After the addition of each glycoprotein solution, impedance changes occur. These impedance changes are reported for each addition dose relative to the baseline impedance (i.e., percent change in modulus of impedance for a given dose with respect to baseline at 1 kHz).

The NanoMonitor has eight sensing sites that are physically isolated from each other. At least three sites should be saturated with a single lectin, and the binding response of one specific glycoprotein should be measured in parallel to assess reproducibility across the sites.

Once the performance metrics (repeatability, reproducibility, accuracy, linearity, etc.) have been evaluated and sensing parameters have been optimized, up to 8 lectin-glycan interactions can be probed at once.

Analyze glycoform variants of fetuin by lectin-based ELISA

8. Serially dilute specific sialylated versions of previously prepared fetuin (Support Protocol) from 10 to 0.156 μg/ml in 0.1 M $NaHCO_3$.
9. Add to 96-well Nunc MaxiSorp plates and incubate overnight at 4°C to allow adsorption of the proteins to the polystyrene.
10. Manually wash off unbound proteins three times with 100 μl of 100 mM TBST.
11. Block the unoccupied sites on the polystyrene surface using SuperBlock blocking buffer.
12. Add 20 μg/ml of biotin-conjugated lectins, SNA and MAA (in PBS), to the glycoprotein-coated plates and incubate with shaking for 2 hr at room temperature.
13. After washing, add extravidin-alkaline phosphatase diluted 1:70,000 in TBST to the plates and incubate for 1 hr at room temperature with gentle shaking.
14. Wash off unbound enzyme at least three times with TBST.
15. Add the substrate for alkaline phosphatase (*p*-nitrophenyl phosphate) to the wells and incubate for 1 hr.
16. Using a microplate spectrophotometer (Spectramax M5), record the absorbance at 405 nm.

Lectin-based ELISA is used as a conventional laboratory technique to assess NanoMonitor's glycosylation analysis capabilities. The above step is optional.

Analyze cancer glycoproteins

17. Culture human pancreatic carcinoma BxPC-3 according to American Type Culture Collection's recommendations (see Key References). Briefly, BxPC-3 cells are cultured in a medium consisting of RPMI 1640 with 10% FBS.

Seeding is done at 15%-20% confluence and cells are harvested when the flask was about 75% confluent (3-4 days, approximately).

18. When the cultures reach approximately 75% confluence, harvest the cells using cell stripper and pellet by centrifugation for 3 min at 43 × *g* (800 rpm for 6 cm rotor radius), room temperature.
19. Extract the total proteins using the M-PER mammalian protein extraction reagent according to the instructions from the manufacturer. Use a protease inhibitor to prevent proteolysis.
20. Quantify proteins using the Qubit Protein Quantitation system or another protein quantification technique and freeze at −20°C until further analysis using NanoMonitor or ELISA.

21. For NanoMonitor analysis follow steps 1-7 above.

COMMENTARY

Background Information

Glycosylation is the enzymatic process in which oligosaccharide chains (glycans) are added to newly synthesized proteins (Nagaraj et al., 2010; Wang, Zhu, Lubman, & Gao, 2019). It is a post-translational modification generated by complex biochemical pathways and is considered a non-template-driven enzymatic process (Reily et al., 2019; Wang et al., 2019). Glycans play an important role in several key biological processes such as cellular growth and differentiation. Glycans can act as the first line of communication between the host and pathogen, and have a role in both the innate and adaptive immune system (Haab & Klamer, 2020; Lin et al., 2020). Specific changes in glycan structure have been linked to several diseases. Aberrant glycosylation has been labeled a "hallmark of cancer" due to its association with carcinogenesis and cancer cell metastasis (Munkley & Elliott, 2016; Reily et al., 2019; Wang et al., 2019). Other disease-associated glycans have utility as biomarkers for various diseases, including cancer (Peiris et al., 2017).

Conventional chemical analysis tools such as NMR, mass spectrometry, etc., have played a crucial role in understanding the composition and structure of glycan chains, including those that can be used as biomarkers. This knowledge is being applied by other techniques to enable routine and rapid screening of clinical samples. In particular, techniques that do not involve extensive purification or chemical modifications to add a fluorophore, nanoparticle, enzymes, or other tags have a lot of practical advantage when analyzing glycan biomarkers. A few emerging techniques include surface plasmon resonance (Foley, Forzani, Joshi, & Tao, 2008; Rosencrantz et al., 2016), fluorescence resonance energy transfer between quantum dots and gold nanoparticles (Kim, Park, Oh, Oh, & Kim, 2009), quartz crystal micro- balance (Lyu, Lim, Lee, Kim, & Lee, 2008; Pei, Anderson, Aastrup, & Ramström, 2005; Yakovleva, Safina, & Danielsson, 2010), and electrochemical (Bothara et al., 2008; La Belle, Gerlach, Svarovsky, & Joshi, 2007; Nagaraj et al., 2010; Reddy et al., 2008; Sadik & Yan, 2007) or piezoelectric biosensors (Belický, Katrlík, & Tkáč, 2016; Serra, Gamella, Reviejo, & Pingarrón, 2008; Silva, 2019). Among these technologies, electrochemical glycan biosensors such as the NanoMonitor are especially appealing owing to high sensitivity, compatibility with microfabrication and miniaturized electronics, mass producibility, and cost effectiveness. The NanoMonitor design incorporates the three basic elements of a typical electrochemical sensor: (i) lectins as biorecognition elements chemically linked to the sensor electrode surface; (ii) an electrochemical transducer that detects electrical current or potential changes that occur at the sensor surface due to lectin-glycan binding; and (iii) electronics to measure and interpret the electrical signals to deduce the presence of target glycans in the sample being analyzed. The principle of NanoMonitor operation is based on electrochemical impedance spectroscopy (EIS), wherein electrical impedance changes at the lectin-glycan-electrode surface in alternating current steady state are measured by imposing a small sinusoidal voltage at a pre-determined frequency. Changes in the amplitude and phase of the sinusoidal signal resulting in the measured impedance changes reflect recognition as well as binding of the glycan with the corresponding lectin ligand. EIS allows glycan-lectin binding events to be probed in a simple, sensitive, label-free and redox mediator−free manner. Another unique feature of NanoMonitor as compared to other EIS-based biosensors is the incorporation of a nanoporous alumina membrane on the sensor surface. This creates nanowells on the sensor surface within which the lectin-glycan binding occurs. This unique three-dimensional environment has been previously investigated extensively to enhance sensitivity and improve binding kinetics (Bothara et al., 2008; Lin et al., 2010).

Critical Parameters

Glycoproteins for NanoMonitor analysis

To test the capabilities of NanoMonitor, two glycoproteins, with the same glycan composition but with a very subtle difference in the linkage of the terminal sugar to the rest of the oligosaccharide chain, were employed. Such differences are known to occur in vivo due to changes in the cellular glycosylation machinery during various diseases including cancer. The protocols developed for the analysis of test glycoproteins are also applicable for the analysis of glycan biomarkers from clinical samples.

Analysis of the glycoforms of fetuin protein with NanoMonitor

The serum protein fetuin was enzymatically modified with sialyltransferases to create two uniquely sialylated versions of the protein that differ only in the chemical linkage of the terminal sialic acid to the glycan chain. The glycoform variants of fetuin represent possible effects of altered cellular glycosylation pathways and were therefore chosen for proof-of-concept studies with the NanoMonitor. The results described here highlight the excellent correlation between the specific binding pattern of the various glycoform variants of fetuin to SNA/MAA lectins on the NanoMonitor as compared to observations with western blots and lectin-based ELISA previously reported. In addition, the detection sensitivity of NanoMonitor is approximately five orders of magnitude higher than that of lectin-based ELISA. The NanoMonitor was able to distinguish between α (2–6) sialylated glycoforms of fetuin and the α (2–3) sialylated version at less than 1 pg/ml protein concentration. Literature suggests that previously the detection of α (2–3)-linked sialic acid with such high sensitivity was only reported for studies that involved conjugation to gold nanoparticles or the use of electrochemical redox probe−based impedance spectroscopy (La Belle et al., 2007) or, to a lesser extent, microarray analysis of fluorescently tagged glycoproteins (Nagaraj et al., 2008). NanoMonitor showed higher sensitivity than a fluorescence resonance−based energy transfer technique where concanavalin A lectin−linked quantum dots and dextran-coated gold nanoparticles were used for the detection of mannose or galactose conjugated to bovine serum albumin in the nanomolar range (Kim et al., 2009). The NanoMonitor is capable of glycan quantification for a wide range of glycoprotein concentrations (< 1 pg/ml to > 10 ng/ml) with significant robustness and repeatability. The reasons for enhanced sensitivity of glycosylation analysis with the NanoMonitor are speculated to be similar to those responsible for enhanced protein detection reported previously (Bothara et al., 2008; Reddy et al., 2008)—and can be attributed, in part, to the effects of the nanoporous alumina membrane.

Analysis of cancer glycoproteins

Preparation of human samples for glycan biomarker analysis with the NanoMonitor is rapid and simple, since the need for sample tagging with a fluorophore or other tags is eliminated. To illustrate this, crude protein extracts from the cultured human pancreatic ductal adenocarcinoma cell line BxPC-3 were analyzed with the protocols described here. Based on interaction with the SNA and MAA lectins on the NanoMonitor, as well as lectin-based ELISA, a significantly higher degree of α (2–3) protein sialylation was observed. This could be indicative of enhanced expression of cellular α (2–3) sialyltransferases as compared to α (2–6) sialyltransferases to confer growth and metastatic advantages upon pancreatic cancer cells (Aubert et al., 2000). Since the NanoMonitor has multiplexed analysis capability, a future version of the device can be configured for the detection of multiple cancer glycan biomarkers simultaneously in human samples using a panel of appropriate lectins or other ligands. Design enhancements to the electronics and optimization of protocols will allow the NanoMonitor to be employed as a cost-effective miniature standalone clinical electronic biosensor device.

Troubleshooting

See Table 1 for a NanoMonitor troubleshooting guide.

Understanding Results

Analysis of glycoforms of fetuin

The NanoMonitor is capable of reliably distinguishing between closely related glycan structures. As a proof of concept, the Basic Protocol 2 describes the detection and analysis of ASF and sialylated glycoforms of fetuin: 3′SF or 6′SF. Fetuin has been routinely used as a model glycoprotein for glycosylation studies by different groups. The binding patterns of these fetuin glycoforms to lectins were previously confirmed using western blots (see supplementary data of the original work at *https://www.futuremedicine.com/toc/nnm/5/3*). The dynamic range for the NanoMonitor was identified by correlation studies with a lectin-based ELISA technique (Duk, Lisowska, Wu, & Wu, 1994). In lectin-based ELISA experiments (discussed in Basic Protocol 2), ASF shows hardly any binding to either SNA or MAA due to the absence of sialic acid (shown in Fig. 2C). 3′SF results in binding to MAA but not SNA (shown in Fig. 2A), while 6′SF results in binding to SNA but not MAA (shown in Fig. 2B). The lower limit of detection of both the sialylated glycoforms of fetuin (3′SF and 6′SF) with lectin-based ELISA was 0.312 μg/ml, higher than that obtained using conventional

Table 1 Troubleshooting Guide Glycosylation Analysis Using NanoMonitor

Problem	Possible cause	Solution
Low protein yield from biological sample (blood, tumor sample etc.)	Inadequate extraction methods	Try other protein extraction methods and buffers
	Protein degradation by proteases	Use appropriate protease inhibitors
Turbid samples or presence of particulates	Presence of cells (red blood cells, white blood cells, platelets, tumor cells etc.) and extracellular matter	Perform sample pre-processing prior to analysis on the sensor or modification of the sensor fluid delivery design to allow microfluidics-based filtration
Poor functionalization and saturation of sensor surface during cross-linking of lectins	Cross linker is not functional	Prepare DSP linker solution carefully as described in the Materials section of Basic Protocol 1 and use immediately
	Low concentration or degradation of lectin stocks	Check and replace with fresh lectin stocks if necessary
Poor potentiostat (Gamry Instruments) connectivity resulting in overload or erroneous measurements	Connection not established between the potentiostat and the software interface (Gamry Instruments)	Check all cable connections After turning on the potentiostat, wait for at least 10 min to ensure stable communication between the hardware and the software If there is an overload signal, check to see that the volume of liquid loaded onto the sensor does not short the contacts to the potentiostat
Poor electrochemical signal associated with the assay	Ionic noise due to noise in the buffer resulting in poor signal to noise ratio	Identify the frequency for EIS analysis that provides the highest electrochemical signal for the glycan-capture probe combination of interest by analyzing the frequency spectra (Bode phase and frequency plots—these can be obtained from the Echem interface of the Gamry Potentiostat)
Variability in measured electrochemical signal between the sensing electrodes	Non-uniform functionalization of the ligands on the sensing electrode	Automate deposition of biomarker capture agents as well as the samples to be screened using noncontact inkjet printing and reduce manual sample handling
	Insufficient time allowed for obtaining the measurement resulting in capturing of the diffusion characteristics of the electrochemical signal	Allow sufficient time equal to or greater than the incubation time to obtain the electrochemical signal from each of the sensing electrodes
Narrow dynamic range of detection	Saturation of sensor response due to high analyte concentrations in the test sample (abnormally elevated cases)	Increase density of capture agents on the sensor surface to increase the surface area to volume ratio for active sensing region
No impedance changes after sample addition	Possible hydrolysis of NHS ester moiety of DSP crosslinker	Hydrolysis of NHS ester is a major competing reaction of the acylation reaction and should be avoided. Hydrolysis increases with increasing pH and occurs more readily in dilute protein or peptide solutions.

(*Continued*)

Table 1 Troubleshooting Guide Glycosylation Analysis Using NanoMonitor, *continued*

Problem	Possible cause	Solution
Inaccurate or irreproducible data	Gamry potentiostat not calibrated properly	During calibration, ensure that the potentiostat is properly grounded. Use the "Calibration" side of the Dummy cell. The cell should be shielded within a Faraday cage to avoid interference from electromagnetic fields Check cell cable integrity and ensure that the cable passes the check

Table 2 Summary of the Performance of the NanoMonitor in Comparison to Lectin-Based ELISA for the Detection of the Glycoform Variants of Fetuin

Performance parameter	NanoMonitor	Lectin-based ELISA
Detection specificity		
3´ sialylated fetuin	≥1 pg/ml	≥0.156 µg/ml
6´ sialylated fetuin	≥100 pg/ml	≥0.312 µg/ml
Detection sensitivity		
3´ sialylated fetuin	1 pg/ml	0.156 µg/ml
6´ sialylated fetuin	1 pg/ml	0.312 µg/ml
Other parameters		
Linear range of detection	1 pg/ml–10 ng/ml	0.312–5 µg/ml
Volume per assay	10 µl maximum	50 µl minimum
Response time per sample	<15 min	Approximately 4 hr
Detection method	Electrochemical impedance	Enzyme-linked colorimetric assay

antibody-based ELISA. This may be because affinity of lectins for carbohydrates (K_d: 10^{-4}–10^{-7}M) is much lower than that for antibody-antigen interactions (K_d: 10^{-8}–10^{-12}M) (Kam & Poon, 2008). However, the precision of detection limits was comparable with the results from other groups (Gornik & Lauc, 2007; Piva, Moreno, & Sharpe-Timms, 2003). Non-specific interactions were observed for concentrations greater than 5 µg/ml (shown in Fig. 2B). The performance parameters of the lectin-based ELISA have been listed for the researcher's reference in Table 2.

Basic Protocol 2 also discusses the use of NanoMonitor to evaluate of interaction of the three fetuin glycoforms (ASF, 3′SF and 6′SF) with the lectins, SNA and MAA, using EIS analysis. After the lectin-conjugation step, the sensor surface is subjected to the baseline dose (also known as the blank dose or "zero dose"), which corresponds to 0 ng/ml glycoproteins (PBS only). Impedance values corresponding to the baseline dose are observed in the range of hundreds of kΩ. Binding of various concentrations of a glycoprotein present in a given sample to the lectin capture probe causes the modulation of interfacial impedance. The output, for a given dose, is reported as percentage change in impedance modulus relative to the baseline dose. The performance metrics of the NanoMonitor for glycoprotein analysis are listed in Table 2.

Figure 2D shows the calibrated dose-response curve of 3′SF with the lectins SNA and MAA on the NanoMonitor. Linear dynamic range was obtained for 1 pg/ml to 1 µg/ml, and a lower limit of detection of 1 pg/ml was observed. Limit of detection was defined as the dose of the fetuin that produces a minimum of 3% change in the impedance from the baseline which is sufficient to reliably distinguish a binding event/interaction from baseline. For MAA, the output percentage change in impedance ranged from 13% to 38%. For the same range of concentrations of 3′SF, the impedance changes were 5% to 8% during its interaction with SNA.

Figure 2E shows the calibrated dose response curve of 6′SF with the lectins SNA

and MAA on the NanoMonitor. Linear dynamic range was obtained for 1 pg/ml to 1 μg/ml, and the lower limit of detection was 1 pg/ml. Specific interaction was observed with SNA, with the output percentage change in impedance ranging from 6% to 33%. For the same range of concentrations of 6′SF, the impedance changes were 1% to 4% during its interaction with MAA.

As expected, no significant binding was observed between ASF and either SNA or MAA. Figure 2F shows that less than 5% change in signal was observed for the ASF concentration range of 1 pg/ml to 1 μg/ml.

Analysis of cancer glycoproteins

Basic Protocol 2 ("Analyze cancer glycoproteins" section) discusses the analysis of binding of total proteins extracted from cultured cancerous (BxPC-3) human pancreatic cells to SNA and MAA. Figure 3 shows the global sialylation pattern of proteins using both NanoMonitor and lectin-based ELISA. For the NanoMonitor, a 65% change to the impedance from the baseline was observed during the interaction of BxPC-3 cell extracts with MAA, while a 9% change in impedance was observed for the interaction of the same extracts with SNA. This is because human pancreatic cancer cells express significantly higher amounts of α (2–3)-linked sialic acids. A similar pattern of binding to MAA and SNA lectins was observed for lectin-based ELISA analysis (Fig. 3).

Time Considerations

In Basic Protocol 1, DSP crosslinker takes 30 min (in dark) and streptavidin binding takes 2 hr incubation time. Subsequent lectin immobilization takes 15 min at room temperature. In Support Protocol 1, sialyltransferase reaction takes place at 37°C overnight. In Basic Protocol 2, EIS analysis takes less than 15 min while lectin-based ELISA analysis typically takes about 4 hr.

Acknowledgments

The authors would like to thank Srivatsa Aithal, Seron Eaton, Manish Bothara, and Peter Wiktor for their original contributions to NanoMonitor: a miniature electronic biosensor for glycan biomarker detection. The authors would also like to thank the Office of Naval Research, Nanoelectronics and Nanometrology Program (N00014.07.1.0457) and the Biodesign Institute at Arizona State University (ASU) for financial support for the original work.

Author Contributions

Antra Ganguly: writing original draft, writing review and editing; **Kai-Chun Lin**: writing review and editing; **Sriram Muthukumar**: writing review and editing; **Vinay Nagaraj**: conceptualization, formal analysis, investigation, methodology, resources, supervision, validation, visualization, writing review and editing; **Shalini Prasad**: conceptualization, formal analysis, investigation, methodology, project administration, resources, software, writing review and editing.

Conflict of Interest

Drs. Shalini Prasad and Sriram Muthukumar have a significant interest in Enlisense LLC, a company that may have a commercial interest in the results of this research and technology. Financial support was received from Office of Naval Research, Nanoelectronics and Nanometrology Program (N00014– 07–1–0457) and the Biodesign Institute at Arizona State University (ASU) for th original work, "NanoMonitor: a miniature electronic biosensor for glycan biomarker detection." The potential individual conflict of interest has been reviewed and managed by The University of Texas at Dallas and played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Data Availability Statement

Data sharing is not applicable to this article, as no new data were gathered or analyzed in this study.

Literature Cited

Belický, Š., Katrlík, J., & Tkáč, J. (2016). Glycan and lectin biosensors. *Essays in Biochemistry*, *60*(1), 37–47. doi: 10.1042/EBC20150005.

Bothara, M., Venkatraman, V., Reddy, R. K. K., Barrett, T., Carruthers, J., & Prasad, S. (2008). Nanomonitors: Electrical immunoassays for protein biomarker profiling. *Nanomedicine*, *3*(4), 423–436. doi: 10.2217/17435889.3.4.423.

Duk, M., Lisowska, E., Wu, J. H., & Wu, A. M. (1994). The biotin/avidin-mediated microtiter plate lectin assay with the use of chemically modified glycoprotein ligand. *Analytical Biochemistry*, *221*(2), 266–272. doi: 10.1006/abio.1994.1410.

Foley, K. J., Forzani, E. S., Joshi, L., & Tao, N. (2008). Detection of lectin-glycan interaction using high resolution surface plasmon resonance. *Analyst*, *133*(6), 744–746. doi: 10.1039/b719321a.

Gornik, O., & Lauc, G. (2007). Enzyme linked lectin assay (ELLA) for direct analysis of transferrin sialylation in serum samples. *Clinical Biochemistry*, *40*(9–10), 718–723. doi: 10.1016/j.clinbiochem.2007.01.010.

Haab, B. B., & Klamer, Z. (2020). Advances in tools to determine the glycan-binding specificities of lectins and antibodies. *Molecular and Cellular Proteomics*, *19*, 224–232. doi: 10.1074/mcp.R119.001836.

Kam, R. K. T., & Poon, T. C. W. (2008). The potentials of glycomics in biomarker discovery. *Clinical Proteomics*, *4*, 67–79. doi: 10.1007/s12014-008-9017-9.

Kim, Y. P., Park, S., Oh, E., Oh, Y. H., & Kim, H. S. (2009). On-chip detection of protein glycosylation based on energy transfer between nanoparticles. *Biosensors and Bioelectronics*, *24*(5), 1189–1194. doi: 10.1016/j.bios.2008.07.012.

La Belle, J. T., Gerlach, J. Q., Svarovsky, S., & Joshi, L. (2007). Label-free impedimetric detection of glycan-lectin interactions. *Analytical Chemistry*, *79*(18), 6959–6964. doi: 10.1021/ac070651e.

Lin, B., Qing, X., Liao, J., & Zhuo, K. (2020). Role of protein glycosylation in host-pathogen interaction. *Cells*, *9*, 122. doi: 10.3390/cells9041022.

Lin, K.-C., Kunduru, V., Bothara, M., Rege, K., Prasad, S., & Ramakrishna, B. L. (2010). Biogenic nanoporous silica-based sensor for enhanced electrochemical detection of cardiovascular biomarkers proteins. *Biosensors and Bioelectronics*, *25*(10), 2336–2342. doi: 10.1016/j.bios.2010.03.032.

Lyu, Y. K., Lim, K. R., Lee, B. Y., Kim, K. S., & Lee, W. Y. (2008). Microgravimetric lectin biosensor based on signal amplification using carbohydrate-stabilized gold nanoparticles. *Chemical Communications*, *39*, 4771–4773. doi: 10.1039/b807438k.

Munkley, J., & Elliott, D. J. (2016). Hallmarks of glycosylation in cancer. *Oncotarget*, *7*(23), 35478–35489. doi: 10.18632/oncotarget.8155.

Nagaraj, V. J., Aithal, S., Eaton, S., Bothara, M., Wiktor, P., & Prasad, S. (2010). NanoMonitor: A miniature electronic biosensor for glycan biomarker detection. *Nanomedicine*, *5*(3), 369–378. doi: 10.2217/nnm.10.11.

Nagaraj, V. J., Eaton, S., Thirstrup, D., & Wiktor, P. (2008). Piezoelectric printing and probing of Lectin NanoProbeArrays for glycosylation analysis. *Biochemical and Biophysical Research Communications*, *375*(4), 526–530. doi: 10.1016/j.bbrc.2008.08.033.

Pei, Z., Anderson, H., Aastrup, T., & Ramström, O. (2005). Study of real-time lectin-carbohydrate interactions on the surface of a quartz crystal microbalance. *Biosensors and Bioelectronics*, *21*(1), 60–66. doi: 10.1016/j.bios.2004.10.006.

Peiris, D., Spector, A. F., Lomax-Browne, H., Azimi, T., Ramesh, B., Loizidou, M., … Dwek, M. V. (2017). Cellular glycosylation affects Herceptin binding and sensitivity of breast cancer cells to doxorubicin and growth factors. *Scientific Reports*, *7*(1), 1–11. doi: 10.1038/srep43006.

Piva, M., Moreno, J. I., & Sharpe-Timms, K. L. (2003). Glycosylation and overexpression of endometriosis-associated peritoneal haptoglobin. *Glycoconjugate Journal*, *19*(1), 33–41. doi: 10.1023/A:1022580813870.

Reddy, R. K., Bothara, M. G., Barrett, T. W., Carruthers, J., & Prasad, S. (2008). Nanomonitors: Protein biosensors for rapid analyte analysis. *IEEE Sensors Journal*, *8*(6), 720–723. doi: 10.1109/JSEN.2008.923177.

Reily, C., Stewart, T. J., Renfrow, M. B., & Novak, J. (2019). Glycosylation in health and disease. *Nature Reviews Nephrology*, *15*, 346–366). doi: 10.1038/s41581-019-0129-4.

Rosencrantz, R. R., Nguyen, V. H., Park, H., Schulte, C., Böker, A., Schnakenberg, U., & Elling, L. (2016). Lectin binding studies on a glycopolymer brush flow-through biosensor by localized surface plasmon resonance. *Analytical and Bioanalytical Chemistry*, *408*(20), 5633–5640. doi: 10.1007/s00216-016-9667-9.

Sadik, O. A., & Yan, F. (2007). Electrochemical biosensors for monitoring the recognition of glycoprotein-lectin interactions. *Analytica Chimica Acta*, *588*(2), 292–296. doi: 10.1016/j.aca.2007.02.046.

Serra, B., Gamella, M., Reviejo, A. J., & Pingarrón, J. M. (2008). Lectin-modified piezoelectric biosensors for bacteria recognition and quantification. *Analytical and Bioanalytical Chemistry*, *391*(5), 1853–1860. doi: 10.1007/s00216-008-2141-6.

Silva, M. L. S. (2019). Lectin biosensors in cancer glycan biomarker detection. *Advances in Clinical Chemistry*, *93*, 1–61. doi: 10.1016/bs.acc.2019.07.001.

Tommasone, S., Allabush, F., Tagger, Y. K., Norman, J., Köpf, M., Tucker, J. H. R., & Mendes, P. M. (2019). The challenges of glycan recognition with natural and artificial receptors. *Chemical Society Reviews*, *48*, 5488–5505. doi: 10.1039/c8cs00768c.

Wang, M., Zhu, J., Lubman, D. M., & Gao, C. (2019). Aberrant glycosylation and cancer biomarker discovery: A promising and thorny journey. *Clinical Chemistry and Laboratory Medicine*, *57*, 407–416). doi: 10.1515/cclm-2018-0379.

Yakovleva, M. E., Safina, G. R., & Danielsson, B. (2010). A study of glycoprotein-lectin interactions using quartz crystal microbalance. *Analytica Chimica Acta*, *668*(1), 80–85. doi: 10.1016/j.aca.2009.12.004.

Key References

Nanomonitor

Bothara et al. (2008). See above.

Nagaraj et al. (2010). See above.

Nagaraj et al. (2008). See above.

EIS

Bhide, A., Muthukumar, S., & Prasad, S. (2018). CLASP (Continuous lifestyle awareness through sweat platform): A novel sensor for simultaneous detection of alcohol and glucose from passive perspired sweat. *Biosensors and Bioelectronics*, *117*, 537–545. doi: 10.1016/j.bios.2018.06.065.

Daniels, J. S., & Pourmand, N. Label-free impedance biosensors: Opportunities and challenges. In *Electroanalysis*, *19*, 1239−1257. doi: 10.1002/elan.200603855.

Ganguly, A., & Prasad, S. (2019) Passively addressable ultra-low volume sweat chloride sensor. *Sensors (Switzerland)*, *19*(20), 4590. doi: 10.3390/s19204590.

Ganguly, A., Lin, K. C., Muthukumar, S., & Prasad, S. (2021). Autonomous, real-time monitoring electrochemical aptasensor for circadian tracking of cortisol hormone in sub-microliter volumes of passively eluted human sweat. *ACS Sensors*, *6*, 63−72. doi: 10.1021/acssensors.0c01754.

Ganguly, A., Rice, P., Lin, K. C., Muthukumar, S., & Prasad, S.A. (2020). Combinatorial electrochemical biosensor for sweat biomarker benchmarking. *SLAS Technology*, *25*(1), 25−32. doi: 10.1177/2472630319882003.

Lisdat, F., & Schäfer, D. (2008). The use of electrochemical impedance spectroscopy for biosensing. *Analytical and Bioanalytical Chemistry*, *391*, 1555–1567. doi: 10.1007/s00216-008-1970-7.

Randviir, E. P., & Banks, C. E. (2013). Electrochemical impedance spectroscopy: An overview of bioanalytical applications. *Analytical Methods*, *5*, 1098–1115. doi: 10.1039/c3ay26476a.

Internet Resources

Gamry potentiostat

https://www.gamry.com/assets/Support-Downloads/Product-Manuals/Reference-600-Operators-Manual.pdf

https://www.gamry.com/potentiostats/reference-600-plus/

https://www.gamry.com/application-notes/EIS/basics-of-electrochemical-impedance-spectroscopy/