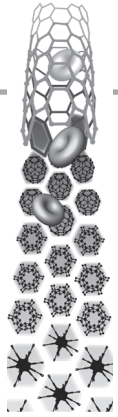

NanoMonitor: a miniature electronic biosensor for glycan biomarker detection

Aim: The goal of our research is to develop an ultrasensitive diagnostic platform called 'NanoMonitor' to enable rapid label-free analysis of a highly promising class of biomarkers called glycans (oligosaccharide chains attached to proteins) with high sensitivity and selectivity. The glycosylation of fetuin – a serum protein – and extracts from a human pancreatic cancer line was analyzed to demonstrate the capabilities of the NanoMonitor. **Material & methods**: The NanoMonitor device consists of a silicon chip with an array of gold electrodes forming multiple sensor sites and works on the principle of electrochemical impedance spectroscopy. Each sensor site is overlaid with a nanoporous alumina membrane that forms a high density of nanowells on top of each electrode. Lectins (proteins that bind to and recognize specific glycan structures) are conjugated to the surface of the electrode. When specific glycans from a test sample bind to lectins at the base of each nanowell, a perturbation of electrical double-layer occurs, which results in a change in the impedance. Using the lectins *Sambucs nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA), subtle variations to the glycan chains of fetuin were investigated. Protein extracts from BXPC-3, a cultured human pancreatic cancer cell line were also analyzed for binding to SNA and MAA lectins. The performance of the NanoMonitor was compared to a conventional laboratory technique: lectin-based enzyme linked immunosorbent assay (ELISA). **Results & discussion:** The NanoMonitor was used to identify glycoform variants of fetuin and global differences in glycosylation of protein extracts from cultured human pancreatic cancerous versus normal cells. While results from NanoMonitor correlate very well with results from lectin-based ELISA, the NanoMonitor is rapid, completely label free, requires just 10 µl of sample, is approximately five orders of magnitude more sensitive and highly selective over a broad dynamic range of glycoprotein concentrations. **Conclusion:** Based on its performance metrics, the NanoMonitor has excellent potential for development as a point-of-care handheld electronic biosensor device for routine detection of glycan biomarkers from clinical samples.

KEYWORDS: cancer glycoprofiling ▪ electrochemical impedance spectroscopy glycoprotein analysis ▪ glycosensor ▪ label-free glycosylation analysis ▪ lectin

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Glycosylation, the enzymatic process of the addition of oligosaccharide chains (glycans) to newly synthesized proteins, is a major class of post-translational protein modification. Glycosylation influences protein structure, function, turnover and interactions with other molecules, and hence has significant impacts on several key biological processes such as cell–cell interactions, cell differentiation, cell development, host–pathogen recognition and immune response. Aberrant glycosylation has been linked to several diseases. For example, during cancer, glycosylation plays a role in early tumor initiation, proliferation, invasion, angiogenesis and metastasis [1]. Cancer-induced alterations of protein glycosylation are considered to be important candidate biomarker for early diagnosis: especially specific glycoforms of tumor biomarkers such as prostate-specific antigen (prostate cancer), CA 125 (ovarian cancer), mucins (MUC1; several cancers), carcinoembryonic antigen (colon cancer) and HER-2 (breast cancer). In 2006, the US FDA approved the use of fucosylated α fetoprotein (AFP-L3) as a marker for detection of early-stage hepatocellular carcinoma and several other glycan biomarkers are in the approval process [2]. In addition to specific glycoproteins, global changes to an individual's glycome are being investigated to identify profiles that can serve as biomarkers [3].

The success of glycans as clinical biomarkers depends on highly sensitive and user-friendly technology for detection of altered protein glycosylation. The conventional laboratory methods for the identification and characterization of glycan structures are based on mass spectrometry and nuclear magnetic resonance. These techniques require expensive instrumentation, advanced expertise, are time consuming, low throughput and are not practical for routine screening of patient samples in a clinical setting. The commercial availability of lectins (proteins that bind to and recognize specific

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glycan structures), glycan-specific antibodies and other ligands (e.g., peptides, aptamers) that bind to glycans has led to the use of western blots, lectin-based ELISAs and microarray-based techniques for the identification of glycan biomarkers. However, none of these techniques are convenient for the rapid and routine screening of a large number of human samples in a high-throughput fashion. Recently, there has been a shift towards exploring 'label-free' methods of glycosylation analysis to address the inherent limitations of traditional research laboratory techniques. These include surface plasmon resonance [4], fluorescence resonance energy transfer between quantum dots and gold nanoparticles [5], quartz crystal microbalance [6,7], electrochemical [8,9] or piezoelectric biosensors [10]. Due to their relative simplicity, high sensitivity, ease of miniaturization, short assay time and low cost, electrochemical sensors for glycan biomarker detection are most promising for transition to point-of-care diagnostic devices.

Electrochemical sensors consist of a biorecognition element (oligonucleotides, antibodies, lectins or other ligands) coupled to an electrode transducer surface. The specific interaction of a biological analyte to its corresponding ligand on the electrode surface is detected by electrical current or potential changes occurring at the transducer/biomolecule interface [11–14]. Electrochemical impedance spectroscopy (EIS) is a commonly used electrochemical technique for interrogating biomolecules since it uses small alternating current amplitude voltage signals without significantly disturbing the properties of the biomolecules or inter-molecular interactions. In the EIS technique, the biosensor measures the electrical impedance of an interface in alternating current steady state by imposing a small sinusoidal voltage at a particular frequency, and measuring the resulting current. The impedance is given by the resulting voltage:current ratio, which, when measured over a range of frequencies is called alternating current impedance EIS. This form of EIS has been used to study electrochemical phenomena (mostly affinity) of various biological interactions [15]. Changes in the amplitude and phase of the sinusoidal signal resulting in the measured impedance changes reflect binding of a particular target–molecular recognition element pair. EIS is used extensively as a nondestructive technique for providing accurate and repeatable measurements of surface conditions such as binding and desorption processes at electrode surfaces. Consequently, EIS allows complex biorecognition events to be probed in a simple, sensitive, label-free and mediator-free manner [16–18]. Hence, EIS has been extensively applied in developing electrochemical biosensors for molecular detection.

The coupling of nanoscale materials such as nanoparticles, nanowires, nanoneedles, nanomembranes, nanotubes or nanorods with electrochemical biosensors offers unique capabilities for ultrasensitive detection of biomolecules. Nanostructures can enhance the surface of electrodes and are more sensitive to the changes produced at the electrical double layer due to the interaction of biomolecules with their ligand [19]. In addition, they provide unique three dimensional environments that are conducive for the interaction of various biomolecules with their ligands. Nanoscale materials such as silicon nanowires have been used to achieve fg/ml sensitivity in detecting prostate-specific antigen [20]. Carbon nanotubes have been used to detect proteins through enzyme-mediated interactions with a detection sensitivity of only 10 protein molecules [21]. Metal and metal-oxide nanoparticles have been used to achieve attomolar sensitivity in detection of specific proteins [22]. A wide range of nanomaterials of varying physical morphology and structures has been used to analyze antigen–antibody interactions, enzyme activity and metabolites for the detection of cancer and other diseases [23]. We have incorporated nanoporous alumina membranes onto microfabricated silicon platforms to generate a microelectronic biosensor known as a NanoMonitor [24]. The pore dimensions and pore density of alumina can be precisely controlled via a simple wet bench-based electrochemical technique known as two-step anodization, which is less expensive than more complex fabrication processes for other nanoporous materials such as silica. We have previously reported that nanowells created by the nanoporous alumina membrane on the sensor surface imparts superior detection capabilities to the NanoMonitor for the identification of cardiovascular biomarkers such as C-reactive protein and myeloperoxidase, endotoxins from pathogens and allergen markers such as Cry-j protein from *Cryptomeria japonica* [24–26]. In the current study we demonstrate the application of NanoMonitor technology to detect subtle but potentially important changes in the glycosylation of proteins using closely related glycoforms of fetuin. We achieve several orders of magnitude higher sensitivity with the NanoMonitor as compared with a conventional technique, lectin-based ELISA. We further demonstrate the ability of our technique to recognize global changes in glycosylation patterns of proteins derived from cancerous versus normal, cultured human pancreas cells.

Material & methods

Fabrication of NanoMonitor & principle of operation

The NanoMonitor is comprised of a microfabricated silicon platform with eight microscale gold-sensing sites (Figure 1A). Each sensing site is comprised of two concentric gold circles, which function as the working and counter-electrodes (Figure 1B). The inner working electrode is 25 µm diameter in contrast to the 125 µm diameter of the outer counter-electrode. Electrical impedance, measured across these two electrodes, changes as a function of the amount of biomolecule bound to the surface. A nanoporous alumina membrane (Whatman Scientific, Kent, UK), with a pore diameter of 20 nm is overlaid on the electrodes (Figure 1C). The alumina membrane has 50% porosity with 10^6 pores/mm^2 and results in the formation of high density of nanowells on a single sensing site. The NanoMonitor is encapsulated by an acrylic microfluidic chamber comprised of eight fluidic channels that are physically isolated from each other. Each channel has an inlet and an outlet port at opposite ends of the channel and the volume per channel is 10 µl (Figure 1A). The chamber is designed so that the channel is flush over the sensing site. In addition, the dimensions of the chamber are such that its perimeter is flush with the perimeter of the nanoporous alumina membrane on each sensing site (Figure 1A) in order to help physically immobilize the alumina membrane onto the sensing site.

Detection of protein glycosylation using the NanoMonitor (Figure 1D) is achieved through EIS, which is based on the principle of double layer capacitive measurement [24,25]. An electrical double layer of approximately 50 nm in thickness is formed at the solid/liquid interface at the base of each nanowell. The 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 [27], the impedance changes are non-inductive in nature. A low voltage (100 mV) alternating current is applied across the sensing site comprised of multiple nanowells and the frequency is scanned from 50 Hz to 3 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 (Gamry 600, Ref, Potentiostat, Gamry instruments, PA, USA). We observed that the maximum change to the impedance as a result of the interaction of glycans with lectins is obtained at 1 kHz.

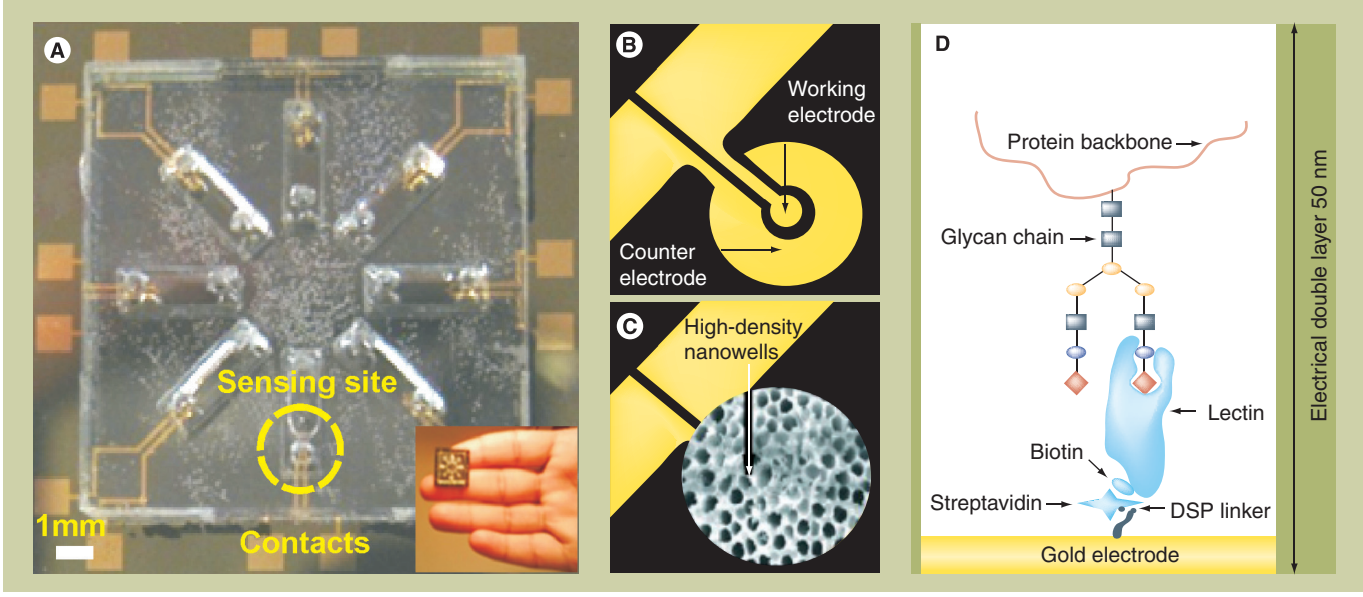

Figure 1. (A) The NanoMonitor consists of a base silicon microplatform with eight gold metal sensing sites to form a hand-held diagnostics device (insert). Individual sensing sites are overlaid with the nanoporous alumina membrane and the microplatform is encapsulated by an acrylic microfluidic chamber to ensure physical isolation between individual sites. **(B)** Each sensing site is comprised 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 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.

Functionalization of electrode surface

Functionalization of the gold electrode was achieved by coating the surface with the crosslinking agent dithiobis succinimidyl propionate, (DSP; Thermo Fisher Scientific Inc., IL, USA). 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 disulphide linkage of DSP chemisorbs rapidly to the gold surface forming monolayers of the DSP molecules on the gold surfaces while the *N*-hydroxysuccinimide groups are available for binding to the primary amine groups of proteins [28]. After incubation of the electrode surface with DSP (4 mg/ml) for 30 min at room temperature, excess unreacted crosslinker was washed off twice with 1 × phosphate-buffered saline (PBS). Next, streptavidin (Thermo Fisher Scientific) was added at a previously determined saturation dose (1 mg/ml in 1 × PBS) to the electrode surface containing the DSP crosslinker. After incubation for two hours, unbound streptavidin was washed off twice with 1 × PBS. Binding to the gold surface and saturation concentrations were previously determined using fluorescently labeled streptavidin [24].

Immobilization of lectins on the sensor surface

Immobilization of lectins to the functionalized gold surface was achieved using the biotin–streptavidin linker chemistry. Various concentrations of biotinylated lectins were tested to determine the saturation concentration, which was 1 mg/ml (data not shown). Biotinylated lectins *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA; EY laboratories, CA, USA) were added to the streptavidin-coated gold surface and incubated for 15 min at room temperature. Unbound lectins were washed off three times with 1× PBS. Nonspecific binding sites on the sensor surface were blocked with SuperBlock blocking buffer (Thermo Fisher Scientific).

Synthesis of glycoform variants of fetuin

Asialofetuin (ASF) was used as a sialic acid acceptor for a sialyltransferase reaction to create fetuin with exclusively 3′-linked terminal sialic acid (3SF) and with exclusively 6′-linked terminal sialic acid (6SF) on the glycan chain of the protein. The reaction, described earlier [29], was performed in a buffer consisting of 100 mM sodium cacodylate, 10 mM $MgCl_2$ and 5 mM $CaCl_2$ (pH 6.0) and catalyzed by recombinant rat α(2–3) or α(2–6) sialyltransferase (Calbiochem, CA, USA). 10 mM CMP-sialic acid (Calbiochem) was the sialic acid donor and 1 mg ASF (Sigma-Aldrich, MO, USA) was the sialic acid acceptor glycoprotein. The sialyltransferase reaction was incubated at 37 °C overnight and the modified glycoprotein was purified using a size exclusion column. The sialylation products were characterized by lectin-based ELISA (see below) and previously by lectin blots as well as microarray analysis [29].

Analysis of protein glycosylation using NanoMonitor

The three glycoforms (ASF, 3SF and 6SF) of the glycoprotein fetuin, to be analyzed on the NanoMonitor, were serially diluted from a 1mg/ml stock solution down to the required concentrations in 1 × PBS. The baseline impedance was first determined by using 1 × PBS (0 pg/ml glycoprotein) followed by measurements with increasing concentration of glycoproteins starting from 1 pg/ml. After the addition of each glycoprotein solution, impedance changes, from baseline impedance, were measured following a 15 min incubation to allow sufficient time for the lectin-glycan binding to occur as well as for the signals to stabilize. Before the addition of the next higher concentration of glycoprotein, the sensing sites were washed twice with 1 × PBS. The NanoMonitor has eight sensing sites that are physically isolated from each other. At least three sites were saturated with a single lectin and the binding response of one specific glycoprotein was measured in parallel to assess reproducibility across the sites.

Analysis of glycoform variants of fetuin by lectin-based ELISA

The synthetic glycoform variants of fetuin were characterized by lectin-based ELISA. Glycoproteins were serially diluted from 10 µg/ml to 0.156 µg/ml in 0.1 M $NaHCO_3$, added to 96-well Nunc MaxiSorp plates (Thermo Fisher Scientific) and incubated overnight at 4°C to allow adsorption of the proteins to the polystyrene. Unbound proteins were washed off with 100 mM tris-buffered saline containing 0.05% Tween (TBST) thrice using the EL×50 automatic plate washer (BioTek Instruments, Inc., VT, USA). Unoccupied sites on the polystyrene surface were blocked using SuperBlock blocking buffer (Thermo Fisher Scientific). Biotinlylated lectins, SNA and MAA (20 µg/ml in PBS), were added to the glycoprotein-coated plates and incubated with shaking for 2 h at room temperature. After washing, extravidin-alkaline phosphatase (Sigma-Aldrich) diluted 1:70,000 in TBST, was

added to the plates and incubated for 1 h at room temperature with gentle shaking. Unbound enzyme was washed at least three times with TBST. The substrate for alkaline-phosphatase, p-nitrophenyl phosphate (SigmaFast PNPP tablets, Sigma-Aldrich) was added to the wells and incubated for one hour. Absorbance was read at 405 nm using a microplate spectrophotometer (Spectramax M5, Molecular Devices, CA, USA).

Analysis of cancer glycoproteins

The human pancreatic carcinoma BxPC-3 and normal human pancreatic duct epithelial cell line HPDE6 (American Type Culture Collection, MD, USA) were cultured according to American Type Culture Collection's recommendations. Briefly, BXPC-3 were grown in RPMI 1640 (Invitrogen Corporation, CA, USA) with 10% FBS (Invitrogen Corporation) and HPDE6 cells were grown in keratinocyte serum-free media (Invitrogen Corporation) with bovine pituitary extract at 50 µg/ml and epithelial growth factor at 0.1 ng/ml. When the cultures reached approximately 75% confluence, the cells were harvested using cell stripper (Mediatech, Inc., VA, USA), pelleted by centrifugation (800 rpm for 3 min at room temperature). Total proteins were extracted using the M-PER mammalian protein extraction reagent (Pierce Chemicals). Complete, Mini; protease inhibitor cocktail tablets (F Hoffmann-La Roche AG, Basel, Switzerland) were used to prevent proteolysis during extraction. Proteins were quantified using the Qubit Protein Quantitation system (Invitrogen Corporation) and frozen at -20°C until analysis by ELISA and NanoMonitor.

Results

Glycoproteins for NanoMonitor analysis

In order to test the ability of the NanoMonitor to identify defined glycan structures, as well as distinguish between closely related ones, ASF and sialylated glycoforms of fetuin; 3SF or 6SF were used. ASF has well characterized glycans and is routinely used as a model glycoprotein for glycosylation studies. The binding patterns of these fetuin glycoforms to lectins were first confirmed by western blots (see supplementary data at www.futuremedicine.com/toc/nnm/5/3). A lectin-based ELISA technique [30] was used to determine the dynamic range of detection of these proteins using the lectins SNA and MAA. SNA recognizes terminal sialic acid on glycans with an α-(2–6) linkage to galactose and MAA recognizes terminal sialic acid on glycans with an α-(2–3) linkage to galactose. In lectin-based ELISA experiments, ASF showed little or no binding to both SNA and MAA due to the absence of sialic acid (Figure 2C). The addition of α-(2–3)-linked sialic acid to ASF to create 3SF resulted in binding to MAA but not SNA (Figure 2A) while the addition of α-(2–6) linked sialic acid resulted in binding to SNA but not MAA (Figure 2B). The lower limits of detection of both the sialylated glycoforms of fetuin with lectin-based ELISA was 0.312 µg/ml. While a linear range of detection was observed up to 5 µg/ml for 6SF, the linearity was less apparent with 3SF. Concentrations higher than 5 µg/ml showed some nonspecific interactions with lectins (Figure 2B). The lower limits of detection observed here for glycoprotiens using lectins are higher compared with typical detection limits for proteins using antibodies during conventional ELISA. This is probably because the affinity of lectins for carbohydrates is much lower than that of antibodies for proteins [31]. However the precision of detection limits we observed for lectin ELISA is well within the range observed by others [32,33].

Analysis of the glycoforms of fetuin protein with NanoMonitor

The interaction of the three fetuin glycoforms (ASF, 3SF and 6SF) with the lectins SNA and MAA was analyzed by EIS using the NanoMonitor as described in the material and methods section. After conjugation of lectins to the sensor surface, change in impedance in response to 0 ng/ml glycoproteins (PBS only) was used to first establish the baseline. Impedance values corresponding to the baseline were typically in the range of hundreds of kΩ. Impedance changes due to the binding of a various concentrations of a glycoprotein were expressed as percentage changes from the baseline measurements. The performance metrics of the NanoMonitor, namely the detection sensitivity/lower limit of detection (the lowest concentration of the glycoprotein that can be consistently detected with the sensor), detection specificity (lowest concentration at which the fetuin glycoforms can be distinguished from one another) and the dynamic range of detection were determined (Table 1).

For the first set of experiments, dilutions of 3SF were prepared, ranging from 1 µg/ml down to 1 pg/ml in the logarithmic scale in 1 × PBS buffer. Figure 2D shows the dose response of 3SF with the lectins SNA and MAA on the NanoMonitor. The interaction of 3SF with MAA was highly specific and the sensitivity of detection (limit of detection) was less than 1 pg/ml. The limit of detection was identified by identifying

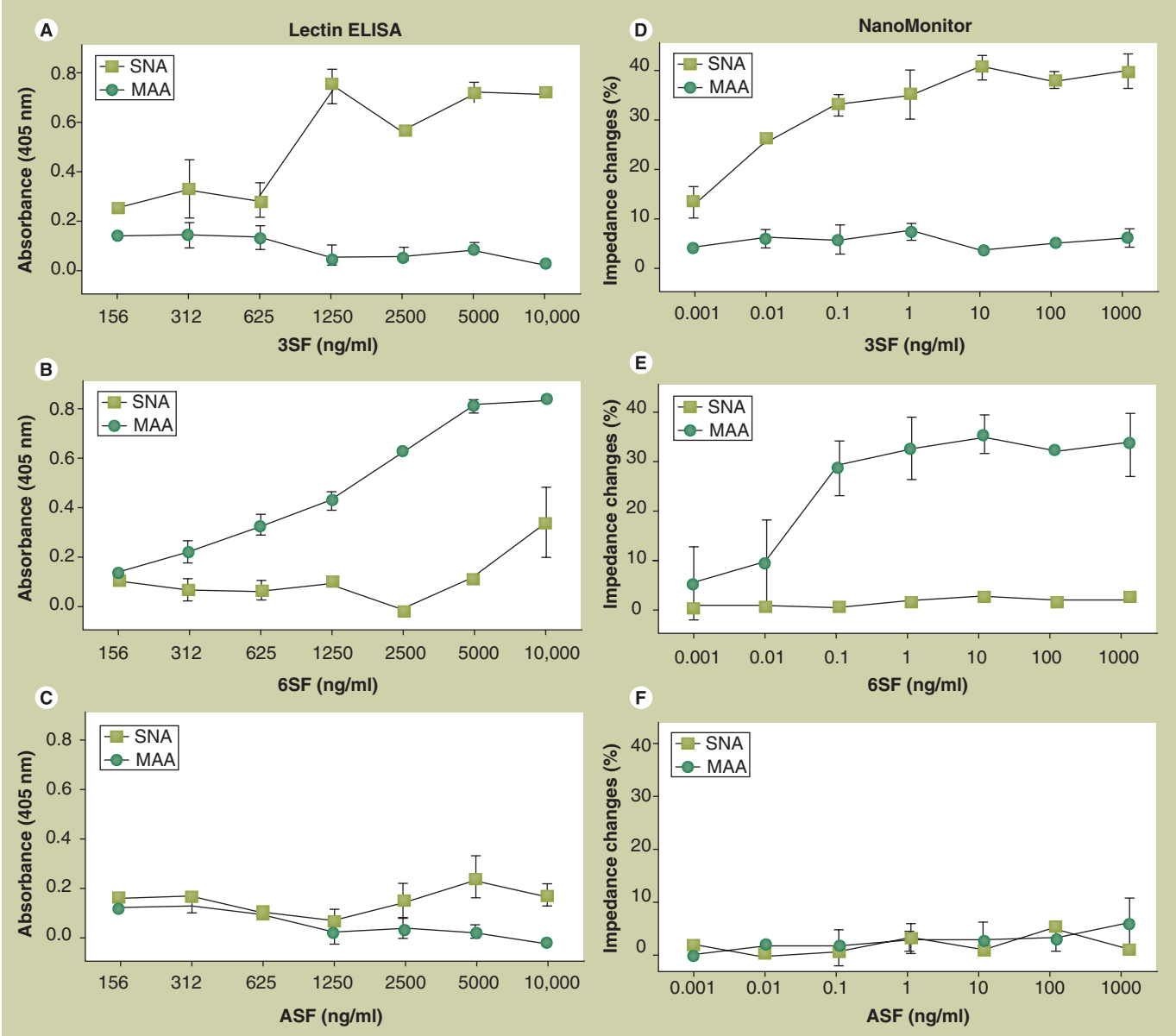

Figure 2. Comparison of the performance of NanoMonitor with that of lectin-based ELISA for the detection of protein glycosylation. Asialofetuin and sialylated glycoforms of fetuin exclusively containing α(2–3) linked or α(2–6)-linked terminal sialic acids were analyzed for binding to SNA lectin (recognizes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

the dose of the fetuin that produced a minimum of 3% change in the impedance from the baseline. This was sufficient to distinguish a binding event/interaction from baseline. Over the range of concentrations from 1 pg/ml to 1 µg/ml, percentage changes of impedance ranged from 13 to 38%. Detection specificity for 3SF was 1 pg/ml. At this concentration, the specific interaction between 3SF and MAA resulted in 13% change in impedance from baseline as compared with 5% change in impedance from baseline for nonspecific interaction with SNA. The actual impedance changes measured from the 1 × PBS baseline over the dose range due to the interaction of 3SF with MAA and SNA are shown in the supplementary data section. For the same range of concentrations of 3SF, the impedance changes were 5–8% during its interaction with SNA.

When 6SF was analyzed for interactions with both the lectins in a range of concentrations from 1 pg/ml to 1 µg/ml we found a very specific interaction with SNA (Figure 2E). The lower limit of

detection of 6SF with SNA lectin was 1 pg/ml, which resulted in a 6% change in impedance from baseline. The maximum change in impedance for 6SF was 33% at 10 ng/ml. The measured impedance changes from 1 × PBS baseline over the dose range for interaction of 6SF with SNA are shown in FIGURE 3B. We were able to distinguish between 6SF and 3SF using SNA at concentrations of 100 pg/ml or higher. Over the range of concentrations tested, 6SF showed impedance changes from 1 to 4% for its interactions with MAA. As expected, ASF showed little or no interaction with SNA and MAA lectins. When a range of concentrations from 1 pg/ml to 1 µg/ml of ASF were analyzed on the NanoMonitor for binding to the lectins less than a 5% change in impedance was observed (FIGURE 2F).

Analysis of cancer glycoproteins

The glycosylation profile of proteins from cancer cells can be very different from that of normal cells and can serve as a potential biomarker [3]. We extracted total proteins from cultured normal (HPDE6) and cancerous (BxPC-3) human pancreatic cells, and analyzed the binding of these proteins to SNA and MAA lectins using the NanoMonitor (see Supplementary Figures 1 & 2 at www.futuremedicine.com/toc/nnm/5/3). While no significant differences were observed with normal cells. we found that human pancreatic cancer cells have a significantly higher amount of α-(2–3)-linked sialic acids based on a greater interaction of protein extracts with MAA as compared with SNA. 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 during interaction of the same extracts with SNA. When these extracts were analyzed by lectin-based ELISA, a similar pattern of binding to MAA and SNA lectins was observed (FIGURE 3).

Discussion

Our research involves the development of an electrochemical sensor called NanoMonitor to analyze protein glycosylation. Subtle changes to the oligosaccharide chains attached to glycoproteins can occur as a consequence of disease-related alterations to the glycosylation machinery *in vivo* resulting in altered glycoforms of proteins [1]. Disease-specific glycoforms of proteins in body fluids such as serum, saliva, urine and others can serve as biomarkers and hold great potential as indicators of an individual's health [31]. To test the ability of NanoMonitor to identify different glycoforms of the same protein, we enzymatically modified the serum protein fetuin 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 aberrant cellular glycosylation pathways and were used for proof-of-concept studies with the NanoMonitor. Instead of antibodies that recognize the protein backbone, lectins that recognize specific glycan structures were used as capture agents on the sensing sites of the NanoMonitor.

The highly specific binding pattern of the various glycoform variants of fetuin to lectins on the NanoMonitor correlated very well with results from Western blots (data not shown) and lectin-based ELISA. However, the detection sensitivity of NanoMonitor is approximately five orders of magnitude higher than that of lectin-based ELISA and we were able to distinguish between α(2–6) sialylated glycoforms of fetuin from the α(2–3) sialylated version at less than 1 pg/ml protein concentrations. Previously, the detection of α(2–3)-linked sialic acid with such high sensitivity has been possible only by conjugation to gold nanoparticles and by using electrochemical

Table 1. Summary of the performance of the NanoMonitor in comparison to lectin-based ELISA for the detection of the glycoform variants of fetuin.

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

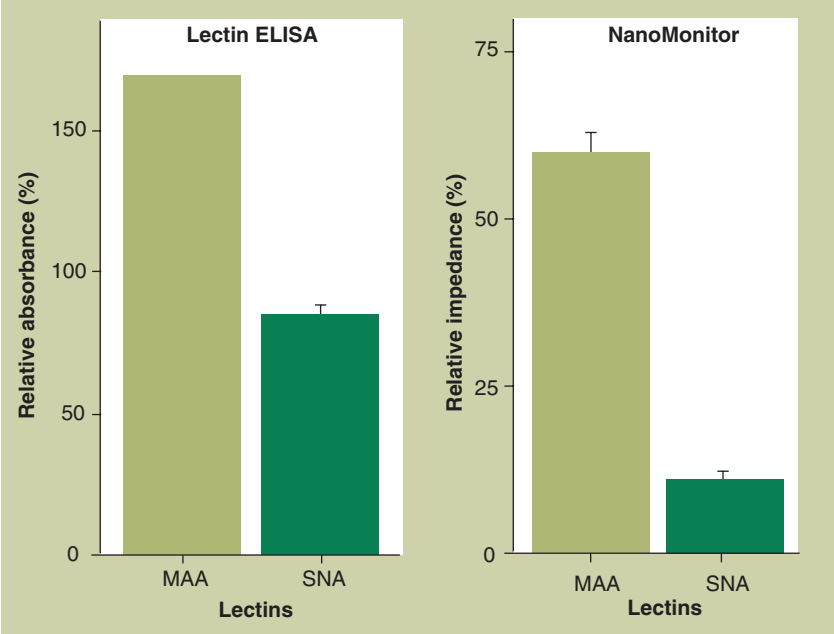

Figure 3. Analysis of global sialylation pattern of proteins from cultured human pancreatic cancer cells (BxPC-3) using lectin-based ELISA and NanoMonitor. The same extract of total proteins from BxPC-3 cells (10 µg/ml) was analyzed for binding to MAA and SNA lectins using lectin-based ELISA and NanoMonitor.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

redox probe-based impedance spectroscopy [9] or to a lesser extent by microarray analysis of fluorescently tagged glycoproteins [29]. NanoMonitor was found to be more sensitive 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 [5]. Other interesting lectin-based sensor platforms such as electrochemical quartz crystal microbalance have been employed for the detection of bacteria (*Escherichia coli*) with Concanavalin A lectin [10]. Instead of detecting glycans, biosensor platforms are also proving to be useful for the identification and quantification of lectins. For example, wheat germ agglutinin and its precursor protein, probarley lectin, were precisely quantified using an amperometric immunosensor with a detection limit of 1 ng/ml [8]. The peanut agglutinin lectin was detected using a surface plasmon resonance technique on a thin gold film surface with a detection sensitivity of 1 µM [4]. NanoMonitor, with its superior detection capabilities can be easily adapted to these and other novel sensitive lectin-based sensor applications.

With the NanoMonitor, we observed significant, robust and repeatable changes to the measured electrical impedance over a broad range of glycoprotein concentrations (< 1 pg/ml to > 10 ng/ml). While the precise reasons for enhanced sensitivity of glycosylation analysis with the NanoMonitor requires further investigation, the mechanisms are probably the same as those that are responsible for enhanced protein detection reported earlier [24–26]. The presence of millions of nanowells at each sensor site created by the alumina membrane may alter the diffusion of molecules through the pores of the wells to the electrode surface, influence lectin binding to the glycans through *in vivo*-like nano-scale confinement of molecules within the nano-wells as well as affect the measured capacitance between the electrodes. When protein biomarkers such as C-reactive protein and myeloperoxidase are analyzed on the NanoMonitor, we observed an increase in impedance of up to 20% from the baseline measurements [24,25]. In comparison, while analyzing fetuin glycoforms we observed an increase in the impedance of up to 40%. This is particularly interesting since the affinity of lectins for glycans (K_d: 10^{-4}–10^{-7}M) is much lower compared with that of antigens for antibodies (K_d: 10^{-8}–10^{-12}M) [31]. The interaction of glycoproteins with lectins on the sensor surface is more complex than that of antigens with antibodies since multiple *N*- and *O*-linked glycans can be present on glycoproteins such as fetuin and the lectin molecules themselves tend to be multimeric proteins with several carbohydrate binding sites. However, our work and other reports [9] of high impedance changes during glycosylation analysis using lectins with EIS suggests that the NanoMonitor can serve as a highly sensitive platform for development as a glycan biomarker detection device.

One of the biggest practical advantages of the NanoMonitor technology is that it is completely label free and does not depend on the use of fluorophore, enzyme or nanoparticle tags. Human samples can be quickly and reliably analyzed on the NanoMonitor without the need for extensive purification or fluorescent labeling. We tested crude total protein extracts from cultured human cancerous and normal pancreatic cells for binding to SNA and MAA lectins. Based on interaction with the lectins on the NanoMonitor as well as lectin-based ELISA protein extracts from human pancreatic cancer, unlike normal human pancreatic cells, showed a significantly higher degree of α(2–3) sialylation. The presence of higher α(2–3)-linked sialic acids in BxPC-3 cells is probably due to the metastatic sialyl-Lewis a and x antigens, a result of the enhanced expression of α(2–3) sialyltransferases as well as α(1–3/1–4)

fucosyltransferases and decreased expression of α(1–2) fucosyltransferases [34]. Sialylation studies also indicate that humans express predominantly α(2–6)-linked sialic acids in the upper respiratory tract compared with α(2–3)-linked sialic acids, which make them more prone to the human influenza A virus rather than the avian influenza A virus. Similarly, the nature of sialic acid linkages found on cell surface proteins of several different tissues determines the susceptibility of host cells to a wide variety of pathogens [35]. The under- or over-expression of naturally occurring glycans as well as the presentation of glycans that are normally restricted to expression during embryonic development have excellent potential for cancer diagnostics [1]. Since the NanoMonitor is configured for multiplexed analysis, the device can be easily adapted for the detection of multiple cancer glycan biomarkers in human samples to a panel of carefully chosen lectins/antibodies to not only identify diseased samples but also to predict the type of cancer. Detection electronics can be integrated directly into the NanoMonitor for a cost effective miniature standalone clinical electronic biosensor device.

Future perspective

From the perspective of basic electrochemistry research with respect to sensor platforms we are interested in understanding how the nanoscale materials, in general, and the nanowells formed by the alumina membrane, in particular, contribute to enhanced sensitivity of detection. To apply the research described in this manuscript to medical diagnostics, the NanoMonitor will be optimized for multiplexed detection of several well-established glycan biomarkers for a range of diseases on a single chip using human body fluids such as serum, saliva, urine and others in a simultaneous manner. Several key engineering- and glycobiology-related challenges will have to be addressed to reach this goal. To overcome specificity issues with certain lectins, the identification and use of other ligands such as anti-glycan antibodies, peptides and aptamers with greater specificity and avidity towards glycan biomarkers is necessary. Microfluidics-based pre-processing protocols may have to be established for the clean-up, concentration or separation of certain types of clinical samples before analysis on the NanoMonitor. To ensure a more robust detection of disease by broadening the biomarker space, the NanoMonitor platform needs to be developed for the simultaneous detection of DNA, RNA, protein, glycan and metabolite biomarkers from the same sample. Automated deposition of biomarker capture agents as well as the samples to be screened using noncontact inkjet printing is necessary to enable high reproducibility, increased density of capture agents on the sensor surface and enhanced sensitivity of detection with minimal sample handling. Electronics will have to be designed to develop the device as a hand-held sensor for commercial use.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- This study was designed to demonstrate the detection of protein glycosylation through electrochemical impedance spectroscopy using the NanoMonitor platform.
- Using a panel of lectins, the performance of NanoMonitor was compared with lectin-based ELISA for the analysis of glycans from synthetic pure glycoproteins as well as complex samples derived from cultured human pancreatic cancer cells.
- Compared to lectin-based ELISA, the NanoMonitor analysis of protein glycosylation is rapid, completely label free, requires a very small volume, is approximately five orders of magnitude more sensitive and highly selective over a broad dynamic range of glycoprotein concentrations.
- NanoMonitor has excellent potential for development as a point-of-care handheld electronic biosensor device for routine detection of glycan biomarkers from clinical samples.

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