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Square-wave voltammetry assays for glycoproteins on nanoporous gold

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Abstract

Electrochemical enzyme-linked lectinsorbent assays (ELLA) were developed using nanoporous gold (NPG) as a solid support for protein immobilization and as an electrode for the electrochemical determination of the product of the reaction between alkaline phosphatase (ALP) and p-aminophenyl phosphate (p-APP), which is p-aminophenol (p-AP). Glycoproteins or concanavalin A (Con A) and ALP conjugates were covalently immobilized onto lipoic acid self-assembled monolayers on NPG. The binding of Con A – ALP (or soybean agglutinin – ALP) conjugate to glycoproteins covalently immobilized on NPG and subsequent incubation with p-APP substrate was found to result in square-wave voltammograms whose peak difference current varied with the identity of the glycoprotein. NPG presenting covalently bound glycoproteins was used as the basis for a competitive electrochemical assay for glycoproteins in solution (transferrin and IgG). A kinetic ELLA based on steric hindrance of the enzyme-substrate reaction and hence reduced enzymatic reaction rate after glycoprotein binding is demonstrated using immobilized Con A–ALP conjugates. Using the immobilized Con A–ALP conjugate, the binding affinity of immunoglobulin G (IgG) was found to be 105 nM, and that for transferrin was found to be 650 nM. Minimal interference was observed in the presence of 5 mg mL⁻¹ BSA as a model serum protein in both the kinetic and competitive ELLA. Inhibition studies were performed with methyl D-mannoside for the binding of TSF and IgG to Con A–ALP; IC₅₀ values were found to be 90 μM and 286 μM, respectively. Surface coverages of proteins were estimated using solution depletion and the BCA protein concentration assay.

Keywords

Enzyme-linked lectin sorbent assay; nanoporous gold; square wave voltammetry; glycoprotein; lectin; self-assembled monolayer

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1. Introduction

Biomolecule immobilization is a fundamental step in the high-throughput screening of biomarkers [1], and the study of protein-protein [2], protein-DNA [3], and other biological interactions. Protein microarrays [4] and DNA microarrays [5] have contributed substantially to proteomics and genomics; however, carbohydrate arrays [6] and lectin arrays [7] for advancing glycomics have not reached their full potential. Protein glycosylation is one of the most common post-translational modifications and plays crucial roles in protein folding, biological activity and proper functioning of glycoproteins [8]. Differential protein glycosylation has been found to be associated with different disease conditions and malignancies [9]. Since at least 50% of mammalian proteins and almost 80% of membrane proteins are glycosylated, carbohydrate-based biomarkers are attractive targets [10, 11]. Cells are covered with glycocalyxes which vary with cell type, and in different stages of cell growth and differentiation [12]. Cell surface carbohydrates are found to change during the course of malignancy [13]. Cell surface carbohydrates are also important for cell-cell communication, cell-pathogen interactions, and metastasis [14]. Data on protein-carbohydrate interactions is important for improved understanding of carbohydrate associated biological processes.

Some of the commonly used techniques in glycoanalysis include mass spectrometry [15], nuclear magnetic resonance spectroscopy (NMR) [16], high performance liquid chromatography (HPLC) [17], and capillary electrophoresis (CE) [18]. These techniques are highly sensitive and can provide detailed information about the structure of carbohydrate units, but require expensive instrumentation. Development of array based methods for the screening of glycoforms is gaining increasing interest among researchers in glycomics [19]. Even though array based methods do not directly provide detailed information about carbohydrate structure, often information on only the terminal or functional carbohydrate units is enough for screening of glycoproteins and other glycoconjugates. A wide range of methods have been applied to the study of glycan-protein interactions including those based on optical transduction (propagating surface plasmon resonance [2], localized surface plasmon resonance [20], fluorescence measurements [21]), piezoelectric (quartz crystal microbalance [22]), electrochemical methods [23] (cyclic voltammetry, electrochemical impedance spectroscopy (EIS) [24], differential pulse voltammetry (DPV) [25], square wave voltammetry (SWV), pulse amperometric detection, microcantilever deflection [26], and flow cytometry [27], amongst others.

Enzyme-linked immunosorbent assays (ELISA) represents a gold standard for immunoassays, whereas similar assays based on lectins [28] as the recognition element for glycans, enzyme-linked lectinosorbent assays (ELLA), are less common [29]. Lectins are used as recognition elements in glycoanalytical techniques including ELLA, immunohistochemistry, and affinity chromatography [30]. Antibodies specific for carbohydrates are not as commonly used because the resulting antibodies are of lower affinity; thus, lectins are the preferred recognition elements in glycoanalysis [30]. Even though ELLA are important techniques for the analysis of glycoforms, they have not been used as routinely as ELISA. This is partly because of the weaker carbohydrate-lectin affinities, the complexity of the carbohydrate distribution in biological systems, absence of a standard and easy to operate technique, and the high background in traditional ELLA because of the nonspecific adsorption of other proteins which are often glycosylated and hence bind to the lectin. Studies have been performed to evaluate blocking agents and significant progress has been made to identify blocking agents that produce the least background in ELLA [31]. The traditional approach of ELLA involves immobilization of glycoproteins or glycoconjugates on 96 well plates by physisorption followed by washing and then incubation with biotinylated lectins and then exposure to avidin or extravidin

labeled alkaline phosphatase or horseradish peroxidase as an enzyme label, for the purpose of detection [32]. ELLA are important for studying the glycan binding of novel lectins or in the study of differences in glycosylation and the identification of glycan biomarkers [33, 34]. The most common approach is physisorption but this is not very efficient as there is always a possibility of loss of protein during washing, denaturation etc. The essential requirements for the development of high-throughput screening of glycoforms include efficient loading onto the surface and retention of activity as well as proper orientation of the recognition moiety on the surface. Thus, covalent coupling onto the surface should be a better approach than physisorption. Self-assembled monolayers (SAMs) on gold are relatively easy to prepare and functionalize for subsequent coupling to proteins. For example, a SAM based ELLA was developed by adsorbing proteins on Ti/Au coated slides with detection performed by assembling biotinylated lectin and avidin/alkaline phosphatase (ALP) on the plates [35]. The amount of lectin bound to the surface was then correlated with the activity of ALP against p-nitrophenyl phosphate determined using measurements of absorbance at 405 nm. Another approach involves the use of lectins covalently attached to enzymes. A majority of these assays are based on absorbance measurements and are done by incubating the surface bound complexes for a significantly long period of time to produce an adequate signal. The long incubation times can lead to complications as many of the enzymatic products are not always stable and errors are introduced due to substrate self-degradation, typical incubation times are 20–30 min but sometimes longer incubation times are reported [29, 36].

Electrochemical assays are attractive because they are not affected by turbidity or background absorbance, involve relatively simple instrumentation, and potentially can be miniaturized [37]. Three different approaches in electrochemical glycobiosensing are common: i) EIS based methods, ii) immobilization of lectin followed by glycoconjugate binding and then labeling with the lectin-enzyme conjugate or lectin-redox agent conjugate, and iii) immobilization of cells onto the surface and then detection with similar probes as in (ii) [23, 38–40]. Electrochemical assays of glycans on living cells have made use of changes in the DPV response of an electroactive SAM upon cell binding [41], or in the DPV response of a lectin-Au-thionine conjugate binding after cell binding to a lectin-modified substrate [40]. Changes in the response of a lectin-enzyme conjugate upon cell binding have also been used [42], as has a method based on competition between mannose units in an SAM and on the cell surfaces [43].

Nanoparticle modified electrodes have also been used to amplify the signal from lectin – glycoprotein binding. A nanoparticle based sensor for lectin-carbohydrate interactions was reported by Wang *et al.* who immobilized lectin onto the mixed SAM modified gold surface and then performed a competitive assay using CdS nanoparticle labeled and unlabeled sugars. The amount of nanoparticles attached to lectin was determined by dissolution followed by stripping analysis [44]. Gold nanoparticles modified with Con A and immobilized in polyvinyl butyral were found to exhibit a response, as determined by EIS, to the sera of patients infected with dengue fever or dengue hemorrhagic fever [45]. Serotypes of dengue fever were distinguished using this detection scheme with *Cratylia morris* (CramoLL) lectin immobilized on iron oxide nanoparticles within the polymer film on a gold electrode. Immobilization of the *Bauhinia monandra* (BmoLL) lectin onto gold nanoparticles dispersed in polyaniline on a gold electrode surface was also able to detect dengue fever glycoproteins via EIS. The same group reported applying these modified electrodes with gold nanoparticles modified with either Con A or CramoLL lectin to the detection of ovalbumin [46, 47]. In a recent study, the binding of Con A to PAMAM dendrimers modified by mannopyranosyl ferrocene units was detected as a reduction in the peak current due to ferrocene oxidation using DPV [48]. The same group also developed mannopyranosyl ferrocene modified gold nanoparticles for DPV detection of Con A binding

[49]. The use of quantum dots (ZnO) conjugated to a glycoprotein (CEA) was demonstrated to enable sensitive detection of CEA by competitive displacement of these conjugates from an electrode surface modified by lectin Con A, followed by SVW detection of zinc stripping peaks [50]. Sensitive detection of Con A was achieved using glucose modified multiwall carbon nanotube – polyaniline composites and DPV detection of the current reduction arising from Con A binding [51]. EIS was applied to detect Con A binding to carbohydrate modified gold nanoparticles on screen-printed carbon electrodes [52]. EIS was recently used to detect Con A binding to a mannose-modified aniline polymer that underwent a conductivity change upon lectin binding [53]. A wider range of studies concerning carbohydrate – protein interactions and their applications in biosensor development has been reviewed [54].

One of the obstacles in studying carbohydrate-protein interaction is the weak binding affinity between carbohydrate and protein, which can be overcome by multivalent interactions between multiple binding sites on proteins, such as lectins, and clusters of the carbohydrate ligands [55]. Multivalency and increased affinity have been achieved on solid surfaces by appropriately controlling carbohydrate density on the surface [56]. Recently, we also showed that the binding affinity of the carbohydrate and lectin on nanoporous gold (NPG) is different than on flat gold [57]. Carbohydrate-protein interaction studies have predominantly been performed on substrate supported gold films [43, 58], glass [59] or polystyrene [60] with a growing number of studies on Au nanoparticles [61–66]. Gold surfaces can be modified by SAMs presenting different terminal functional groups, which can be used in conjugation reactions for the attachment of biomolecules [67].

Nanoporous gold (NPG) is prepared by selectively leaching less noble metal(s) such as Ag from an alloy with typically 20% – 50% gold [68, 69]. It consists of interconnected pores and ligaments which increase the surface to volume ratio tremendously. The increased surface area of NPG can be used to enhance the sensitivity of assays by increasing enzyme loading and amplifying analytical signals [70, 71]. The development of NPG in array formats has recently been reported [72–74], and this strengthens the prospects for application of NPG in screening technologies. A general review of the analytical applications of NPG has recently appeared [75].

The aim of the present study is to explore some of the possibilities for developing ELLA on NPG. We report here the application of NPG for development of electrochemical ELLA, in both kinetic and competitive formats, using SWV. Scheme 1 summarizes the essential aspects of the electrode modifications used for lectin-enzyme and glycoprotein immobilization. We also demonstrate that glycoproteins immobilized onto NPG exhibit a differential response to the binding of a lectin-enzyme conjugate (Con A or soybean agglutinin bound to alkaline phosphatase) as detected by SWV detection of the product formed from the action of alkaline phosphatase on the substrate p-aminophenyl phosphate (p-APP). The present study demonstrates that NPG surfaces offer new possibilities for the development of assays for glycoconjugates, and prospects for application in high-throughput screening using electrochemical methods. The present study represents the first application of NPG to electroanalytical detection of glycoprotein-lectin interactions.

A selection of biologically significant glycoproteins studied as biomarkers for various diseases including transferrin, immunoglobulin G, fetuin, asialofetuin, carcinoembryonic antigen, and prostate specific antigen, are used in these experiments. Serum transferrin consists of two biantennary glycan units and glycosylation changes in transferrin have been reported for disease conditions including hepatocellular carcinoma and sepsis [76, 77]. Carbohydrate deficient transferrins are regarded as biomarkers for alcoholism [78]. Human IgG is the most prominent class of proteins in human serum and changes in glycosylation

are observed in disease states [79]. Fetuin is regarded as a biomarker for vascular calcification and low levels of circulating fetuin has been found to be associated with peripheral arterial disease in type 2 diabetes [80, 81]. In humans, the homologue of this protein (fetuin-A) is now referred to as α -2-HS-glycoprotein. The properties and biological significance of fetuins has recently been reviewed [82]. Carcinoembryonic antigen is a 180 kDa glycoprotein involved in cell attachment and is a biomarker for colorectal cancer, almost 50% of the total weight of CEA is associated with the glycan part [83]. Prostate specific antigen is a biomarker for prostate cancer; it is also heavily glycosylated and its different glycosylation patterns have been reported as related to malignant versus benign conditions [84]. Concanavalin A is one of the most commonly used lectins in glycan assays [85]. Concanavalin A is a lectin specific for α -D-mannopyranoside and α -D-glucopyranoside [86]. It has been reported to bind to biantennary mannose containing glycoproteins with higher affinity compared to the glycoproteins with triantennary mannose units [87, 88]. Soybean agglutinin (SBA) is a lectin from *Glycine max* and binds to the GalNAc/Gal carbohydrate moiety, and binds less strongly to D-galactose [89]. SBA exists as a tetramer over a wide range of pH up to pH 10.8 but becomes a monomer below pH 2.5 [90, 91].

2. Materials and methods

2.1. Materials

Gold wire (0.2 mm diameter, 99.99%) was obtained from Electron Microscopy Sciences (Fort Washington, PA). Transferrin (> 97%), immunoglobulin G (IgG) from rabbit serum, fetuin from fetal calf serum (lyophilized powder), asialofetuin (type I) also from fetal calf serum, Concanavalin A, soybean agglutinin, and bovine serum albumin (98%) all were purchased from Sigma-Aldrich (St. Louis, MO). Carcinoembryonic antigen (CEA) and prostate specific antigen (PSA) were purchased from Fitzgerald (North Acton, MA). Alkaline phosphatase labeling kits were purchased from Dojindo Molecular Technologies Inc. (Rockville, Maryland). p-Aminophenyl phosphate (p-APP) was obtained from Gold Biochem (St. Louis, MO). Sodium carbonate (enzyme grade, >99%), N-hydroxysuccinimide (NHS, 97%), sulfuric acid (certified ACS plus), nitric acid (trace metal grade), hydrogen peroxide (50%), and sodium bicarbonate (certified ACS) were all from Fisher Scientific (Pittsburg, PA). Potassium dicyanoargentate ($K[Ag(CN)_2]$) (99.96%) and potassium dicyanoaurate ($K[Au(CN)_2]$) (99.98%), ethanol (HPLC/spectrophotometric grade), acetonitrile (HPLC grade), lipoic acid, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (>99%), glycine (99%), methyl- α -D-mannopyranoside (> 99%), sodium bicinchoninate (98%), sodium tartrate (> 99%), sodium hydroxide (99.99%), cupric sulfate (99%), zinc chloride (98%), magnesium chloride (99%), and sodium chloride (99%), glycine (> 99%), calcium chloride (99.5%), manganese chloride (> 99%) were obtained from Sigma-Aldrich (St. Louis, MO). All reagents were used without further purification. Milli-Q water (18.2 M Ω) was used for preparation of all aqueous solutions. In some of the experiments, a thiolated α -mannoside (8-mercaptooctyl α -D-mannopyranoside) was used, whose synthesis has already been reported [57]. The synthesis of 3,6-dioxa-8-mercaptooctanol (PEG₃-SH), adapted from a prior report [92], is included in the supporting information file.

2.2. Procedures

2.2.1. Preparation of nanoporous gold coated gold wires—Solutions of 50 mM $K[Ag(CN)_2]$ and $K[Au(CN)_2]$ in 0.25 M Na_2CO_3 were prepared. The solution used for electrodeposition was prepared by combining 4.9 mL of the solution of $K[Ag(CN)_2]$ with 2.1 mL of the $K[Au(CN)_2]$ solution followed by degassing with argon for 10 min. Electrodeposition was done using a three electrode arrangement in a glass cell containing 7

mL of solution, a platinum wire counter-electrode, and a Ag|AgCl (sat'd. KCl) reference electrode. A gold wire of length 5.0 mm and diameter 0.2 mm served as the working electrode. Electrodeposition was carried out for a period of 10 minutes at a potential of -1.0 V (vs. Ag|AgCl in KCl (sat'd.)). Dealloying was carried out by immersing the electrodeposited Au+Ag alloy coated gold wire in concentrated nitric acid for 17 hours followed by rinsing with Milli-Q water and ethanol.

2.2.2. Characterization of NPG—The surface morphology and general structure of NPG was studied by scanning electron microscopy (JEOL JSM-6320F field emission SEM). Electrochemical surface area was determined by the gold oxide stripping method by scanning from 0 V to 1.5 V and back to 0 V (vs. Ag|AgCl) at 100 mV sec^{-1} . The charge under the oxide reduction peak was integrated to estimate the surface area of the NPG covered gold wires, using the reported conversion factor of $450 \mu\text{C cm}^{-2}$ [93].

2.2.3. Preparation of self-assembled monolayers—SAMs of lipoic acid were prepared by immersing 10 wires at a time into a 10 mM lipoic acid solution, in ethanol, of 10 mL total volume. The wires were left for approximately 17 hours (overnight) and then rinsed with ethanol twice followed by rinsing with acetonitrile. SAMs of mercaptododecanoic acid (MDDA), 1:5 molar solution ratio MDDA + mercaptooctanol (HS-C₈-OH), 1:5 molar solution ratio MDDA + PEG₃-SH, and of a 1:5 molar solution ratio of the thiolated α -mannoside and PEG₃-SH were prepared in a similar manner.

2.2.4. Protein immobilization onto self-assembled monolayers—NPG coated wires modified with lipoic acid SAMs, or those containing mercaptododecanoic acid, were immersed into a solution of EDC (5 mM) and NHS (5 mM) in acetonitrile for 5 hours. After 5 hours, the wires were rinsed with acetonitrile, dried and then rinsed twice with 10 mM phosphate buffered saline (pH 7.4) and immersed into the Con A-ALP conjugate solution (containing 50 μg of conjugate), in 10 mM PBS buffer (100 μL) of pH 7.4 in a small glass vial at 4 °C for 17 hours followed by rinsing with PBS buffer for use in the kinetic assays. For the competitive immunoassays and screening of conjugate binding, NPG coated gold wires with NHS ester groups were prepared as above and then these wires were incubated with 50 mg of glycoproteins in 100 μL of PBS buffer for 17 hours at 4 °C. Inhibition studies with methyl- α -D-mannopyranoside were performed with immobilized glycoprotein on the NPG surface and incubated with 50 $\mu\text{g mL}^{-1}$ Con A-ALP conjugate and different concentrations of methyl- α -D-mannopyranoside.

2.2.5. Preparation of lectin alkaline phosphatase (Con A-ALP or SBA-ALP) conjugates—100 μg of the Con A was labeled with pre-activated alkaline phosphatase (NH₂ reactive ALP) provided in the Dojindo labeling kit (LK12–10) following the instructions provided. Briefly, 100 μg Con A was added to the microcentrifuge filter provided with the kit, washed twice with washing buffer and then 30 μL of ALP dissolved in reaction buffer was added to the tube and incubated at 37 °C for 2 hours. 170 μL of storage buffer was added to the tube, mixed well and then the ALP-antibody conjugate was stored at -20 °C until use. The conjugate of soybean agglutinin (SBA) with ALP was prepared in a similar manner.

2.2.6. Electrochemical enzyme-linked kinetic lectin assay—Square wave voltammetric (SWV) measurements were done using a PARSTAT 2273 (Princeton Applied Research, Oak Ridge, TN) and the PowerPULSE software. The optimal parameters for the SWV were obtained by varying pulse width, pulse height and step height used in the square wave voltammetric analysis of a p-aminophenol (p-AP) standard solution (1 mM). The best parameters determined for the square wave voltammetric measurement of the oxidation of p-

aminophenol were: pulse height 50 mV, pulse width 0.2 sec, and step height 2 mV. The potential was scanned from -0.1 V to 0.2 V at a rate of 5.0 mV sec^{-1} . SWV scans record the forward current (I_f), reverse current (I_r), and the difference current (ΔI) vs. the applied potential.

In the kinetic enzyme-linked lectin assay, the peak difference current for p-AP oxidation on each wire was measured before incubating with glycoprotein and again after incubating with glycoprotein. The reported peak difference current is that found after subtraction of the background current in the square wave voltammogram using the PowerPULSE software. The incubation period with glycoprotein used was 2 hours and was carried out in PBS buffer (pH 7.4, 10 mM) containing 1 mM each Ca^{2+} and Mn^{2+} . The NPG covered electrodes were then removed from the incubation solution, rinsed with glycine buffer, and then put into 3 mL of glycine buffer (pH 9.0, 100 mM). The substrate, p-aminophenyl phosphate (p-APP) was introduced via micropipette into the stirred solution (argon degassed) containing the Con A-ALP conjugate modified NPG covered Au wires and allowed to react for a period of 2 minutes prior to conducting the potential scan. p-APP concentrations less than $100 \mu\text{M}$ were not enough to obtain satisfactory currents, and thus $200 \mu\text{M}$ was chosen as a working substrate concentration. Each measurement was repeated three times using three different electrodes. The absolute value of the difference in the maximum peak difference currents $|\Delta I (\text{before incubation}) - \Delta I (\text{after incubation})|$ was plotted vs. the glycoprotein concentration to obtain the response plots.

2.2.7. Electrochemical enzyme-linked competitive lectin assay—For the competitive immunoassay, the NPG covered gold wire electrodes modified with immobilized glycoprotein were allowed to compete for the Con A-ALP conjugate in solution with variable concentrations of free glycoproteins in solution for 17 hours (overnight) in PBS buffer (10 mM, pH 7.4) in presence of 1 mM each Ca^{2+} and Mn^{2+} at 4°C . The concentration of Con A-ALP conjugate used was $25 \mu\text{g mL}^{-1}$ and $50 \mu\text{g mL}^{-1}$ for the assay performed for TSF and IgG, respectively. After incubation with conjugate, the NPG coated wires with bound conjugate were incubated with 0.2 mM p-APP in glycine buffer (pH 9.0, 100 mM) for 2 minutes and then a square-wave voltammetry scan was conducted.

2.2.8 Inhibition studies with methyl α -D-mannopyranoside— IC_{50} values using methyl α -D-mannopyranoside were calculated using Graphpad Prism 5.04 software. The model used was response (Y) vs. $\log$ [inhibitor], with four parameters (Bottom, Top, $\log(\text{IC}_{50})$, and Hillslope) in the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(\log(\text{IC}_{50}) - X) * \text{Hillslope}})^{-1}$ where $X = \log$ [inhibitor]. In this equation, Y represents the difference in SWV peak difference current and $X = \log$ [methyl α -D-mannoside]. In these experiments, NPG covered Au wires modified with lipoic acid bound glycoproteins were incubated with methyl α -D-mannopyranoside of the given concentration in the presence of $50 \mu\text{g mL}^{-1}$ of conjugate for 17 hours. After removal and gentle rinsing with buffer, the peak difference current from the SWV was then determined after 2 minutes incubation with p-APP. The electrode was then placed in buffer containing $50 \mu\text{g mL}^{-1}$ conjugate and 1 mM methyl α -D-mannopyranoside and then after incubation for two additional hours, the peak difference current from the SWV was measured again after 2 minutes incubation with p-APP. Analysis of the difference between these two peak difference currents as a function of concentration of methyl α -D-mannopyranoside was used to determine the IC_{50} values for transferrin and IgG.

2.2.9. Reductive desorption of lipoic acid SAM—Reductive desorption of lipoic acid SAM was carried out in a 0.5 M NaOH solution, argon degassed for 30 minutes. CV scans were performed in 3 mL of solution in a three electrode cell arrangement as mentioned

before for other CV measurements. The CV scan was performed between 0 and -1.5 V (vs. Ag|AgCl) at a scan rate of 20 mV sec^{-1} .

2.2.10. BCA assay estimation of the amount of protein immobilized on NPG—

The concentrations of transferrin, IgG, CEA, and Con A-ALP conjugate solutions before and after incubation with NHS-activated lipoic acid modified NPG covered Au wires was determined using the BCA (bicinchoninic acid) assay [94]. *Reagent A*: 1 g bicinchoninate (BCA), 2 g sodium carbonate, 0.16 g sodium tartrate, 0.4 g sodium hydroxide, and 0.95 g sodium bicarbonate were dissolved into 100 mL Milli-Q water and the pH was then adjusted to 11.25 with 10 M sodium hydroxide. *Reagent B*: 0.4 g cupric sulfate was dissolved in 10 mL Milli-Q water. A standard working solution was prepared by mixing 100 volumes of reagent A and 2 volumes of reagent B. Standard Con A samples containing 0.2 to 50 mg protein was prepared. 2.5 mL of standard working reagent was added with 50 μL protein and incubated at 60°C for 30 min. Samples were cooled and absorbance measurement was recorded at 562 nm using a Cary-50 UV-visible spectrophotometer. The amount of protein immobilized on the NPG surface was calculated from the difference in protein concentration in solution before and after exposure to the NPG surface modified with an activated SAM.

3. Results and discussion

3.1. Characterization of nanoporous gold electrodes

Surface characterization of the NPG covered gold wires was performed by SEM and the surface area was then determined by cyclic voltammetry. Fig. 1a shows the top view of the NPG covered gold wire. SEM shows the expected network of pores and ligaments. The surface areas of NPG and of gold wire were determined using the gold oxide stripping method. Fig. 1b shows the cyclic voltammogram for NPG coated gold wire measured in $0.5 \text{ M H}_2\text{SO}_4$ at a scan rate of 100 mV sec^{-1} . A CV measured using the gold wire as the working electrode was similar in appearance but with much smaller currents. The geometric surface area of the gold wire electrodes used is 0.032 cm^2 , whereas true surface area was found to be 0.040 cm^2 indicating a roughness factor of 1.25. The true surface area of the NPG coated gold wires (12.5 cm^2) used in this study is approximately 300 times higher than that of the bare gold wire alone.

3.2. Square-Wave Voltammetric Detection of the Activity of a Lectin–Enzyme Conjugate Linked to Self-Assembled Monolayers

A comparative study of NPG covered gold wire and uncoated gold wire for potential application in electrochemical ELLA was done by preparing lipoic acid SAMs on both surfaces followed by immobilization of the Con A-ALP conjugate onto the EDC/NHS activated SAMs. SWV studies were performed by incubating these modified electrodes in 1 mM p-APP in glycine buffer (pH 9.0, 100 mM) for 2 minutes prior to recording the SWV sweep. Fig. 2a shows the SWVs of the Con A-ALP conjugate modified electrodes; the SWV for the modified gold wire shows no discernible peak difference current after incubation with 1 mM p-APP, whereas a significant peak difference current was observed after incubation with 1 mM p-APP for the modified NPG covered Au wire electrode. This observation shows the increased sensitivity of the higher surface area NPG covered Au wire electrode compared to that of the gold wire electrode alone for use in an electrochemical assay using the immobilized enzyme-lectin conjugate.

The use of self-assembled monolayers of lipoic acid allows easy immobilization of the conjugate onto the NPG surface. A key property of lipoic acid is that it forms a relatively disordered SAM on the NPG surface with a significant presence of defects. These defects provide sites for the electrooxidation of the p-aminophenol generated after the enzymatic

reaction of alkaline phosphatase and p-APP substrate. Several different SAMs were studied for possible application for protein immobilization and assay development. Fig. 2b shows SWVs of Con A-ALP conjugate immobilized onto different SAMs on NPG. SAMs of mercaptododecanoic acid (MDDA), mercaptododecanoic acid + mercaptooctanol (HS-C₈-OH), and MDDA + 3,6-dioxa-8-mercaptooctanol (HS-(CH₂CH₂O)₂-CH₂CH₂OH) were studied for possible application in the electrochemical ELLA. SWV scans in the absence of p-APP substrate for these SAMs can be found in the supporting information file. Sweeps in the absence of p-APP are generally featureless but may show different background current levels. Among the SAMs studied, those of lipoic acid resulted in SWV peak difference currents due to oxidation of p-aminophenol that were larger than those of the other SAMs studied. The p-aminophenol product does not easily access the NPG surface through the other SAMs to undergo electrooxidation, and hence the peak current is smaller. The SWV background current is larger for the less ordered lipoic acid SAM, possibly due to it having a higher monolayer capacitance than for the better ordered MDDA containing SAMs. Lipoic acid was thus chosen as a suitable molecule for SAM formation in the present assay approaches, since it allows for both protein immobilization and electrochemical detection of the enzyme product.

3.3. Michaelis-Menten kinetics of the Con A-ALP conjugates on NPG

The activity of the Con A – alkaline phosphatase conjugate on the lipoic acid SAM modified NPG was measured to further study the suitability of the surface for conjugate immobilization. The ALP kinetic behavior on the NPG surface was studied by varying the concentration of substrate and recording the SWV difference currents. p-APP concentrations of 0.01 mM to 1.0 mM were studied, and the results are shown in Fig. 3 for an NPG electrode modified by the Con A-ALP conjugate exposed to these substrate concentrations for a period of 2 minutes each. A period of 2 minutes was found to be sufficient to obtain the maximal difference current and longer times of exposure to substrate up to 30 minutes did not increase the difference current. The peak difference current is the difference between the maximum value of $-\Delta I$ and a background difference current as determined at the peak potential. A set of individual SWV over the above range of substrate concentration is presented in the supporting information file as an example. There was an increase and trend towards saturation in the peak difference current with the increase in substrate concentration which finally reached a plateau, consistent with Michaelis-Menten kinetic behavior. The peak difference current of the SWV measurement is related to the initial reaction rate and hence to the reaction kinetics. The K_m value was determined by fitting the data to the Michaelis-Menten equation and it was found to be 200 μ M. This value was of interest because most enzyme electrodes are operated near K_m values. The SWV sweeps resulted in a prominent peak for the oxidation of p-aminophenol in this concentration range. In contrast, attempts to measure current peaks due to oxidation of p-aminophenol using linear sweep voltammetry were much less successful due to the high background currents arising from charging of the electric double layer. The oxidation of p-AP, the ALP reaction product, to p-quinoneimine occurs near a potential close to 0 V (vs. Ag|AgCl) on NPG. The surfaces of NPG are more electroactive than relatively smooth gold surfaces. Catalytic activity depends on nanostructure which leads to the oxidation potential depending upon the defects, curvature and roughness of the surface. The lower oxidation potential observed for p-AP in this study compared to the oxidation potential of p-AP on relatively flat gold surfaces reported elsewhere might be related to the nanostructure of NPG[95]. For example, a negative shift in oxidation potential of methanol on NPG has been reported by Zhang et al. [96] The peak difference current for SWV has been reported to be proportional to the velocity of the reaction and can be used to create a Michaelis-Menten plot[97, 98]. The rate of a reaction on the electrode surface can be obtained by dividing the peak difference current by the product nF , where, n is the number of electron involved in the redox process and F is

Faraday's constant[97]. A K_m value of $56 \pm 5 \mu\text{M}$ was reported for free alkaline phosphatase in pH 9.0 TRIS buffer solution using p-APP as the substrate[37]. In another similar study with IgG-ALP conjugates on NPG we have found the K_m value to be $290 \mu\text{M}$. In our prior study using IgG-ALP conjugates where the monoclonal IgG binds free PSA, and the substrate used was p-nitrophenyl phosphate with UV-visible detection of the p-nitrophenolate product at 410 nm, an increase in K_m from $210 \mu\text{M}$ to $300 \mu\text{M}$ was observed upon immobilization using EDC activated lipoic acid monolayers. The conjugation of the IgG to the enzyme itself in solution was found to increase the measured value of K_m from $40 \mu\text{M}$ to $210 \mu\text{M}$ [68]. Similar shifts in K_m value are thus expected for the Con A-ALP conjugate on the NPG surface.

An increase in K_m for the ALP enzyme is expected both as a consequence of conjugation to Con A and immobilization within NPG for a number of reasons. Conjugation to Con A will introduce steric hindrance to access of the substrate to the active site of ALP. Immobilization of the Con A – ALP conjugate in NPG will introduce an additional further increase in K_m . Increases in K_m have often been reported for immobilization of enzymes within porous supports and have been attributed to restricted conformational freedom on the surface, adoption of orientations that hinder access of substrate to the active site, conformation changes upon immobilization, and diffusional restrictions on the substrate within the porous network[99]. A higher value of K_m , $410 \mu\text{M}$, for alkaline phosphatase covalently attached to nylon mesh using BSA and glutaraldehyde as cross-linkers was reported by Pariente et al.[100]. An approximately three-fold increase in the K_m value for type III adenosine deaminase and a five-fold increase for type V enzyme immobilized by BSA glutaraldehyde crosslinking onto the Teflon® membrane of an ammonia sensor was reported[101]. A ten-fold increase in K_m was reported for alkaline phosphatase immobilized on nylon mesh using p-nitrophenyl phosphate and p-aminophenyl phosphate substrates [100]. Shulga et al. reported an increase in K_m for acetylcholinesterase immobilized by physisorption on NPG[102]. Wilson et al. reported a similar increase in K_m for ALP and hydroquinone diphosphate substrate[103]. A decrease in turnover number was reported when enzyme was conjugated to IgG. Enzymatic activity and K_m value is also shown to be dependent on the pore size of the porous materials used for enzyme immobilization. A pore size dependent change in enzymatic activity and K_m values were reported for 'gigaporous' (314 nm), macroporous (104 nm) and mesoporous (14.7 nm) polystyrene microspheres for the lipase from *Burkholderia cepacia* immobilized by strong hydrophobic interactions[104]. Brinker et al. showed that the change in enzymatic activity and affinity for the corresponding substrate can be enzyme dependent; their study on glucose-6-phosphate dehydrogenase and horseradish peroxidase showed that enzymatic activity of immobilized glucose 6-phosphate was 36% and the enzymatic activity of HRP was 73% of that of the free enzyme in solution. Miller et al. reported that the enzymatic activity of free glutathione-S-transferase was 4 times higher than the activity of glutathione-S-transferase immobilized in porous silicon[105]. Stevenson et al. reported an increase in K_m and decrease in enzymatic activity of HRP immobilized on glass coverslips[97].

Optimal conditions for the activity of Con A – ALP conjugate on the NPG surface were determined by varying buffer pH. Fig. 4 shows the profile of enzymatic activity versus pH for the conjugate on the NPG surface, measured as peak difference current versus pH. As reported in the literature, the activity of alkaline phosphatase is maximum around pH 9.0 [106]. There was almost no activity observed in pH 7.4 phosphate buffer; therefore, glycine buffer at pH 9.0 was chosen as the optimal buffer for this study. Since alkanethiol SAMs are reported to be less stable at basic pH, and most of the protein binding studies are performed in phosphate buffer close to neutral pH, phosphate buffer at pH 7.4 was chosen for the incubation of electrodes with the conjugates and for longer storage [107, 108].

The use of other enzymes in the Con A – enzyme conjugate, such as horseradish peroxidase, could also be considered. Alkaline phosphatase was chosen as it is one of the most widely used enzymes which is robust and has lower substrate specificity as almost any phosphate group containing compound can act as a substrate for this enzyme. ALP can act against a number of other reported substrates that can be oxidized at an electrode surface. Some of the possible substrates include p-nitrophenyl phosphate which is converted to p-nitrophenol by alkaline phosphatase and undergoes oxidation at +1.0 V, at such a high potential substantial background due to oxidation of components in biological samples would reduce the bioanalytical applicability of the assay, also at this high potential oxidation of gold might weaken Au-S bonds and hence desorb SAMs from the surface. Phenyl phosphate is converted to phenol at +0.7 V vs. Ag|AgCl but it has been reported to form a passivating polymeric film on the electrode surface [109]. Naphthyl phosphate is converted to naphthol and undergoes oxidation at 0.4 V vs. Ag|AgCl [110]. Ascorbic acid phosphate is converted to ascorbic acid at 0.32 V vs. Ag|AgCl but no redox peak was observed in our study for this substrate, also, the presence of ascorbic acid in biological samples makes it a relatively less attractive substrate for biological assays [111]. Hydroquinone diphosphate is a relatively new substrate for alkaline phosphatase which gives hydroquinone under dephosphorylation by alkaline phosphatase. Hydroquinone undergoes oxidation at 21 mV on a flat gold surface [103]. Hydroquinone diphosphate is not commercially available so p-aminophenyl phosphate, which is available, was the substrate of choice, which is one of the most favored substrates for ALP in electrochemical assays [110, 112]. The product p-aminophenol is oxidized near 0 V (vs. saturated calomel) on the screen printed carbon electrode [113]. Some of the advantages of p-APP over other substrates that make p-APP an attractive substrate include the low oxidation potential, less electrode fouling and electrochemical reversibility, and it is commercially available [110].

3.4. Kinetic enzyme-linked lectinsorbent assay on NPG

The kinetic enzyme-linked lectinsorbent assay is based on the difference in the rate of enzymatic reaction of ALP conjugated to Con A, before and after glycoprotein binding to the Con A. Thus, we chose a 200 μM substrate concentration for the kinetic assay, a concentration which falls in the pseudo-linear range. The presence of a large glycoprotein molecule bound to the lectin close to the ALP causes steric hindrance for the access of the p-APP substrate to the active site of ALP. The binding of the glycoprotein will reduce the initial rate of enzymatic conversion of p-APP to the oxidizable p-aminophenol product. The enzyme reaction rate is assumed to be proportional to the peak difference current of the square wave voltammogram. The difference in SWV peak difference current before and after incubation is the response variable for the assay. We measure the peak difference current after a fixed incubation time which is assumed to be proportional to the relative rates of the enzymatic reaction of ALP before and after glycoprotein binding to the Con A. The concentration of p-APP of 200 μM is close to the K_m value, and p-APP concentrations less than 100 μM did not produce current peaks of sufficient magnitude in the SWV scans. For the kinetic glycoprotein assay, the incubation time for conjugate and glycoproteins is important so a study was performed by varying the incubation time with the glycoprotein and the conjugate immobilized NPG. Saturation of the peak current was reached within 1 hour and thus 2 hours was chosen for the incubation time to be sure peak current saturation was reached. The same information was used for the incubation time for the competitive assay studies presented below. Even though 2 hours of incubation time was used to ensure complete equilibrium for the assay, signal saturation was generally obtained within 60 minutes.

Enzyme kinetics and hence the rate of conversion of substrate to product depends on the access of the substrate to the active site of the enzyme. When relatively large and bulky

molecules bind to the lectin conjugated to the enzyme, access of the substrate to the active site is hindered and results in a decrease in activity and hence a decrease in initial velocity of the reaction. Kinetic assays are not frequently found in the literature [114–116]. A competitive type enzyme multiplied immunoassay (EMIT) for digoxin was reported by Chang et. al based on the difference in enzymatic activity of glucose-6-phosphate dehydrogenase (G6PDH) covalently attached to the digoxin. The enzymatic activity of G6PDH was reported to decrease when digoxin specific antibody bound to the digoxin and as more unlabeled digoxin were added more enzyme bound digoxin was released from the antibody and hence enzymatic activity increased[116]. Rosenthal et. al. evaluated EMIT and compared with the standard radioimmunoassay for digoxin and has found excellent agreement between the two methods [117].

As a proof of concept of this assay we chose the glycoproteins IgG and transferrin. When glycoprotein binds to Con A – ALP conjugate, access of the p-APP to the enzyme active site is hindered and results in a decrease in the reaction velocity. Fig. 5(a–b) shows the binding isotherms determined for the binding of IgG and transferrin to the immobilized Con A-ALP conjugate. As expected, there was a decrease in enzymatic activity with the increase in concentration of glycoprotein which is observed in terms of the increased difference in peak difference current. The binding affinity of Con A to IgG was found to be $K_d = 105$ nM. A K_d value for binding of IgG and Con A was not found, but it has been reported to bind to Con A through the oligosaccharide presented on the Fc region[118]. Con A immobilized onto a monolithic polymer cryogel was used to bind human IgG from plasma [119]. The binding affinity of Con A to transferrin was found to be $K_d = 650$ nm. The binding affinity of Con A to transferrin in solution was reported to be 1–2 μ M [120], and a value of $K_d = 1.2$ mM was found in SPR experiments of transferrin binding to Con A immobilized on a lectin chip[121]. The binding affinity of Con A to glycoprotein depends upon the glycan structure and multivalent interactions. Binding affinity has been shown to be higher for multivalent mannose units compared to similar monovalent units in the glycoconjugates [122]. Thus, the overall affinity of a glycoprotein-lectin interaction is dependent upon the mannose linkage, whether it is biantennary or triantennary, as well as the numbers and spacing of terminal mannose units which can possibly lead to multivalent binding hence increasing affinity. Two other proteins asialofetuin and fetuin were also studied for the kinetic assay but these proteins did not produce any significant change in the peak current, this we believe is because of the relatively lower binding affinity and especially the smaller sizes of these proteins (45–55 kDa) as compared to IgG (~160 kDa) or transferrin (~80 kDa). The kinetic assay described based on lectin – enzyme conjugates is thus expected to be more responsive for larger size glycoproteins. Based on the average of the standard deviations (σ) for the determinations at each protein concentration, a detection limit based on $3 \times \sigma$ would be a difference of 0.50 on the y-axis for the IgG data representing a detection limit of 0.02 μ M. A similar consideration for the transferrin data ($3 \times \sigma = 0.63$) gives a detection limit of 0.14 μ M. The response in each case is non-linear, with the range over which transferrin can be detected before the response approaches saturation being wider (up to ~3–4 μ M than that over which IgG can be detected (up to ~1 μ M). The level of transferrin in human serum typically falls between 2–3 g L⁻¹, or 2.5 – 3.8 μ M[123]. Thus, detection of variant glycoforms of transferrin of lower concentration seems at least worth considering using electrochemical ELLA provided the transferrin fraction can be isolated and comparison with other methods such as mass spectrometry made. The overall concentrations of all immunoglobulin G in human serum is typically 7 – 16 g L⁻¹, or 47 – 107 μ M [124].

3.5 Square-Wave Voltammetric Detection of Lectin-Enzyme Conjugate Binding to Immobilized Glycoproteins

A selection of glycoproteins (transferrin, IgG, fetuin, asialofetuin, PSA and CEA) were covalently immobilized onto the lipoic acid SAMs on the NPG surface by EDC/NHS coupling. Bovine serum albumin (BSA) and LPA modified NPG were used as negative controls. These protein modified wires after incubation with the Con A-ALP conjugate were then incubated with the ALP substrate p-APP and then SWV was performed. Fig. 6a shows a bar plot of peak heights of the SWVs for the different glycoproteins. The glycoproteins immobilized on NPG covered Au wires were incubated with $50 \mu\text{g mL}^{-1}$ Con A-ALP conjugate in PBS (pH 7.4, 10 mM) containing 1 mM Ca^{2+} and Mn^{2+} , for 24 hours, and then SWV scans were performed in the presence of 1 mM p-APP in pH 9.0 glycine buffer, the SWV scans were performed after 2 minutes incubation with p-APP. Data were also acquired after 2 hours of incubation of the immobilized glycoproteins with Con A – ALP conjugate and show a similar pattern of response but with lower current magnitudes, as shown in Fig. 6b. While the 24 hour incubation produces larger currents, the 2 hour incubation time would be more attractive for practical reasons. Each value used to determine the average peak heights in Fig. 6 is the peak value of $-\Delta I$ from an individual SWV from which a background current has been subtracted (see supporting information file). The background current level varies somewhat between different electrodes, and is partly a function of the capacitance of the SAM (see supporting information for individual examples).

Different peak difference currents were observed depending on the amount of conjugate bound onto the glycoprotein attached to the SAM on the NPG surface, which is anticipated to vary with the structure of the glycans present on the immobilized glycoproteins, the number and orientation of the glycans presented by the surface, and any contribution from non-specific adsorption of conjugate. The maximum peak difference current from Con A – ALP was obtained for CEA and the smallest peak currents were observed with fetuin and asialofetuin, second in response to CEA are transferrin and PSA, followed by IgG. BSA immobilized on NPG and the LPA SAM on NPG alone both showed very small peak currents. The very small response from the LPA SAM indicates that the Con A – ALP conjugate does not strongly adsorb non-specifically on this surface. Significantly larger peak currents were observed with the bare NPG incubated with the Con A-ALP conjugate due to the non-specific adsorption of protein on the gold surface. A thiolated α -mannoside modified NPG was also used for the study but an almost negligible signal was obtained using either the pure thiolated α -mannoside SAM or a 1:5 mixed SAM of the thiolated α -mannoside and 3,6-dioxa-8-mercaptiooctanol. This could be because either the conjugate did not bind to the SAM or the oxidation of the p-AP product was blocked by the SAM. It is possible that the Con A – ALP conjugate encounters some steric interference in its interaction with mannose on the surface that is not present for binding of Con A alone. Con A is reported to bind to the mannose units of glycoproteins and its binding affinity is higher with biantennary glycoproteins as compared to triantennary glycan units. Transferrin has biantennary mannose units in its glycan structure and has been reported to bind to Con A [121, 125]. All subclasses of IgG possess a single N-linked glycan that exhibits significant heterogeneity around the core N-acetylgalactosamine unit such as addition of fucose, galactose or sialic acid units [126]. CEA is highly glycosylated and almost 50% of its total weight is carbohydrate and has been shown to bind to Con A [127]. Fetuin contains 3 O-linked glycans and 3 N-linked glycans per molecule [128]. A full analysis of the N-linked glycans of fetuin revealed 23 different structures of varying degree of sialylation, extent of branching (biantennary vs. triantennary), and position of a galactose residue [129]. The O-linked glycans were proposed to be a tetrasaccharide or a trisaccharide variant with one sialic acid instead of two, and including a galactose unit and an N-acetylgalactosamine unit [130]. Asialofetuin can be produced from fetuin by removal of the terminal sialic acid

groups, leaving the terminal galactose units [131]. Comparable frequency changes for binding of fetuin, asialofetuin and transferrin were reported in a quartz crystal microbalance based study. Safina et al. reported strong binding and relatively large SPR signals for Con A binding to TSF compared to its interaction with fetuin and asialofetuin, thus the relative signal intensity and observed binding affinity and hence relative differentiation between two glycoproteins is also affected by the method of study[132]. We have also seen higher binding affinity and hence higher peak current for CEA and TSF.

Similar experiments carried out with the soybean agglutinin – ALP conjugate ($50 \mu\text{g mL}^{-1}$, incubation time of 2 hours) give a different pattern of results, as seen in Fig. 6c (see supporting information for representative set of SWV data). The highest peak difference current is also found for CEA, and the peak difference current for PSA is intermediate in value, but there is a greater difference in current seen between fetuin and asialofetuin. Soybean agglutinin binds preferentially to terminal N-acetylgalactosamine units, and less strongly to galactose residues. This preference is consistent with the stronger response from conjugate binding to asialofetuin than to fetuin. The glycans presented by CEA also have terminal galactose units and a terminal N-acetylgalactosamine unit, along with terminal fucose units and mannose units within the highly branched glycan structure [133]. The terminal carbohydrate units of CEA can vary with the source of the CEA sample [134]. CEA is a much larger protein than either fetuin or asialofetuin and overall contains a greater fraction of glycan structures. PSA contains a single N-linked glycan and is about 8% carbohydrate by mass, and displays variable sialylation[135, 136]. The binding to BSA is absent as expected since BSA presents no attached glycans, and binding to the LPA SAM is also fairly small. It is hypothesized based on these initial results that an NPG array format with use of a larger number of lectin – ALP conjugates each responding differently to an immobilized glycoprotein could be used as a method of electrochemical profiling of glycosylation.

3.7. Competitive assay on NPG

Lectin-carbohydrate interactions were subsequently studied in a competitive format assay, in a format using immobilized glycoproteins for screening lectin-enzyme conjugate binding, and in a kinetic assay approach based on the rate of enzymatic reaction before and after lectin-glycoprotein binding. ELLA are traditionally performed by adsorbing the glycoproteins of interest onto the surface and then allowing either lectins labeled with enzymes or fluorophores, or biotinylated lectins to bind. If a biotinylated lectin is allowed to bind, then it is subsequently reacted with a streptavidin or avidin labeled enzyme. The amount of lectin bound to the surface bound glycoprotein is then detected by enzymatic reaction with the product formation followed spectrophotometrically.

Competitive ELLA was performed on the NPG surface using the glycoproteins TSF and IgG. Glycoproteins were covalently immobilized on the NPG surface and allowed to compete for binding to Con A–ALP conjugate in solution with free glycoprotein in solution. A decrease in amount of the bound conjugate with an increase in amount of free glycoprotein in solution is observed. A different response depending upon the binding affinity, and size of the proteins and hence number of conjugates immobilized on the NPG surface was observed for the different glycoproteins used in this study. Fig. 7 (a–b) shows response plots for the competitive assays for transferrin and IgG. Binding of conjugate to transferrin and hence a significant signal was observed with $12 \mu\text{g mL}^{-1}$ of conjugate at 2 hours incubation whereas, at this conjugate concentration and incubation time, a relatively small signal was obtained for IgG on the NPG surface. An incubation time of 17 hours (overnight) was also evaluated. An increase of almost 50 % in signal for the competitive response (SWV peak difference current) was found for the 17 hours incubation time. The competitive response for transferrin was obtained using $25 \mu\text{g mL}^{-1}$ conjugate with $200 \mu\text{M}$

p-APP, and that for IgG was obtained using $50 \mu\text{g mL}^{-1}$ conjugate and 1 mM p-APP. In addition to the affinity of glycoprotein with a lectin, the response will also depend on the relative size of the protein, orientation on the surface and extent of glycosylation. Based on the average of the standard deviations (σ) for the determinations at each protein concentration, a detection limit based on $3 \times \sigma$ would be a difference of 1.8 on the y-axis for the IgG data representing a detection limit of 0.03 nM. A similar consideration for the transferrin data ($3 \times \sigma = 0.65$) gives a detection limit of 0.4 nM. The response in each case is non-linear, with the range over which transferrin can be detected before the signal flattens being wider (up to ~ 10 nM than that over which IgG can be detected (up to ~ 1 nM).

3.8. Inhibition studies by methyl α -D-mannopyranoside (Me- α -D-Man)

Methyl α -D-mannopyranoside is a ligand for Con A, and binding of Con A to other glycoproteins and glycoconjugates has been reported to be inhibited by Me- α -D-Man. As a test of the binding specificity, inhibition studies were performed using NPG covered wires modified with lipoic acid and covalently bound glycoprotein. These electrodes were incubated with different concentrations of Me- α -D-Man together with $50 \mu\text{g mL}^{-1}$ Con A-ALP conjugate for 17 hours. Peak difference current ($-\Delta I$) from the SWV at each concentration of Me- α -D-Man was subtracted from the peak difference current ($-\Delta I$) observed after subsequent incubation for 2 hours in the presence of 10 mM Me- α -D-Man. With the increase in amount of the Me- α -D-man there was less conjugate bound to the surface and less enzymatic activity resulting in smaller peak difference currents (Fig. 8). IC_{50} values obtained by these methods for TSF and IgG were near $90 \mu\text{M}$ and $286 \mu\text{M}$, respectively. Inhibition constant (K_i) of Me- α -D-Man to Con A binding to the a mannose substituted glycolipid SAM on a gold surface has been reported to be $92 \pm 6 \mu\text{M}$ [137]. The observed IC_{50} values depend on the relative binding affinity of the solid phase ligand and the free ligand in solution, hence the IC_{50} values reported here are in good agreement with the literature reports [6].

3.9. Effect of BSA on ELLA

The effect of an interfering protein was studied using bovine serum albumin (BSA) as a model major serum protein and 5 mg mL^{-1} BSA ($75 \mu\text{M}$) was added to the incubating solution along with $2 \mu\text{M}$ IgG or $2 \mu\text{M}$ TSF, for the kinetic ELLA. The addition of 5 mg mL^{-1} BSA had no effect on the difference in peak current. Fig. 9a shows the bar plot for the study of effect of BSA on kinetic enzyme-linked lectinsorbent assay. As a control experiment, 5 mg mL^{-1} BSA was used alone, instead of glycoprotein, in the kinetic assay and no change in signal was observed; therefore, the drops seen in peak current are due to the binding of glycoprotein to Con A and the consequent reduced enzymatic reaction rate. Similar studies were also performed in the competitive assay as well and added BSA was found to have no real effect (Fig. 9b).

3.10. BCA assay and amount of protein immobilized on the NPG surface

The amount of protein immobilized on the NPG surface was determined by the BCA assay and solution depletion. Table I summarizes the surface coverages of the different proteins immobilized on the NPG surface in moles cm^{-2} . A calibration plot was obtained using known concentrations of Con A and the amount of protein immobilized on NPG surface was determined from the difference in amount of protein initially present in solution and remaining in solution after immobilization onto the NPG surface. The surface coverage of lipoic acid on the NPG electrodes was estimated by reductive desorption of the SAMs in 0.5 M NaOH solution. The average ($n = 5$) surface coverage for lipoic acid SAMs was found to be $2.42 \times 10^{-10} \text{ mol cm}^{-2}$ [57]. The number of lipoic acid molecules was estimated from the charge passed under the reductive desorption peak and the gold surface area of the NPG modified electrode was taken as the 12.5 cm^2 average determined from the oxide stripping

experiments. The thiol atom surface coverage for alkanethiol SAMs on flat gold surfaces has been reported to be $7.6 \times 10^{-10} \text{ mol cm}^{-2}$ and for lipoic acid it is $7.1 \times 10^{-10} \text{ mol cm}^{-2}$ of thiols, which is equivalent to $3.5 \times 10^{-10} \text{ mol cm}^{-2}$ of lipoic acid molecules cm^{-2} , assuming two thiol groups per lipoic acid molecule [138]. This indicated that the surface coverage of lipoic acid on NPG surface is approximately 70% and is thus less than a monolayer. It is also possible that for some of the lipoic acid molecules only one Authiolate bond has formed.

Estimation of the surface coverage of the conjugate on the lipoic acid modified SAMs was done by solution depletion measurements and the BCA protein concentration assay. The amount of either Con A-ALP conjugate or glycoprotein (transferrin, IgG) immobilized onto the surface was estimated by using a solution depletion measurement of protein concentration and the BCA protein concentration assay. Ten NPG wires modified with a lipoic acid SAM, and a terminal $-\text{COOH}$ group activated by EDC/NHS, were added to the $50 \mu\text{g}$ of Con A – ALP conjugate or to $50 \mu\text{g}$ of the glycoproteins dissolved in $100 \mu\text{L}$ PBS buffer. The wires were incubated for 17 hours at 4°C . The amount of protein remaining in the incubation solution was then determined. The difference between the amount of protein initially present in solution and the final amount left in solution gave the estimated amount of protein immobilized onto the NPG wires. Table I presents the amounts of these biomolecules found on average immobilized on an NPG wire as determined from the BCA assay. Values for CEA and PSA of $3.0 \times 10^{-13} \text{ mol cm}^{-2}$ and $9.7 \times 10^{-13} \text{ mol cm}^{-2}$, respectively, were previously reported. Given the surface coverage of lipoic acid, equivalent to $2.42 \times 10^{-10} \text{ mole cm}^{-2}$ and the surface coverage in mole cm^{-2} of either the Con A – ALP conjugate (4.6×10^{-13}), transferrin (7.1×10^{-13}), IgG (4.7×10^{-13}), CEA (3.0×10^{-13}) or PSA (9.7×10^{-13}), one protein is estimated to be attached for approximately every 280–800 lipoic acid molecules. This shows that the conjugation of protein to the surface is dependent upon the type of protein, although association is most probably by bonding to the most easily accessible lysine residues. Transferrin is immobilized in larger amount compared to IgG and Con A – ALP conjugate. This is also consistent with the lower signal observed for IgG in the competitive assay. It is evident from these data that a small fraction of the lipoic acid molecules are conjugated to the much larger proteins. The area occupied by these glycoproteins or by the conjugate is subject to variability considering that they can occupy a range of orientations. Hence, any estimate of fractional surface coverage is approximate in the absence of orientational information.

4. Conclusions

The presented electrochemical assays makes use of the high surface area of NPG and of its ability to be used as an electrode for SWV detection of an oxidizable enzyme product, in this case p-aminophenol (p-AP). The use of SWV is required to overcome the large double layer charging on the NPG electrode. The assay approach can in principle be applied to a range of glycoproteins, especially if different lectins are employed. Given that NPG can be used to create elements in an electrode array, the response of a collection of different immobilized lectin-enzyme conjugates to glycoprotein binding in kinetic or competitive strategies could be envisioned as the basis for an approach to glycoprotein fingerprinting. Binding of different lectin-enzyme conjugates to array elements presenting a glycoprotein bound to an SAM surface could be envisioned as an additional strategy for glycoprotein fingerprinting. Although glycans would not be directly identified, the response patterns could potentially indicate general glycan types or the likely presence of certain terminal sugars. Significant further development would be needed to test and achieve such goals.

A feature of this assay strategy that should be noted is that access of the p-AP product to the gold surface where it is oxidized must be possible, and as such well-packed monolayers with

very high coverage of protein may not be conducive to the assay since they would block the oxidation of p-AP. As such, disordered monolayers presenting sites at which p-AP can be oxidized are a desired feature for these assays. The p-AP produced by the enzyme action prior to the SWV sweep is likely building up inside the NPG pores and beginning to diffuse out into the bulk solution although most of the current likely arises from reactions within the NPG pore structure. The oxidation current observed should be arising from oxidation of p-AP within the NPG surface and interior and also from some p-AP located around the NPG electrode some short distance into the bulk solution.

Enzyme-linked lectin assays are important for the high throughput screening of glycoproteins. NPG electrodes can be prepared in a range of sizes and formats, and are suitable for miniaturization, fabrication of electrode arrays, or use in flow-through electrochemical devices. The properties of NPG electrodes present opportunities for the development of new electrochemical assay formats. The greatly enhanced surface to volume ratio of NPG facilitates strategies such as the one described here which relies upon the enhancement in peak current in a square wave voltammetry sweep following product formation within a nanoporous electrode.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Sanchez-Carbayo M. Expert. Opin. Med. Diag. 2008; 2:249–262.
- [2]. Szabo A, Stolz L, Granzow R. Curr. Opin. Struct. Biol. 1995; 5:699–705. [PubMed: 8574707]
- [3]. Brockman JM, Frutos AG, Corn RM. J. Am. Chem. Soc. 1999; 121:8044–8051.
- [4]. Templin MF, Stoll D, Schrenk M, Traub PC, Vöhringer CF, Joos TO. Drug Discov. Today. 2002; 7:815–822. [PubMed: 12546969]
- [5]. Schena M, Shalon D, Davis RW, Brown PO. Science. 1995; 270:467–470. [PubMed: 7569999]
- [6]. Houseman BT, Mrksich M. Chem. Biol. 2002; 9:443–454. [PubMed: 11983333]
- [7]. Zheng T, Peelen D, Smith LM. J. Am. Chem. Soc. 2005; 127:9982–9983. [PubMed: 16011345]
- [8]. Lis H, Sharon N. Eur. J. Biochem. 1993; 218:1–27. [PubMed: 8243456]
- [9]. Ohtsubo K, Marth JD. Cell. 2006; 126:855–867. [PubMed: 16959566]
- [10]. Apweiler R, Hermjakob H, Sharon N. Biochimica et Biophysica Acta: General Subjects. 1999; 1473:4–8.
- [11]. Cheng, Y.; Ni, N.; Peng, H.; Jin, S.; Wang, B. Carb. Recog. John Wiley & Sons, Inc.; 2011. Carbohydrate Biomarkers; p. 133-156.
- [12]. Stevens MM, George JH. Science. 2005; 310:1135–1138. [PubMed: 16293749]
- [13]. Dabelsteen E. The J. Path. 1996; 179:358–369.
- [14]. Varki A. Glycobiology. 1993; 3:97–130. [PubMed: 8490246]
- [15]. Dell A, Morris HR. Science. 2001; 291:2351–2356. [PubMed: 11269315]
- [16]. Castric P, Cassels FJ, Carlson RW. J. Biol. Chem. 2001; 276:26479–26485. [PubMed: 11342554]
- [17]. Campbell MP, Royle L, Radcliffe CM, Dwek RA, Rudd PM. Bioinformatics. 2008; 24:1214–1216. [PubMed: 18344517]
- [18]. Balaguer E, Neusüss C. Anal. Chem. 2006; 78:5384–5393. [PubMed: 16878873]

- [19]. Blixt O, Head S, Mondala T, Scanlan C, Huflejt ME, Alvarez R, Bryan MC, Fazio F, Calarese D, Stevens J, Razi N, Stevens DJ, Skehel JJ, van Die I, Burton DR, Wilson IA, Cummings R, Bovin N, Wong C-H, Paulson JC. Proc. Natl. Acad. Sci. U. S. A. 2004; 101:17033–17038. [PubMed: 15563589]
- [20]. Yonzon CR, Jeoung E, Zou S, Schatz GC, Mrksich M, Van Duyne RP. J. Am. Chem. Soc. 2004; 126:12669–12676. [PubMed: 15453801]
- [21]. Park S, Shin I. Angew. Chem. Int. Ed. 2002; 41:3180–3182.
- [22]. Shen Z, Huang M, Xiao C, Zhang Y, Zeng X, Wang PG. Anal. Chem. 2007; 79:2312–2319. [PubMed: 17295446]
- [23]. Sanchez-Pomales G, Zangmeister RA. Int. J. Electrochem. 2011; 2011:1–11.
- [24]. La Belle JT, Gerlach JQ, Svarovsky S, Joshi L. Anal. Chem. 2007; 79:6959–6964. [PubMed: 17658764]
- [25]. Xue Y, Ding L, Lei J, Ju H. Biosens. Bioelectron. 2010; 26:169–174. [PubMed: 20591645]
- [26]. Gruber K, Horlacher T, Castelli R, Mader A, Seeberger PH, Hermann BA. ACS Nano. 2011; 5:3670–3678. [PubMed: 21388220]
- [27]. Guasch RM, Guerri C, O'Connor J-E. Cytometry. 1995; 19:112–118. [PubMed: 7743891]
- [28]. Sharon N, Lis H. Science. 1972; 177:949–959. [PubMed: 5055944]
- [29]. Gornik O, Lauc G. Clin. Biochem. 2007; 40:718–723. [PubMed: 17320850]
- [30]. Rüdiger H, Gabius H-J. Glycoconj. J. 2001; 18:589–613. [PubMed: 12376725]
- [31]. Thompson R, Creavin A, O'Connell M, O'Connor B, Clarke P. Anal. Biochem. 2011; 413:114–122. [PubMed: 21320462]
- [32]. Love KR, Seeberger PH. Angew. Chem. 2002; 41:3583–3586. [PubMed: 12370898]
- [33]. Wu A, Wu J, Singh T, Chu K-C, Peumans W, Rougé P, Van Damme E. J. Biomed. Sci. 2004; 11:874–885. [PubMed: 15591785]
- [34]. Gabius HJ. Biochem. Soc. Trans. 2008; 36:1491–1496. [PubMed: 19021582]
- [35]. Shankar SP, Chen II, Keselowsky BG, García AJ, Babensee JE. Journal of Biomedical Materials Research Part A. 2010; 92A:1329–1342. [PubMed: 19353560]
- [36]. Köhn M, Benito JM, Ortiz Mellet C, Lindhorst TK, García Fernández JM. ChemBioChem. 2004; 5:771–777. [PubMed: 15174159]
- [37]. Thompson RQ, Barone GC Iii, Halsall HB, Heineman WR. Anal. Biochem. 1991; 192:90–95. [PubMed: 2048739]
- [38]. Zhang J-J, Cheng F-F, Zheng T-T, Zhu J-J. Anal. Chem. 2010; 82:3547–3555. [PubMed: 20369831]
- [39]. Ding L, Cheng W, Wang X, Xue Y, Lei J, Yin Y, Ju H. Chem. Comm. 2009:7161–7163. [PubMed: 19921016]
- [40]. Zhang X, Teng Y, Fu Y, Xu L, Zhang S, He B, Wang C, Zhang W. Anal. Chem. 2010; 82:9455–9460. [PubMed: 20954719]
- [41]. Shao Z, Li Y, Yang Q, Wang J, Li G. Anal. Bioanal. Chem. 2010; 398:2963–2967. [PubMed: 20865403]
- [42]. Cheng W, Ding L, Lei J, Ding S, Ju H. Anal. Chem. 2008; 80:3867–3872. [PubMed: 18407618]
- [43]. Ding L, Cheng W, Wang X, Ding S, Ju H. J. Am. Chem. Soc. 2008; 130:7224–7225. [PubMed: 18489098]
- [44]. Dai Z, Kawde A-N, Xiang Y, La Belle JT, Gerlach J, Bhavanandan VP, Joshi L, Wang J. J. Am. Chem. Soc. 2006; 128:10018–10019. [PubMed: 16881623]
- [45]. Andrade CAS, Oliveira MDL, de Melo CP, Coelho LCBB, Correia MTS, Nogueira ML, Singh PR, Zeng X. J. Colloid Interface Sci. 2011; 362:517–523. [PubMed: 21816406]
- [46]. Oliveira MDL, Correia MTS, Coelho LCBB, Diniz FB. Colloids Surf. B. Biointerfaces. 2008; 66:13–19. [PubMed: 18573642]
- [47]. Oliveira MDL, Nogueira ML, Correia MTS, Coelho LCBB, Andrade CAS. Sensors Actuators B: Chem. 2011; 155:789–795.
- [48]. Martos-Maldonado MC, Casas-Solvas JM, Quesada-Soriano I, García-Fuentes L, Vargas-Berenguel A. Langmuir. 2013; 29:1318–1326. [PubMed: 23286545]

- [49]. Martos-Maldonado MC, Thygesen MB, Jensen KJ, Vargas-Berenguel A. *Eur. J. Org. Chem.* 2013;2793–2801.
- [50]. Yang C, Xu C, Wang X, Xiao Z, Gu B. *Electrochim. Acta.* 2011; 60:50–54.
- [51]. Hu F, Chen S, Wang C, Yuan R, Wang C. *Biosens. Bioelectron.* 2012; 34:202–207. [PubMed: 22387041]
- [52]. Loaiza OA, Lamas-Ardisana PJ, Jubete E, Ochoteco E, Loinaz I, Cabanero G, García I, Penades S. *Anal. Chem. (Wash.).* 2011; 83:2987–2995.
- [53]. Wang Z, Sun C, Vegesn Giri, Liu Haiying, Liu Yang, Li Jinghong, Zeng X. *Biosens. Bioelectron.* 2013; 46:183–189. [PubMed: 23563436]
- [54]. Zeng X, Andrade CAS, Oliveira MDL, Sun X-L. *Anal. Bioanal. Chem.* 2012; 402:3161–3176. [PubMed: 22200920]
- [55]. Lee YC, Lee RT. *Acc. Chem. Res.* 1995; 28:321–327.
- [56]. Cloninger MJ. *Curr. Opin. Chem. Biol.* 2002; 6:742–748. [PubMed: 12470726]
- [57]. Pandey B, Tan YH, Fujikawa K, Demchenko AV, Stine KJ. *J. Carbohydr. Chem.* 2012; 31:466–503. [PubMed: 23519474]
- [58]. Reuel NF, Mu B, Zhang J, Hinckley A, Strano MS. *Chem. Soc. Rev.* 2012; 41:5744–5779. [PubMed: 22868627]
- [59]. Kuno A, Uchiyama N, Koseki-Kuno S, Ebe Y, Takashima S, Yamada M, Hirabayashi J. *Nat Meth.* 2005; 2:851–856.
- [60]. Pei Z, Anderson H, Aastrup T, Ramström O. *Biosens. Bioelectron.* 2005; 21:60–66. [PubMed: 15967351]
- [61]. Tsai C-S, Chen C-T. *ChemplusChem.* 2012; 77:314–322.
- [62]. Ding L, Ji Q, Qian R, Cheng W, Ju H. *Anal. Chem. (Wash.).* 2010; 82:1292–1298.
- [63]. Wang X, Ramstrom O, Yan M. *Anal. Chem. (Wash.).* 2010; 82:9082–9089.
- [64]. Sanchez-Pomales G, Morris TA, Falabella JB, Tarlov MJ, Zangmeister RA. *Biotechnol. Bioeng.* 2013; 109:2240–2249. [PubMed: 22488121]
- [65]. Bertok T, Sediva A, Katrlík J, Gemeiner P, Mikula M, Nosko M, Tkac J. *Talanta.* 2013; 108:11–18. [PubMed: 23601864]
- [66]. Marradi M, Chiodo F, Garcia I, Penade's S. *Chem. Soc. Rev.* 42:4728–4745. [PubMed: 23288339]
- [67]. Samanta D, Sarkar A. *Chem. Soc. Rev.* 2011; 40:2567–2592. [PubMed: 21264402]
- [68]. Shulga OV, Zhou D, Demchenko AV, Stine KJ. *Analyst.* 2008; 133:319–322. [PubMed: 18299744]
- [69]. Stine, KJ.; Jefferson, K.; Shulga, OV. *Nanoporous Gold for Enzyme Immobilization Enzyme Stabilization and Immobilization.* Minter, SD., editor. Vol. vol. 679. Humana Press; 2011. p. 67-83.
- [70]. Rogers KR. *Anal. Chim. Acta.* 2006; 568:222–231. [PubMed: 17761264]
- [71]. Duan C, Meyerhoff ME. *Microchimica Acta.* 1995; 117:195–206.
- [72]. Seker E, Berdichevsky Y, Begley MR, Reed ML, Staley KJ, Yarmush ML. *Nanotechnology.* 2010; 21:125504. [PubMed: 20203356]
- [73]. Samanman S, Thammakheta C, Kanatharan P, Buranachai C, Thavarungkula P. *Electrochim. Acta.* 2013; 102:342–350.
- [74]. Wang D, Ji R, Albrecht A, Schaaf P. *Beilstein Journal of Nanotechnology.* 2012; 3:651–657. [PubMed: 23019561]
- [75]. Collinson MM. *ISRN Analytical Chemistry.* 2013 article number 692484.
- [76]. Landberg E, Pahlsson P, Lundblad A, Arnetorp A, Jeppsson JO. *Biochem. Biophys. Res. Commun.* 1995; 210:267–274. [PubMed: 7755600]
- [77]. Yamashita K, Koide N, Endo T, Iwaki Y, Kobata A. *J. Biol. Chem.* 1989; 264:2415–2423. [PubMed: 2536709]
- [78]. Stibler H. *Clin. Chem.* 1991; 37:2029–2037. [PubMed: 1764777]
- [79]. Malhotra R, Wormald MR, Rudd PM, Fischer PB, Dwek RA, Sim RB. *Nat. Med.* 1995; 1:237–243. [PubMed: 7585040]

- [80]. Eraso LH, Ginwala N, Qasim AN, Mehta NN, Dlugash R, Kapoor S, Schwartz S, Schutta M, Iqbal N, Mohler ER, Reilly MP. *Diabetes Care*. 2010; 33:408–410. [PubMed: 19910501]
- [81]. Komaba H, Fukagawa M. *Kidney Int*. 75:874–876. 0000. [PubMed: 19367310]
- [82]. Mori K, Emoto M, Inaba M. Recent Patents on Endocrine, Metabolic & Immune Drug Discovery. 2011; 5:124–146.
- [83]. Wanebo HJ, Rao B, Pinsky CM, Hoffman RG, Stearns M, Schwartz MK, Oettgen HF. *N. Engl. J. Med*. 1978; 299:448–451. [PubMed: 683276]
- [84]. Peracaula R, Tabarés G, Royle L, Harvey DJ, Dwek RA, Rudd PM, de Llorens R. *Glycobiology*. 2003; 13:457–470. [PubMed: 12626390]
- [85]. Geisbrecht ER, Montell DJ. *Nat. Cell Biol*. 2002; 4:616–620. [PubMed: 12134162]
- [86]. Edelman GM, Cunningham BA, Reeke GN, Becker JW, Waxdal MJ, Wang JL. *Proc. Nat. Acad. Sci. USA*. 1972; 69:2580–2584. [PubMed: 4506778]
- [87]. Sparbier K, Koch S, Kessler I, Wenzel T, Kostrzewa M. *Journal of biomolecular techniques : JBT*. 2005; 16:407–413. [PubMed: 16522863]
- [88]. Krusius T, Finne J, Rauvala H. *FEBS Lett*. 1976; 72:117–120. [PubMed: 62678]
- [89]. Manimala JC, Roach TA, Li Z, Gildersleeve JC. *Angew. Chem*. 2006; 118:3689–3692.
- [90]. Lotan R, Siegelman HW, Lis H, Sharon N. *J. Biol. Chem*. 1974; 249:1219–1224. [PubMed: 4855992]
- [91]. Sinha S, Surolia A. *Biophys. J*. 2005; 88:4243–4251. [PubMed: 15792973]
- [92]. Raehm L, Kern J-M, Sauvage J-P, Hamann C, Palacin S, Bourgoin J-P. *Chem. Eur. J*. 2002; 8:2153–2162. [PubMed: 11981900]
- [93]. Guo Y-G, Zhang H-M, Hu J-S, Wan L-J, Bai C-L. *Thin Solid Films*. 2005; 484:341–345.
- [94]. Noble, JE.; Bailey, MJA. Chapter 8 Quantitation of Protein. In: Richard, RB.; Murray, PD., editors. *Methods Enzymol. Vol. Volume 463*. Academic Press; 2009. p. 73-95.
- [95]. Borkowska Z, Tymosiak-Zielinska A, Nowakowski R. *Electrochim. Acta*. 2004; 49:2613–2621.
- [96]. Zhang J, Liu P, Ma H, Ding Y. *J. Phys. Chem. C*. 2007; 111:10382–10388.
- [97]. Lyon JL, Stevenson KJ. *Anal. Chem*. 2006; 78:8518–8525. [PubMed: 17165848]
- [98]. Kergaravat SV, Pividori MI, Hernandez SR. *Talanta*. 2012; 88:468–476. [PubMed: 22265528]
- [99]. Li ZF, Kang ET, Neoh KG, Tan KL. *Biomaterials*. 1998; 19:45–53. [PubMed: 9678849]
- [100]. Pariente F, Hernández L, Lorenzo E. *J. Electroanal. Chem*. 1992; 342:73–87.
- [101]. Bradley CR, Rechnitz GA. *Anal. Chem*. 1984; 56:664–667.
- [102]. Shulga OV, Jefferson K, Khan AR, D'Souza VT, Liu J, Demchenko AV, Stine KJ. *Chemistry of Materials*. 2007; 19:3902–3911. [PubMed: 18820734]
- [103]. Wilson MS, Rauh RD. *Biosens. Bioelectron*. 2004; 20:276–283. [PubMed: 15308232]
- [104]. Li Y, Gao F, Wei W, Qiu J-B, Ma G-H, Zhou W-Q. *J. Mol. Catal. B: Enzym*. 2010; 66:182–189.
- [105]. DeLouise LA, Miller BL. *Analytical Chemistry*. 2005; 77:1950–1956. [PubMed: 15801723]
- [106]. Bowers GN, McComb RB. *Clin. Chem*. 1966; 12:70–89. [PubMed: 4952423]
- [107]. Widrig CA, Chung C, Porter MD. *J. Electroanal. Chem*. 1991; 310:335–359.
- [108]. Dammer U, Hegner M, Anselmetti D, Wagner P, Dreier M, Huber W, Güntherodt HJ. *Biophys. J*. 1996; 70:2437–2441. [PubMed: 9172770]
- [109]. Wang J, Martinez T, Yaniv DR, McCormick LD. *J. Electroanal Chem*. 1991; 313:129–140.
- [110]. Frew JE, Foulds NC, Wilshire JM, Forrow NJ, Green MJ. *J. Electroanal. Chem*. 1989; 266:309–316.
- [111]. Gyurcsanyi RE, Bereczki A, Nagy G, Neuman MR, Lindner E. *Analyst*. 2002; 127:235–240. [PubMed: 11913867]
- [112]. Lin Y-Y, Liu G, Wai CM, Lin Y. *Anal. Chim. Acta*. 2008; 612:23–28. [PubMed: 18331854]
- [113]. Pemberton RM, Hart JP, Stoddard P, Foulkes JA. *Biosens. Bioelectron*. 1999; 14:495–503. [PubMed: 10451917]

- [114]. Tsang, VCW.; Wilson, BC.; Peralta, JM. [30] Quantitative, single-tube, kinetic-dependent enzyme-linked immunosorbent assay (k-ELISA). In: John, HVV.; Langone, J., editors. *Methods Enzymol. Vol. Volume 92*. Academic Press; 1983. p. 391-403.
- [115]. Treloar PH, Nkohlkwo A.a.T. Kane JW, Barber D, Vадgama PM. *Electroanalysis*. 1994; 6:561–566.
- [116]. Chang JJ, Crowl CP, Schneider RS. *Clin. Chem*. 1975; 21:967.
- [117]. Rosenthal AF, Vargas MG, Klass CS. *Clin. Chem*. 1976; 22:1899–1902. [PubMed: 184987]
- [118]. Malaise MG, Franchimont P, Bouillene C, Houssier C, Mahieu PR. *Clin. Exp. Immunol*. 1987; 68:543–551. [PubMed: 3652523]
- [119]. Babac C, Yavuz H, Galaev IY, Pi kin E, Denizli A. *React. Funct. Polym*. 2006; 66:1263–1271.
- [120]. Young NM, Leon MA. *Biochim. Biophys. Acta*. 1974; 365:418–424. [PubMed: 4429675]
- [121]. Katrlík J, Skrabana R, Mislovicová D, Gemeiner P. *Colloids Surf., A*. 2011; 382:198–202.
- [122]. Dam, TK.; Brewer, CF. Multivalent Protein–Carbohydrate Interactions: Isothermal Titration Microcalorimetry Studies. In: Jo, MLJ.; Holt, M.; Gary, KA., editors. *Methods Enzymol. Vol. Volume 379*. Academic Press; 2004. p. 107-128.
- [123]. Yua Y, Xu J, Liu Y, Chen Y. *J. Chromatogr. B*. 2012; 902:10–15.
- [124]. Gonzalez-Quintela A, Alende R, Gude F, Campos J, Rey J, Meijide LM, Fernandez-Merino C, Vidal C. *Clin. Exp. Immunol*. 2007; 151:42–50. [PubMed: 18005364]
- [125]. Kerckaert J-P, Bayard B. *Biochem. Biophys. Res. Commun*. 1982; 105:1023–1030. [PubMed: 7092889]
- [126]. Sethuraman N, Stadheim TA. *Curr. Opin. Biotechnol*. 2006; 17:341–346. [PubMed: 16828275]
- [127]. David GS, Reisfeld RA. *J. Natl. Cancer Inst*. 1974; 53:1005–1010. [PubMed: 4473556]
- [128]. Nilsson B, Norden NE, Svensson S. *J. Biol. Chem*. 1979; 254:4545–4553. [PubMed: 86542]
- [129]. Green ED, Adelt G, Baenziger J, Wilson S, Van Halbeek H. *J. Biol. Chem*. 1988; 263:18253–18268. [PubMed: 2461366]
- [130]. Spiro RG, Bhoyroo VD. *J. Biol. Chem*. 1974; 249:5704–5717. [PubMed: 4137945]
- [131]. Gupta D, Brewer CF. *Biochemistry*. 1994; 33:5526–5530.
- [132]. Safina G, Duran IB, Alasel M, Danielsson B. *Talanta*. 2011; 84:1284–1290. [PubMed: 21641439]
- [133]. Yamashita K, Totani K, Kuroki M, Matsuoka Y, Ueda I, Kobata A. *Cancer Res*. 1987; 47:3451–3459. [PubMed: 3581081]
- [134]. Chandrasekaran EV, Davila M, Nixon DW, Goldfarb M, Mendicino J. *J. Biol. Chem*. 1983; 258:7213–7222. [PubMed: 6406498]
- [135]. Tabares G, Radcliffe CM, Barrabes S, Ramirez M, Aleixandre RN, Hoesel W, Dwek RA, Rudd PM, Peracaula R, De Llorens R. *Glycobiology*. 2006; 16:132–145. [PubMed: 16177264]
- [136]. Meany DL, Zhang Z, Sokoll LJ, Zhang H, Chan DW. *Journal of Proteome Research*. 2009; 8:613–619. [PubMed: 19035787]
- [137]. Mann DA, Kanai M, Maly DJ, Kiessling LL. *J. Am. Chem. Soc*. 1998; 120:10575–10582.
- [138]. Dong Y, Abaci S, Shannon C, Bozack MJ. *Langmuir*. 2003; 19:8922–8926.

Highlights

- First application of nanoporous gold for detecting glycoprotein-lectin interactions
- New kinetic assay for glycoprotein detection using square-wave voltammetry
- Differential binding of lectin-enzyme conjugate to immobilized glycoproteins
- Competitive glycoprotein assay on nanoporous gold
- New methods enabled by high surface area

Scheme 1.

The electrode modification steps used to produce NPG modified with lipoic acid SAMs onto which either glycoprotein or lectin-enzyme conjugate is then immobilized. Immobilized glycoprotein on NPG is then used either for competitive assay for glycoprotein by competition with glycoprotein in solution and Con A – ALP conjugate, or for assay of the binding of the Con A – ALP (or soybean agglutinin – ALP) conjugate itself. Immobilized Con A – ALP conjugate is the basis for the described kinetic assay.

Fig. 1.

(a) SEM images of NPG covered gold wire in cross-sectional view at 1 kX magnification, (b) SEM image of NPG covered gold wire (top view) at 100 kX magnification, (c) cyclic voltammogram of NPG covered gold wire performed in 0.5 M H_2SO_4 between 0 and 1.5 V (vs. Ag|AgCl) at a scan rate of 100 mV sec^{-1} .

Fig. 2.

(a) Square-wave voltammograms (SWV) of Con A-ALP conjugate immobilized on gold wire and on NPG. Con A-ALP conjugate was immobilized on lipoic acid modified gold wire and NPG, SWV were recorded with 1 mM p-APP in glycine buffer (pH 9.0, 100 mM) after 2 min incubation with the substrate p-APP, (b) SWV of Con A-ALP conjugate immobilized on different SAMs on NPG. SAMs of (1) lipoic acid (LPA), (2) mercaptododecanoic acid (MDDA), (3) a 1:5 mixed SAM of MDDA and mercaptooctanol (HO-C₈-SH) and (4) a 1:5 mixed SAM of MDDA and 3,6-dioxa-8-mercaptooctanol (PEG₃-SH) were prepared and Con A-ALP conjugate immobilization was done by EDC/NHS coupling. SWV was then recorded with 1 mM p-APP in pH 9.0 glycine buffer, also after 2 min incubation with the substrate. SWV was conducted using a pulse height of 50 mV, a pulse width of 0.2 sec, a step height of 2 mV and scan rate of 5.0 mV sec⁻¹ (vs. Ag|AgCl reference electrode).

Fig. 3.

Difference current peak heights from SWV scans (pulse height of 50 mV, pulse width of 0.2 sec, step height of 2 mV and scan rate of 5.0 mV sec⁻¹, Ag|AgCl reference electrode) vs. p-APP concentrations from 0.01 mM to 1.0 mM in pH 9.0 glycine buffer (100 mM) for the Con A – ALP conjugate immobilized on lipoic acid SAMs fit to the Michaelis-Menten equation. Error bars represent standard error of three measurements.

Fig. 4.

Effect of pH on the enzymatic activity of ALP determined using SWV peak difference currents. Different pH glycine buffers (100 mM, of pH 8.0, 8.5, 9 and 10) and a pH 7.4 PBS buffer (100 mM) were used with 200 μM p-APP. SWV was conducted using a pulse height of 50 mV, a pulse width of 0.2 sec, a step height of 2 mV and scan rate of 5.0 mV sec^{-1} (vs. Ag|AgCl reference electrode).

Fig. 5. Kinetic ELLA response on NPG. Different concentrations of IgG (a) and of transferrin (b) were incubated for 2 hours (in PBS, pH 7.4, 10 mM with 1 mM Ca^{2+} and 1 mM Mn^{2+}) with Con A-ALP conjugate immobilized on lipoic acid modified NPG and the difference in SWV peak difference current before and after incubation was plotted to obtain the response plot. SWV was conducted using a pulse height of 50 mV, a pulse width of 0.2 sec, a step height of 2 mV and scan rate of 5.0 mV sec^{-1} (vs. Ag|AgCl reference electrode) in pH 9.0 glycine buffer (100 mM). Error bars represent standard error of three measurements.

Fig. 6.

(a) Bar plot for SWV response due to Con A – ALP conjugate binding to different glycoproteins on NPG. Glycoproteins were immobilized on NPG and incubated with $50 \mu\text{g mL}^{-1}$ conjugate for 24 hrs (in PBS, pH 7.4, 10 mM with 1 mM Ca^{2+} and 1 mM Mn^{2+}) and then incubated with 1 mM p-APP for 2 minutes. (b) Bar plot for SWV response to Con A – ALP conjugate binding using a 2 hour incubation time, with other conditions same as in (a). (c) Bar plot for SWV response due to soybean agglutinin – ALP conjugate binding to different glycoproteins on NPG. Glycoproteins were immobilized on NPG by covalent linking to lipoic acid SAMs and then incubated with $50 \mu\text{g mL}^{-1}$ conjugate for 2 hrs and then incubated with 1 mM p-APP for 2 minutes. The glycoproteins are transferrin (TSF), immunoglobulin G (IgG), fetuin (FET), asialofetuin (ASF), carcinoembryonic antigen (CEA), and prostate specific antigen (PSA). Response to immobilized bovine serum albumin (BSA), to a lipoic acid (LPA) SAM, and to a 1:5 molar ratio mixed SAM of 8-mercaptooctyl α -D-mannopyranoside (MAN) and 3,6-dioxo-8-mercaptooctanol (PEG₃-SH) is also shown. SWV was conducted using a pulse height of 50 mV, a pulse width of 0.2 sec, a step height of 2 mV and scan rate of 5.0 mV sec^{-1} (vs. Ag|AgCl reference electrode) in pH 9.0 glycine buffer (100 mM). Error bars represent standard error of three measurements.

Fig. 7.

Competitive ELLA on NPG. Glycoproteins were covalently immobilized on the NPG surface by linkage to lipoic acid SAM and then incubated with Con A – ALP conjugate (in PBS, pH 7.4, 10 mM with 1 mM Ca^{2+} and 1 mM Mn^{2+}) and glycoprotein. The SWV peak difference current was measured after incubation for 17 hours with glycoprotein in these solutions. (a) TSF assay done using $25 \mu g mL^{-1}$ of conjugate, followed by incubation in 1 mM p-APP for 2 minutes. (b) IgG assay done using $50 \mu g mL^{-1}$ of conjugate, followed by incubation in 1 mM p-APP for 2 minutes. SWV peak difference current ($-\Delta I$) was determined using different concentrations of glycoprotein in solution. SWV was conducted using a pulse height of 50 mV, a pulse width of 0.2 sec, a step height of 2 mV and scan rate of $5.0 mV sec^{-1}$ (vs. Ag|AgCl reference electrode) in pH 9.0 glycine buffer (100 mM). Error bars represent standard error of three measurements.

Fig. 8.

Inhibition studies of the effect of Me-α-D-Man on Con A binding to glycoprotein. Different concentrations of Me-α-D-Man were added to the $50 \mu\text{g mL}^{-1}$ Con A – ALP conjugate solution (in PBS, pH 7.4, 10 mM with 1 mM Ca^{2+} and 1 mM Mn^{2+}), TSF (a) and IgG (b) and incubated for 17 hours. Wires were removed from the incubating solution and SWV measurements were done, followed by incubation again for 2 hours in 1 mM Me-α-D-Man. The peak difference current at the indicated concentration of Me-α-D-Man was subtracted from the peak difference current in the presence of 10 mM Me-α-D-Man and this defines $\Delta(\text{peak difference current})$ as plotted on the y-axis. SWV was conducted using a pulse height of 50 mV, a pulse width of 0.2 sec, a step height of 2 mV and scan rate of 5.0 mV sec^{-1} (vs. Ag|AgCl reference electrode) in pH 9.0 glycine buffer (100 mM). Error bars represent standard error of three measurements.

Fig. 9.

Effect of BSA on ELLA assays. (a) 5 mg mL^{-1} BSA was added to the incubation solution (PBS, pH 7.4, 10 mM with 1 mM Ca^{2+} and 1 mM Mn^{2+}) along with glycoprotein and, after incubated for 2 hrs, SWV measurements were done. BSA was also used as a control protein for the study of specific response of glycoprotein binding to Con A. (b) Effect of BSA on a competitive ELLA, 5 mg mL^{-1} BSA was added along with the Con A – ALP conjugate and glycoprotein solution (also in PBS, pH 7.4, 10 mM with 1 mM Ca^{2+} and 1 mM Mn^{2+}) for 2 hrs and then SWV measurements were done. SWV was conducted in both cases using a pulse height of 50 mV, a pulse width of 0.2 sec, a step height of 2 mV and scan rate of 5.0 mV sec^{-1} (vs. Ag/AgCl reference electrode) in pH 9.0 glycine buffer (100 mM). Error bars represent standard error of three measurements.

Table 1

Surface coverage of proteins on NPG

Proteins	*Wt. in ($\mu\text{g cm}^{-2}$)	Molar Mass (kDa)	Mol cm^{-2}	Molecules cm^{-2}
Con A-ALP (Conjugate)	0.080	104 + 69**	4.6×10^{-13}	2.8×10^{11}
TSF	0.057	80	7.1×10^{-13}	4.3×10^{11}
IgG	0.075	160	4.7×10^{-13}	2.6×10^{11}
CEA	0.054	180	3.0×10^{-13}	1.0×10^{11}

* Surface coverage was determined by BCA assay based on the solution depletion studies. Amount of protein left in the incubation solution was subtracted from the amount initially present in the solution to obtain amount immobilized on the NPG surface, wt. represent protein immobilized in 10 NPG wires.

** Calculation is based on the assumption of one ALP per Con A molecule.