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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 α-D-mannopyranoside for the binding of TSF and IgG to Con A–ALP; IC_{50} 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.

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

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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 lectinsorbent 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