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Electrochemical detection of protein glycosylation using lectin and protein-gold affinity interactions

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Abstract

We report a new method for the electrochemical detection of glycosylation on proteins, which relies on lectin-protein interaction on a bare gold electrode. The target protein isolated by immunoaffinity is directly adsorbed onto a gold surface and its glycosylation status is retrieved by subsequent addition of specific lectins. The adsorption and subsequent recognition process is monitored electrochemically in the presence of [Fe(CN)₆]³⁻/⁴⁻ redox system. By decoupling target protein capture from glycosylation read-out steps, this approach circumvents unwanted antibody-lectin crosstalk while enabling specific glycosylation detection of a glycoprotein in serum-spiked samples in less than 1h.

Keywords

Electrochemical detection, cancer biomarkers, protein glycosylation, lectins, protein-gold affinity interaction,

Introduction

Glycosylation is a widespread protein modification that has recently been linked to the onset of cancer and could serve as cancer biomarkers.¹⁻⁴ Current immuno-based techniques for glycosylation detection typically rely on sandwich approaches using combinations of lectin to detect the glycan and antibody to detect the protein.⁵⁻⁹ Since antibodies are also glycosylated, unwanted antibody-lectin interactions can lead to false positive read-outs and must be carefully assessed for each combination of lectin and antibody.¹⁰⁻¹²

Over the past few years several electrochemical biosensors have been developed for cost effective, rapid, sensitive and accurate quantification of glycans in clinical samples.¹³⁻¹⁶ However, most of these methods used lectins to detect overall changes in the glycan profile of a given sample without adding antibodies to monitor a specific glycoprotein to which the glycan is attached. This limits its utility for diagnostic applications which demand quantification of the specific glycans attached to a particular protein biomarker. More recently, we have combined a sandwich approach with electrochemistry detection to enable specific glycosylation profiling of protein biomarkers in spiked serum.¹⁷ Although this approach offered specific glycosylation detection with femtomolar sensitivity in serum-spiked samples, it could still suffer from antibody-lectin cross-reaction unless each combination of lectin and antibody
is carefully assessed. In addition, the process still involved numerous steps and tedious chemical modification of the gold electrode surface for the preparation of sandwich complexes.

In this report, we have introduced a new electrochemical approach for specific glycosylation detection that avoids antibody-lectin cross-reaction and also circumvents the need for any gold surface modification. This results in a rapid and easy approach to glycosylation detection on glycoproteins.

Experimental

Reagents and materials

Unless otherwise stated, the reagents used for the experiments were of analytical grade and purchased from Sigma Aldrich (Australia). Lyophilized chicken egg albumin (#A5503) and lectin wheat germ agglutinin (WGA) was purchased from sigma and Narcissus pseudonarcissus lectin (NPL), Sambucus nigra lectin (SNA), Phaseolus vulgaris lectin L (LPHA) were from Vector Laboratories. Monoclonal anti chicken egg albumin antibody produced in mouse was from Biolegend (# 520402). NHS-PEG-Biotin (PG2-BNNS-5k) was from Nanocs Inc, Dynabeads streptavidin (# 65001) from Invitrogen. UltraPure™ DNase/RNase-free distilled water (Invitrogen, Australia) was used to carry out the experiments.

Antibody biotinylation

100 μl (0.5 mg/ml) of mouse anti chicken ovalbumin was incubated with 0.3 μl of NHS-PEG-Biotin linker (reconstituted at 100mM in DMSO) for 1 hour at room temperature. The excess linker was removed using zeba spin column (MWCO 40KDa, ThermoFisher, 87767) following manufacturer’s instructions. Briefly, storage buffer of the column was removed by spinning the column at 1500 RPM for 1 min. The column was washed with 300 μl of PBS in the same manner. Finally, protein (up to 130 μl) was added carefully and column was spun at 1500 RPM for 2 mins. The flow through was collected and protein concentration was determined using Nanodrop at 280 nm.

Immuno-precipitation using magnetic beads

Different concentrations of chicken ovalbumin (100ng/μL, 200ng/μL and 2000ng/μL) were spiked in to 500 μL human serum (collected from healthy volunteers with consent and ethics approved by the University of Queensland Human Ethics Committee). 2 μl of biotinylated
antibodies (0.5 mg/ml) were then added to the serum/ovalbumin mixture (500 ug/ml) and incubated for 1 hour at 4°C with continuous shaking. 25 ul of prewashed streptavidin-dynabeads were added to the mixture and incubated for 1 hr at room temperature with continuous agitation. The supernatant was removed and the beads washed twice with buffer (25mM Tris.HCl pH 7.4, 150 mM NaCl, 1%NP-40, 1 mM EDTA, 5% glycerol) followed by a single wash with PBS. Captured proteins were eluted by incubating with 100 μL of glycine HCL, pH 2.7, for 15 mins at room temperature with vigorous shaking. Finally, the eluate was collected and protein concentration estimated using Nanodrop and then used for further analysis.

**Label free detection using differential pulse voltammetry**

All electrochemical experiments were carried out using CH1040C (CH Instruments) with a three electrode system consisting of a gold working electrode (2mm in diameter) Pt counter electrode, and Ag/AgCl reference electrode (all electrodes are from CH Instruments, USA). Differential pulse voltammetric (DPV) experiments were conducted in PBS solution containing 2.5mM [K₃Fe(CN)₆] and 2.5mM [K₄Fe(CN)₆] electrolyte solution. DPV signals were obtained with a potential step of 5 mV, pulse amplitude of 50 mV, pulse width of 50 ms, and pulse period of 100 ms. For the detection of glycosylated protein, the gold electrodes were cleaned first by polishing with Alumina polishing powder (CH Instruments) followed by ultrasonication with acetone and deionised water for 5 minutes and then dried under the flow of nitrogen. DPV signals of clean electrodes were measured in electrolyte solution to get the baseline current. The electrodes were incubated in 5 μL of samples for 20 minutes, and then washed three times with 1 ml of milliQ water. The relative DPV currents (i.e., %ᵢᵣ, percent difference of the DPV signals generated for sample with respect to the baseline current) due to the adsorption of protein were then measured by using equation 1. Mercaptohexanol (MCH, 200 μM) was then added to the surface of gold electrode and incubated for 20 minutes to block the unbound sites. The difference in relative DPV signals between MCH back filler and protein was calculated by using equation 2. Finally, 5 μL of lectin (100 ng/ μL) was added on the electrode and further incubated for 20 minutes before the final readout. The difference in relative DPV signals between MCH and lectin was calculated by using equation 3.

\[
\% \text{ iᵣ}^{\text{protein}} (\% \text{ Change in DPV}_{\text{protein}}) = \frac{I_{\text{Baseline}} - I_{\text{protein}}}{I_{\text{Baseline}}} \times 100 \quad \text{...} \quad \text{...} \quad \text{...} \quad (1)
\]
\[
\% \text{I}_\text{Filler} \quad (\% \text{Change in } \text{DPV}_{\text{Filler}}) = \frac{I_{\text{protein}} - I_{\text{Filler}}}{I_{\text{protein}}} \times 100 \quad \ldots \quad \ldots \quad \ldots \quad (2)
\]

\[
\% \text{I}_\text{Lectin} \quad (\% \text{Change in } \text{DPV}_{\text{Lectin}}) = \frac{I_{\text{Filler}} - I_{\text{Lectin}}}{I_{\text{Filler}}} \times 100 \quad \ldots \quad \ldots \quad \ldots \quad (3)
\]

where, \( I_{\text{Baseline}} \) is the peak current of the blank electrode, \( I_{\text{protein}} \) is the peak current after applying the protein sample, \( I_{\text{Filler}} \) is the peak current after addition of the blocking agent and \( I_{\text{Lectin}} \) is the peak current after addition of lectin.

**Results and discussion**

Scheme 1 explains the basic principle of this assay. Briefly, the target protein is captured from the clinical sample using antibodies anchored onto the surface of magnetic beads. This enables decoupling of the antibody-capture step from the glycosylation reading step, avoiding antibody-lectin cross-reactions. Since proteins have a natural strong affinity towards gold surfaces, we directly adsorbed the purified protein onto a bare gold electrode surface after elution from the magnetic beads. This offered a simple way to avoid gold-surface modification procedures. Finally, we blocked all gold surface-vacant sites with a backfiller molecule (i.e., mercaptohexanol (MCH)) then added a selected lectin to allow specific binding to the protein’s glycosylated sites. In our method, all these steps can be monitored using differential pulse voltammetry (DPV) in the presence of a \([\text{Fe(CN)}_6]^{3/-4}\) redox system. The addition of subsequent layers on the sensor surface acts as a barrier for the interfacial electron transfer reaction of the \([\text{Fe(CN)}_6]^{3/-4}\) process, which results in a decrease in DPV current response.\(^{18, 22}\)

To demonstrate the applicability of the method, we selected ovalbumin\(^9\) as a model protein. This protein has multiple lectin binding sites and, since it is not present in humans, it is suitable as an external protein for quantification in the background of human serum samples.\(^{23, 24}\) We first evaluated ovalbumin adsorption onto the gold surface by testing different protein concentrations (i.e., 100 ng/μL, 200 ng/μL, 500 ng/μL and 1 μg/μL) (Fig. 1 ESI). In the presence of \([\text{Fe(CN)}_6]^{3/-4}\) solution, we observe that the adsorbed protein effectively reduces the Faradaic current in comparison to the current generated for bare gold surface. Protein concentrations higher than 200 ng/μL appears to saturate the gold electrode inhibiting the generation of electrical current. In contrast, 100 ng/μL and 200 ng/μL ovalbumin generated a much lower current reduction with respect to the bare electrode (\( \% \text{I}_{\text{Protein}} = 20\% \) and 50\%).
respectively, see ESI for detail calculation). We selected the 200 ng/µL concentration for our experiments since a % \( i_{\text{protein}}^{\text{Protein}} = 50\% \) indicates that there was a large amount of protein on the surface, while still enabling access to \([\text{Fe(CN)}_6]^{3/4-}\) system at the electrode surface for monitoring subsequent steps.

To read the glycosylated status of the adsorbed ovalbumin, we added WGA lectin (wheat germ agglutinin)\(^9\) at 100 ng/μL concentration and used streptavidin as a control for this step (Fig. 1A). Previous to this step, we blocked any empty space available on the gold surface using MCH as back-filler. MCH is a short chain alkane with a thiol and a hydroxyl end that allows it to adsorb in the gold surface at an upright conformation with the thiol end at the bottom and the hydroxyl group at the top. This orientation of MCH perfectly blocks larger nonspecific molecules such as DNA or proteins\(^{20, 25, 26}\) to bind either with the exposed gold surface or to the MCH molecule itself but still allows the \([\text{Fe(CN)}_6]^{3/4-}\) solution to come through the small gap to reach the gold surface for generating Faradaic current. The MCH concentration was optimized through comparison of current reduction values upon addition of either lectin or streptavidin control (Fig. 2 ESI). At the optimised concentration of 200 µM MCH, we observe a significant reduction of the current (\( % \, i_{\text{lectin}} = 22\% \)) when the lectin WGA is added, whereas no current reduction when streptavidin is added. This data suggest that our lectin-based glycosylation read-out is specific to the ovalbumin glycan site and not due to any lectin-gold interaction, with the adsorbed ovalbumin retaining the glycoslation structure recognised by WGA.

To further test the specificity of the method in detecting the glycosylation type of the glycoprotein, we performed profiling using four lectins with different known glycan specificities, namely, WGA, SNA (Sambucus nigra lectin), LPHa (Phaseolus vulgaris lectin L) and NPL (Narcissus pseudonarcissus lectin). The lectins were chosen for their different glycan binding profiles. WGA has a broad binding profile including β-GlcNAc, GalNac and terminal sialic acid, while SNA interacts with α-2, 6 sialic acid, LPHa interacts with tri/tetra-antennary β 1-6 GlcNAc and NPL interacts Man α 1-6 Man\(^5,9,27\). All these glycans have been reported to bind ovalbumin\(^5,9,27\). All lectin measurements were statistically different from the negative control, streptavidin, indicating specific recognition via glycans (Fig. 1B). As expected based on the glycan specificity, WGA generates the highest current difference (i.e., \( % \, i_{\text{lectin}} = 22\) ), while lower current differences were observed for the other lectins (i.e., \( % \, i_{\text{lectin}} = 8\) for SNA; \( % \, i_{\text{lectin}} = 5\) for LPHa and \( % \, i_{\text{lectin}} = 10\) for NPL). These results indicate that this
method may potentially enable multiplex detection of glycosylation types by exploiting the glycan specific nature of lectins. Furthermore, the glycosylation specificity of lectins might help to segregate between different glycosylation processes, hence contributing to wider the potential applications of the developed method.

Having confirmed the differential detection through lectin binding, next we optimised the parameters for protein adsorption. Protein adsorption is essentially a kinetic process; hence we assumed that variables such as time and pH could potentially enhance the adsorption process. At first, we optimised the time required to achieve the 50% current reduction with the lowest ovalbumin concentration. As shown in Fig. 2A, we achieved the desired DPV current reduction (i.e., \( \%i_{\text{Protein}} = 50\% \)) after 55 minutes from 100 ng/μL starting protein concentration. However, longer adsorption times did not result in higher surface coverage (data not shown), presumably due to dissociation of the protein adsorbed onto gold electrode surface. We next investigated the effect of pH during protein adsorption over the 2.7-9 pH range. We found that the adsorption of ovalbumin was significantly increased at pH 2.7 compared to pH 7.0 and pH 9.0 (Fig 3 ESI). Since the isoelectric point of ovalbumin is 4.9, we believe that pH values below 4.9 would charge the protein positively thereby increasing its affinity towards the gold surface. Given the high adsorption capability of the protein a pH 2.7, we tested whether under this condition we could generate the desired DPV current reduction (i.e., \( \%i_{\text{Protein}} = 50\% \)) from a much lower starting protein concentration, and also within a shorter adsorption time-frame. As shown in Fig 2B, at pH 2.7, 5 ng/μL of ovalbumin adsorbed for 15 mins were sufficient to obtain the optimum current reduction (i.e., \( \%i_{\text{Protein}} = 50\% \)). The significant reduction on protein concentration and adsorption time obtained under this condition strongly suggest that this method might have applicability for rapid diagnostic applications.

Finally, to demonstrate the full applicability of our method for glycosylation analysis in biological samples, we spiked 200 ng/μL of ovalbumin in a human serum sample and sequestered it from this complex protein matrix using magnetic beads coupled to anti-ovalbumin antibodies. After releasing the captured target protein from the beads, 5 ng/μL of this purified ovalbumin was adsorbed onto the gold surface at the optimized conditions and its glycosylation status was detected using the WGA lectin (streptavidin was also used as a control in this step). The ovalbumin adsorption generated the expected \( \%i_{\text{Protein}} = 50\% \) (Fig 2C) indicating that the purified protein was capable of reaching the optimum adsorption level. Fig 2C also shows that the adsorbed ovalbumin had significant binding with WGA lectin (\( \%i_{\text{Lectin}} \)).
but negligible binding with the streptavidin. This data demonstrates an on-electrode detection limit of 5 ng/µL purified glycoprotein, which is comparable to previous studies.\textsuperscript{5,16} Future improvement on the methodology, such as electrode miniaturisation and integration of the capture and detection steps into a microfluidics device will further increase the sensitivity and utility of the method.

Conclusions

We have developed a new method for detecting specific glycosylated forms of protein biomarkers by using direct protein-gold interaction coupled with lectin detection. Our two step assay which prevents the cross reaction between antibodies and lectin avoiding the possibility of false positive result. The simple coupling method without complicated surface modification is highly amenable for multiplexing. Further, this label free and inexpensive electrochemical method of detection may potentially be combined with microfluidics for multiplex detection of glycosylated proteins for clinical applications.

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Notes and References

Figure Captions

**Scheme 1** Scheme of label free detection of glycosylation of proteins using direct protein-gold interaction and electrochemical readout. Adsorption of purified glyco-protein on bare gold electrode and subsequent MCH back filler and lectin binding steps (bottom) hinder the interfacial electron transfer of a [Fe(CN)$_6$]$^{3-}/4-$, resulting in attenuation of the electrochemical current (top, right).

**Fig.1** Mean values of the percentage current difference ($\% i_r$) for the detection of glycosylated ovalbumin in buffer. (A) Detection of 200ng/µL of ovalbumin by using WGA lectin or streptavidin. (i) DPV signals for ovalbumin sample in buffer with WGA lectin- Test sample; (ii) DPV signals for ovalbumin sample in buffer with streptavidin- Negative control. (B) Detection of 200ng/µL ovalbumin by using multiple lectins WGA, SNA, LPHa and NPL with negative control streptavidin. Each bar in (A) – (B) represents the average of three separate trials ($n = 3$). Each bar represents the standard deviation of measurements (relative standard deviation (%RSD) was found to be < 10% for $n = 3$). Statistical significance were determined by pairwise comparisons between 2 conditions using student’s t-test. *, $p = 0.005$ to 0.05; **, $p = 0.0005$ to 0.005, ***, $p = 0.00005$ to 0.0005.

**Fig.2** Optimization of binding parameters. (A) Mean $%i_r^{Protein}$ values for adsorption of 100 ng/µL and 50 ng/µL ovalbumin at pH 7. Ovalbumin concentration 100 ng/µL, time 55 mins shows optimal electrode adsorption (red dotted lines) (B) Mean $%i_r^{Protein}$ values for the adsorption of 5 ng/µL ovalbumin at pH 2.7 over a 5-20 mins period. The time 15 min shows optimal electrode adsorption. (c) Mean $%i_r^{lectin}$ values generated measuring the glycosylation read-out of 5 ng/µL glycosylated ovalbumin spiked in serum sample using WGA lectin or streptavidin control; (i) DPV signals for ovalbumin with WGA lectin- positive control; (ii) DPV signals for ovalbumin with streptavidin – negative control. Each data point represents the average of three separate trials, and error bars represent the standard deviation of measurements ($% \text{ RSD} = < 10\%$ for $n = 3$).
Figures

Scheme 1
Figure 1

(A) Bar graph showing the % $i_r$ of WGA and Strep with Ovalbumin 200 [ng/µL].

(B) Graph showing the % $i_r$ of different lectins (Strep, SNA, LPHA, NPL, WGA) with significance levels indicated.
Figure 2
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