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Detection of human asialo-α_1-acid glycoprotein using a heterosandwich immunoassay in conjunction with the light addressable potentiometric sensor

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Highly specific detection of human α_1-acid glycoprotein (AGP) and asialo-α_1-acid glycoprotein (asialo-AGP) was made possible by use of a sandwich immunoassay. The glycoproteins were sandwiched between biotinylated and fluoresceinated polyclonal rabbit anti-human AGP antibodies. Additionally, asialo-AGP could be distinctly detected, apart from AGP, via the formation of a heterosandwich immunoassay using biotinylated polyclonal rabbit anti-human AGP and the lectin, fluoresceinated ricin toxin. Streptavidin was added to the formed immunocomplexes and the immunocomplexes captured on a biotinylated nitrocellulose membrane. The signal generator, urease conjugate of an anti-fluorescein antibody, was then bound to the complex on the membrane. The rate of pH change under microvolume conditions (0.6 µl) was monitored using a silicon chip-based, light addressable potentiometer sensor. Results indicated that AGP and asialo-AGP can be detected to the 2 pg level when two antibodies are used to form the immunocomplex. Asialo-AGP can be detected down to 250 pg when the heterosandwich immunoassay is used; this assay exhibited no response up to 10 ng for native AGP or asialofetuin. Both immunoassays can be used to quantify the level of AGP and asialo-AGP in solution. Although the assay presented is very specific for AGP, asialo-AGP and terminal galactose, it is readily adaptable for the detection of any glycoprotein and terminal carbohydrate (or branched structure) by use of a protein-specific antibody and various lectins.

Keywords: α_1-acid glycoprotein, asialo-α_1-acid glycoprotein, heterosandwich immunoassay, light addressable potentiometric sensor

Introduction

Detection of specific penultimate carbohydrates on glycoproteins has become of recent importance in the biotechnology field in the downstream processing of drugs and biopharmaceuticals [1–11]. This is of particular importance because of cytotoxic side effects and decreased efficacy. Thus a method is needed that requires a minute amount of sample and can assess glycoprotein production in solution without sample degradation. The Threshold® Immunoassay System, using the light addressable potentiometric sensor (LAPS) technology, provides such a system. Although the carbohydrate sequence will not be determined, the quantity of a given penultimate carbohydrate residue (or branched chain structure) on any specific glycoprotein can be determined.

The Threshold System is particularly amenable to glycoprotein detection [12, 13]. All the binding events occur in solution phase. Furthermore, the sample need not be degraded or modified prior to analysis. The detection limit for many analytes is at the attomole level, and finally, we have previously shown that Threshold can be applied to the detection of glycoproteins [14].

In this paper we show that the quantity of glycoprotein (in various forms) can be determined using a polyclonal antibody-based immunoassay. Furthermore, a heterosandwich immunoassay has been developed for the specific detection of glycoproteins using a specific antibody and lectin. The antibody will sequester, specifically, a given protein and the lectin can be used to probe the carbohydrate residues (or branched structures) on the

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exterior of the molecule. The specific example used in this paper was human α_1-acid glycoprotein (analyte), rabbit anti-human AGP, and the galactose specific lectin, ricin. The results show that this unique assay works exceedingly well and can detect asialo-AGP to the 250 pg level while eliciting no response for native AGP or asialofetuin to the 10 ng level.

Materials and methods

Human α_1-acid glycoprotein was obtained from Sigma Chemical Company, St Louis, MO (purified from Cohn fraction VI). The asialo form of the glycoprotein was obtained by treatment of the native material with neuraminidase-coupled agarose beads (Sigma Chemical Company) in phosphate buffer, pH 5.0. FITC-ricin (F-ricin; 1.7 fluorescence/ricin) and polyclonal rabbit anti human AGP (IgG) were also purchased from Sigma Chemical Company. The antibody was biotinylated using biotin-DNP-NHS reagent (product of Molecular Devices Corp.) and purified to give a product which contained 3.6 molecules biotin per antibody. Production of fluoresceinated antibody was accomplished using procedures similar to those described for the biotinylated antibody (using Fluorescein-NHS from Molecular Devices Corp.) to give a product which contained 1.0 molecules fluorescein per antibody. The wash buffers, assay buffers, anti-fluorescein antibody-urease conjugate, and the biotin-BSA coated nitrocellulose capture membranes are components of the Immuno-Ligand Assay detection kit (Molecular Devices Corp). Asialofetuin was obtained as described earlier [14].

The assay format was as follows. For the polyclonal antibody immunoassay format, 150 ng each of b- and F-Ab per test were mixed with the analyte; streptavidin (2 µg) was added so that the final volume was 250 µl per test. For the heterosandwich immunoassay, each test contained 50 ng of F-ricin and 50 ng b-Ab (total and not specific), 2 µg of streptavidin and varying quantities of either AGP, asialo-AGP, or asialofetuin in 250 µl. The mixture was incubated for 1 h at room temperature and then filtered through the prewashed nitrocellulose membrane mounted on the Threshold workstation. The captured sample was then rinsed with 0.5 ml of wash buffer followed by 200 µl volume of anti-fluorescein antibody-urease conjugate (containing 2 µg of conjugate). Lastly, the membrane was rinsed once more with 0.5 ml wash buffer. Each membrane contained eight assay spots so that eight individual AGP/asialo-AGP/asialofetuin concentrations could be assayed at one time. Typically for the standard detection curves, three spots were used per AGP/asialo-AGP/asialofetuin concentrations (to obtain data in triplicate).

Detection of the complex was made possible by placing the membrane ('sticks') containing the assay spots into a silicon chip-based reader filled with wash buffer and the substrate (urea). A plunger pushes the membrane against the silicon chip to create a micro-volume chamber (0.6 µl) and the rate of pH change is detected, the output given in $\mu V\,s^{-1}$ [13]. The rates are directly related to the quantity of complex captured and, hence, the amount of analyte present.

Electrophoresis of AGP and asialo-AGP was performed on the Beckman Paragon® serum protein electrophoresis system (Brea, CA) using barbitol buffer, pH 8.2. The SPE gels were run for 1 h at 100 V and stained with Paragon blue stain. Ionspray-mass spectrometry was performed on a Perkin Elmer-Sciex API-3.

Results and discussion

Glyco-biopharmaceuticals are the main products of genetically engineered proteins from mammalian cells [7, 8], and range in size from hormones to antibodies. It is often essential that the carbohydrate composition of the glycoprotein be uniform and close to completion. Any deviation from this could result in loss of function, early blood stream clearance, or modified function, and in some cases result in toxicity [1–11]. There are very few methods on the market that allow screening of glycoproteins in their intact form. Many methods require digestion or chemical modification of the glycoprotein in order to release the oligosaccharide or glycopeptide. This takes time and results in some sample degradation.

A glycoprotein with a mixture can be determined using the protein-epitope based polyclonal immunoassay format shown in Fig. 1. This format is independent of the carbohydrate present and will allow quantitation of a

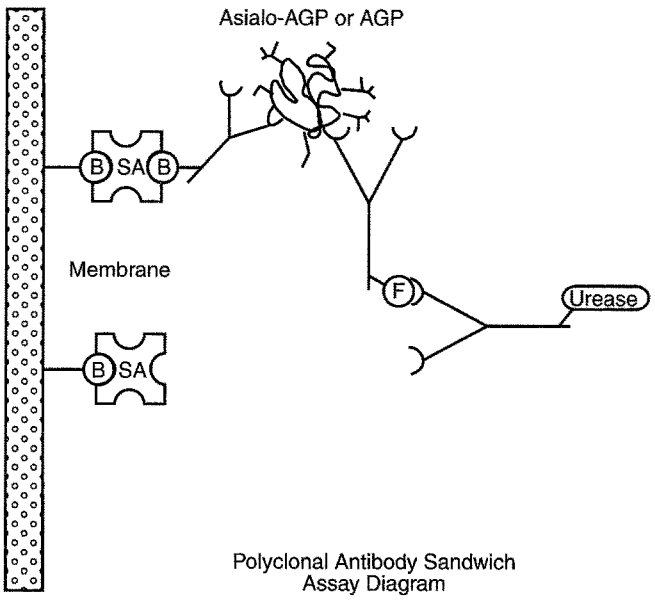

Figure 1. Diagram of the polyclonal antibody immunoassay format. B, biotin; SA, streptavidin; F, flourescein.

specific glycoprotein (all glycoforms). Determination of the presence and quantitation of a specific glycoform will require a separate assay which probes for specific carbohydrate that may be present. Such an assay has been developed whereby an antibody is used to anchor the protein portion of the glycoprotein while another antibody or lectin probes for terminal carbohydrate structure. Lectins are attractive for probing the terminal carbohydrates because a large assortment are available with different specificities. We call this type of immunoassay the heterosandwich immunoassay. In order to determine whether this system would allow low level detection of glycoproteins, we designed an assay for the detection of asialo-AGP using a biotinylated polyclonal rabbit anti-human AGP antibody (to anchor the glycoprotein) and galactose-specific F-ricin (to probe the carbohydrate portion of the native and asialo-AGP). The assay format is shown in Fig. 2. As shown in Figs 1 and 2, addition of streptavidin allows the immunocomplex to be captured on the biotinylated membrane. The urease-conjugated anti-fluorescein antibody provides a signal for detection.

Alpha-1-acid glycoprotein is found in human serum in various concentrations; elevated concentrations and varying degrees of glycosylation are found in some disease states [15–19]. It has been demonstrated that drugs and other compounds may bind to AGP in the serum and the glycoprotein also interacts with steroids and nucleic acids [19–21]. This polymorphic glycoprotein contains five N-linked complex carbohydrate chains and displays several glycoforms even at a single site. The oligosaccharides present all contain sialic acid at the terminus which may play a role in the biological function of this glycoprotein. In the native form, ricin binding would be inhibited due to the sialic acid groups present. Asialo-AGP, on the other hand, should contain a considerable number of terminal galactose residues that can bind ricin. Note that the heterosandwich immunoassay system shown in Fig. 2 detects any number of terminal galactose residues as the sandwich is formed using a protein specific antibody which should not block access to the terminal galactose residues. Also note that, in this assay system, AGP is captured on the membrane due to Ab binding, but no signal is generated because of the inability of ricin to bind to AGP.

The asialo form of AGP was prepared by incubation of AGP with neuraminidase attached to agarose beads. Electrophoresis of the native material showed a single band that migrated to the anode, as one would expect for a protein with a very low p_I. The neuraminidase treated sample, on the other hand, barely moved from the gel application point, an indication that a large number of negatively charged sialic acid groups had been removed. Furthermore, free NeuAc could be detected in the asialo-AGP sample (after digestion) using ionspray mass spectrometry.

Figure 3 shows the standard curve for the detection of AGP and asialo-AGP using the polyclonal antibody protein-based immunoassay procedure described in the methods section. Both AGP and asialo-AGP appear to elicit similar responses. The lower limits of detection for the system was 2 pg for the glycoprotein. This detection limit could be lowered substantially if the background

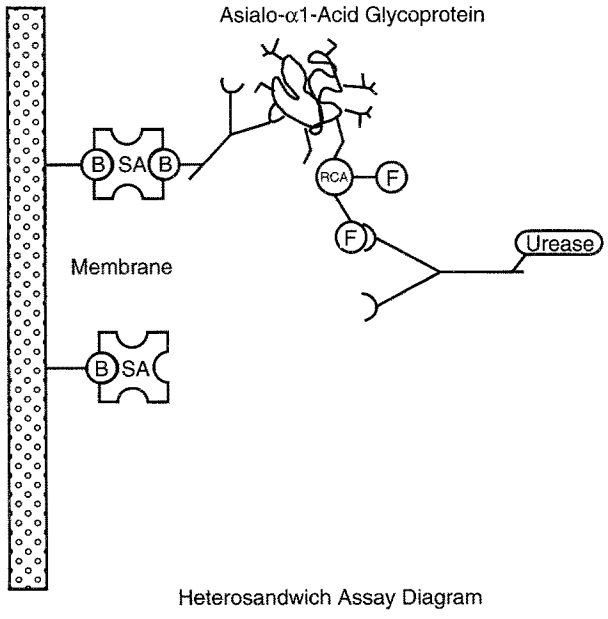

Figure 2. Diagram of the heterosandwich immunoassay format. See Fig. 1 for abbreviations; RCA, ricin.

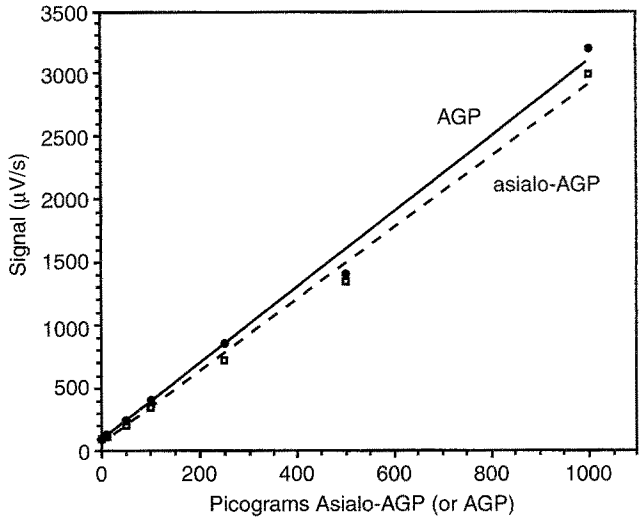

Figure 3. Plot of the LAPS response in the polyclonal immunoassay format as a function of increased concentrations of AGP or asialo-AGP. Each sample (in 250 μl buffer) contained 150 ng each of F-Ab (F:Ab ratio of 1.0) and b-anti-AGP antibody, 2 μg of streptavidin, and varying quantities of AGP or asialo-AGP. Details are given in the Methods section.

signal (due to nonspecific binding) could be reduced. This could be accomplished by affinity purification of the anti-AGP antibody (typically 80% is nonspecific). However, the present results indicate that the assay is sensitive and sialic acid independent.

Figure 4 shows the standard curve for the detection of AGP and asialo-AGP using the procedure described in Methods. An additional glycoprotein, asialofetuin, was also tested. Although our antibody was not specific for asialofetuin, we tested this glycoprotein in our assay system because it contains terminal galactose residues to which ricin will bind [14]. Neither AGP nor asialofetuin appear to elicit a response in the heterosandwich immunoassay. However, asialo-AGP gives a linear response as a function of concentration and is readily detectable at the nanogram level. The detection limit, based on two times the standard deviation above the zero analyte value, is about 250 pg for asialo-AGP. The limit of detection could be lowered if the antibody was affinity purified and, by using another lectin, had a higher affinity for galactose. Both of these changes would allow a reduction in the amount of protein present in the assay and thereby reduce signal due to nonspecific binding.

In an attempt to eliminate some of the background signal, we used only the F-ricin β-chain in the assay (instead of the entire molecule which is composed of α and β chains). The assay results are shown in Fig. 5. After normalizing the signal output by accounting for the difference in the degree of labelling of the two ricin forms, we found the signal to be about 30% lower than expected due to a number of reasons. One is the instability of the isolated chain. Another may be that

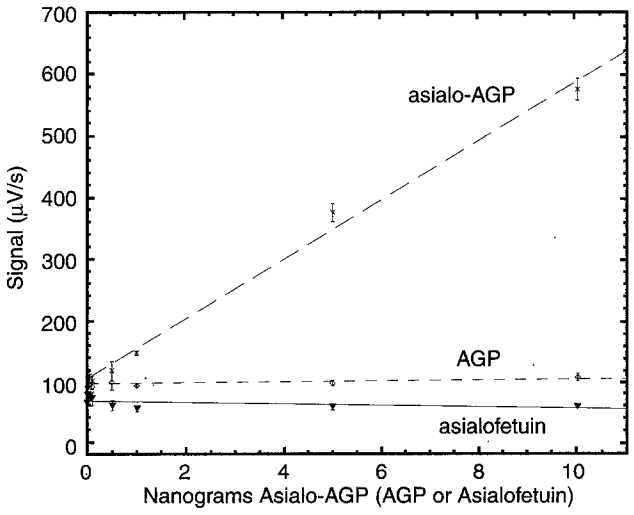

Figure 4. Plot of the LAPS response as a function of increased concentrations of AGP or asialo-AGP. Each sample (in 250 µl buffer) contained 50 ng of F-ricin (F:ricin ratio of 1.7), 50 ng of total b-anti-AGP antibody, 2 µg of streptavidin, and varying quantities of AGP, asialo-AGP, or asialofetuin. Details are given in the Methods section.

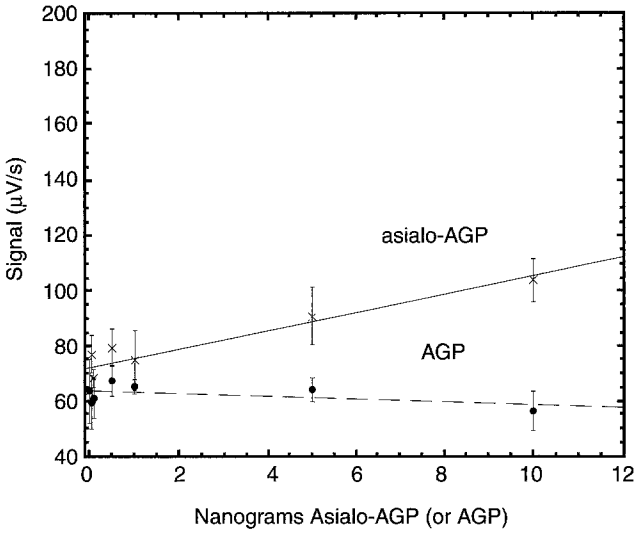

Figure 5. Same conditions as in Fig. 4 except F-ricin beta chain was used instead of the intact ricin. The F:ricin β-chain molar ratio was 0.9.

removal of the alpha chain exposes a fluorescein reaction site that is crucial for lectin binding. Nonetheless, the assay works with β-ricin chain and may be safer to use (non toxic).

Another problem that could arise in a complex mixture is the presence of additional terminal galactose-containing glycoproteins. The heterosandwich assay system would still be viable, but the quantity of ricin used in the assay would have to be modified. This is because the additional terminal galactose-containing glycoproteins present would compete with the specific glycoprotein (asialo-AGP) for the limited amount of F-ricin. To determine the quantity of F-ricin needed in the modified assay mixture, a fixed amount of the mixture (containing fixed amounts of antibody and analyte) are titrated with varying amounts of F-ricin until a plateau is reached. The plateau value for ricin (or a value greater than this) can now be used in the modified assay mixture for the detection of glycoproteins. Such a titration is shown in Fig. 6. For this experiment, 10 ng each of asialo fetuin and asialo-AGP were incubated with fixed amounts of b-antibody and varying quantities of F-ricin. As can be seen, the asialofetuin interferes with the detection of asialo-AGP by binding some of the F-ricin molecules (compare the signal output for the 50 ng level of F-ricin in Fig. 6 with the signal in Fig. 4 at the 10 ng per test for asialo-AGP). Further addition of F-ricin results in a plateau and this result can be used to establish the new F-ricin:b-antibody ratio that is required for the hetero-sandwich immunoassay. It should be noted that if an extremely large quantity of another galactose containing protein is present, use of the assay may not be feasible. Use of the immunoassay detection system shown in Fig. 3 in conjunction with the modified (corrected F-ricin:

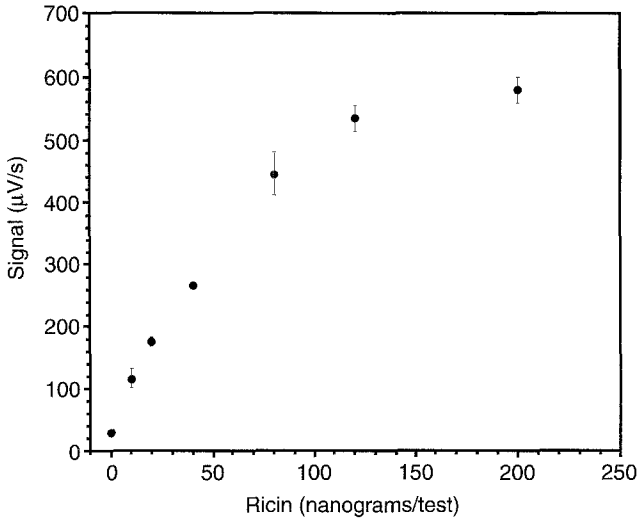

Figure 6. Effects of F-ricin on the LAPS response in a hetero-sandwich assay containing asialofetuin and asialo-AGP. Each sample (in 250 μl buffer) contained 50 ng of total b-anti-AGP antibody, 10 ng each of asialofetuin and asialo-AGP, 2 μg of streptavidin, and varying quantities F-ricin.

b-Ab) heterosandwich immunoassay system shown in Fig. 4 would allow detection and quantitation of a specific glycoform. Note that this approach requires specific standards and the value obtained for the glycoform would be an 'average value'; for instance, the assay cannot distinguish whether AGP molecules have 30% of their sialic acids residues removed (uniformly) or 30% of the AGP molecules have 100% of their sialic acid residues removed.

We have shown that the LAPS based immunoassay detection system can be used to detect picogram quantities of a specific glycoprotein and the terminal carbohydrate residues present on that glycoprotein. The system can be used to detect any glycoprotein and terminal carbohydrate (or branched structure) present in an assay mixture because of the protein-epitope specificity of the antibody used. The LAPS based Threshold System is already well known for the detection of contaminants in biopharmaceuticals and lends itself to this additional application in downstream processing of biopharmaceuticals.

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