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Magnetic bead-based fluorometric detection of lectin–glycoprotein interactions

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ABSTRACT

A sandwich-type bioassay was developed to detect the lectin–glycoprotein interactions using fluorescein isothiocyanate (FITC) as the label. The bioassay was optimized using the well-described lectins and their target glycoprotein. The biotinylated lectins from *Triticum vulgaris* (wheat germ, WGA) and *Arachis hypogaea* (peanut, PNA) were immobilized on streptavidin-coated superparamagnetic iron oxide microparticles (beads) and utilized for the isolation of target biomolecules (Invertase from *Saccharomyces cerevisiae*) from complex matrices. The secondary lectin layer was labeled with biotin, which allowed the binding of ExtrAvidin®-conjugated FITC. The fluorescence signal of FITC was measured at an emission wavelength of 519 nm. By optimizing the experimental conditions, changes in the fluorescence signals displayed linear concentration dependence until 50 μg/mL invertase. The fluorescence intensity peaked, when the WGA and PNA concentrations were at 50 μg/mL. The proof-of-concept study shows that the versatile and simple bioanalytical technique is a promising candidate for the rapid screening of lectin–glycoprotein interactions.

1. Introduction

Lectins are proteins that are able to recognize and bind non-covalently to specific soluble carbohydrates or their functional groups without causing any structural changes in the bound carbohydrate [1]. They have been implicated in having roles as diverse as modulating intracellular traffic, endocytosis, cell–cell recognitions, signal transduction, inflammation processes, and cancer cell metastasis [2–5]. In addition, lectins are structurally complex and their binding epitopes are often not well-described [5]. In this report, two lectins with distinct binding motifs were used to develop the versatile fluorometric technique. *Triticum vulgaris*, also known as wheat germ agglutinin (WGA), is composed of two subunits with a combined molecular weight of 43.2 kDa [6]. WGA is known to selectively bind to the sugar *N*-acetyl-D-glucosamine and to sialic acid [7–10]. *Arachis hypogaea*, also known as peanut agglutinin (PNA), is composed of four subunits with a combined molecular weight of 110 kDa [11–13]. Rhodes et al. [14] have recently reviewed the lectin–epithelial interactions in the human colon. PNA is known to have increased binding affinity towards the Thomsen-Friedenreich antigen, which is a highly expressed oncofetal carbohydrate antigen observed in the colonic epithelium under inflammatory conditions such as ulcerative colitis, Crohn's disease and colon cancer [15].

Invertase (β-D-fructofuranosidase fructohydrolase, E.C. 3.2.1.26) is a highly efficient enzyme that has been described as specific for converting sucrose to glucose and fructose [16,17]. The hydrolized sugar mixture obtained by invertase has the advantage of being colorless in contrast to the colored products obtained by acid hydrolysis [18,19]. Invertase is commonly used to liquidify the sugar found inside confections. Chu et al. [20] reported that endo-β-N-acetylglucosaminidase H treated external invertase from *Saccharomyces cerevisiae* (yeast) was restored to 40% of its original activity, while internal invertase remained completely inactive. The observed differences was attributed to the presence of this oligosaccharide moiety in the external regions of invertases. In this report, we aimed to investigate the interactions of two lectins WGA or PNA with these oligosaccharide chains found on the yeast invertase.

The affinity of lectins towards carbohydrates and glycoproteins has been exploited in numerous biosensor designs [21–30]. Surface-plasmon resonance (SPR) was combined with HPLC profiling with fluorescence detection for ligand-fishing in complex mixtures of oligosaccharides [22]. Linman et al. [23] have recently reported an SPR study of protein-carbohydrate interactions using biotinylated sialosides. Blagoi et al. [24] described a FRET-based approach between fluorescein (donor)-labeled lectins, adsorbed on the surface of polymeric beads, and polymeric dextran molecules labeled with Texas Red (acceptor). Nakata et al. [26] modified Concanavalin A, a glucose- and mannose-selective lectin, with fluorescein in the proximity of its sugar binding site and followed the changes in the fluorescence signal upon binding with the targeted sugars in test tubes and in cells. Sato and Anzai [27] observed

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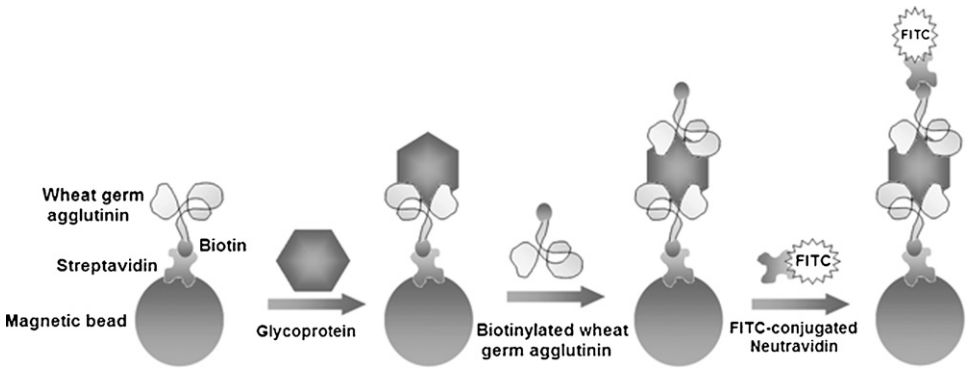

Fig. 1. Schematic illustration of the sandwich-type fluorometric bioassay for the investigation of lectin–glycoprotein interactions. Streptavidin-coated magnetic beads were modified with the biotinylated primary lectins. The attachment of the target glycoprotein was followed by the attachment of the secondary lectin onto the glycoprotein. The biotinylated ends of the secondary lectins allowed the binding of Extravidin®-conjugated FITC dye onto the complex. The fluorescence measurements were taken at an excitation wavelength of 494 nm and an emission wavelength of 519 nm.

that the fluorescence of fluorescein isothiocyanate-labeled Concanavalin A (FITC-Con A) was quenched by forming an FITC-Con A-glycogen conjugate and dequenched upon addition of sugars to the conjugate solution due to disaggregation of the conjugate. Recently, Liu et al. [28] reported the determination of trace glucose and disease biomarkers using affinity adsorption solid substrate-room temperature phosphorimetry based on WGA labeled with dendrimers-porphyrin dual luminescence molecule. Gamella et al. [29] reported electrochemical applications to recognize and quantify microorganisms using lectin adsorptive affinity impedance. The voltammetric evaluation for the binding of WGA to glucosamine-modified magnetic beads was reported by Sugawara et al. [30].

In this report, a fluorometric technique was developed in combination with magnetic separations. Magnetic beads are extensively used in biosensing procedures because of their ability to isolate the target biomolecule from complex matrices [30–33]. As illustrated in Fig. 1, the biotinylated primary lectins (WGA and PNA), when in the presence of streptavidin conjugated magnetic beads, readily bind to the streptavidin due to the strong affinity between the streptavidin and biotin entities. Then, a carbohydrate-containing target biomolecule (Invertase) was introduced to the lectin-modified magnetic beads to allow for the possible interaction between the lectin and the sugar moieties. The biotinylated secondary lectins were added with the purpose of binding to the added carbohydrate moieties, effectively creating the sandwich-type complex. Finally, the addition of FITC-conjugated with egg white avidin (Extravidin®) to the biotinylated secondary lectin (farthest from the magnetic beads) allowed us to detect the presence of the lectin–glycoprotein–lectin complex.

2. Experimental

2.1. Reagents

Streptavidin-coated superparamagnetic Fe_3O_4 microparticles (magnetic beads), Invertase glycoprotein standard from *S. cerevisiae*, Extravidin®-Fluorescein isothiocyanate (FITC) conjugate, the biotinylated lectins from *T. vulgaris* (wheat germ) and *A. hypogaea* (peanut) were purchased from Sigma–Aldrich (USA). Extravidin®-FITC was provided as a liquid in 0.01 M phosphate buffered saline, containing 10% (v/v) 0.5 M carbonate buffer, pH 9.5 with 15 mM sodium azide as a preservative. A 50 mM phosphate buffer (PBS, pH 7.4) comprised of KH_2PO_4 and K_2HPO_4 was used in all incubation and washing steps.

2.2. Apparatus

Fluorescence measurements were performed using the Nanodrop 3300 fluorospectrometer (Thermo Scientific, USA). The computer software used for the analysis of spectrograms was Nanodrop 3300 version 2.6.0. Separations of the magnetic beads and the surrounding solution were performed using the DynaMag-Spin (Invitrogen, USA).

2.3. Procedure

2.3.1. Preparation of the primary lectin conjugated magnetic beads

The stock solution (1 mg/mL) of magnetic beads was shaken vigorously to create a homogenous dispersion of the beads. An aliquot (100 μL) of the magnetic bead stock, followed by 100 μL of various dilutions of the biotinylated lectin (WGA or PNA) in PBS were placed in a vial. Then, the mixture was subjected to slow shaking at 300 rpm for 1 h to allow for the binding of the biotinylated lectins with the streptavidin-coated beads. The mixture was then washed three times with 200 μL aliquots of blank PBS. The washing procedure consisted of separating the magnetic beads from the solution by putting the vial against the magnet of the DynaMag-Spin, which caused the magnetic beads to aggregate against the wall of the vial. A micropipette was used to remove the supernatant solution without disturbing the aggregated magnetic beads. Then, 200 μL of blank PBS was placed back in the vial, and the vial was taken out of the DynaMag-Spin. The beads were dispersed with vortexing on a shaker at 1000 rpm for 10 s. This process constituted one 'wash' of the magnetic beads and repeated for three times for each washing procedure. These three repetitive washing steps ensured the suppression of non-specifically adsorbed species on the magnetic beads. As a result, the primary lectin-modified magnetic beads were obtained.

2.3.2. Preparation of the lectin–target–lectin complex

The 200 μL of primary lectin-modified magnetic beads was separated into two 100 μL aliquots. 100 μL target solution consisting of various dilutions of the glycoprotein invertase were prepared with PBS. Then, 100 μL of the lectin-modified magnetic bead solution was added to the 100 μL target solution, and the 200 μL was subjected to 1 h of shaking at 300 rpm. The solution was then washed three times with blank PBS as described above previously to suppress the amount of the non-specifically adsorbed biomolecules. The 200 μL solution was placed in the magnet and the solution was removed using a micropipette from the magnetic beads. 100 μL

of the same biotinylated lectin was added to the magnetic beads to form the second layer of lectins in the lectin–target–lectin complex. 100 μL of additional PBS was added, bringing the total volume to 200 μL once again. The solution was shaken for 1 h at 300 rpm, and then was washed three times using blank PBS as described above. The resulting complex of lectin–target–lectin sandwich created free biotin residues on the outer layer ready for the binding with the Extravidin®-conjugated fluorescent dye.

2.3.3. Preparation of the Extravidin®-FITC dye conjugation

The aliquot of (50 μL) Extravidin®-conjugated FITC solution was added to the 50 μL of magnetic bead solution to form the desired final concentration of the fluorescent dye. The 100 μL magnetic bead and FITC dye solution was shaken for 1 h at 300 rpm. Note that the remaining 150 μL magnetic bead solution was used as a blank against which the fluorescence will be measured. The beads were again washed three times using blank PBS as described above to suppress non-specifically adsorbed dye compounds.

2.3.4. Fluorescence spectroscopy

The magnetic bead solution (2 μL) without the FITC-modification was loaded onto the Nanodrop 3300 platform, and was used as the blank (background signal). The magnetic bead solution (2 μL) labeled with the FITC dye was then loaded onto the Nanodrop and measured at an excitation wavelength of 494 nm and an emission wavelength of 519 nm. The magnetic bead and FITC dye solution was placed on the magnet, and 2 μL of the remaining solution was also measured. Then, the separated magnetic beads were washed with three 100 μL aliquots of 50 mM phosphate buffer solution, and then 2 μL of this solution was measured. The RFU intensity of this final measurement indicated the amount of binding that occurred between the ligands. Analytical evaluations were based on these end-point fluorescence measurements. The statistical data were obtained by repeating the measurements for four times ($n = 4$) for each experimental condition.

3. Results and discussion

Fluorescence intensity output allowed us to determine the sandwich-type affinity binding between the biotinylated lectins and the targeted biomolecules. First, the magnetic beads with the lectin–target–lectin sandwich-type complex prior to the addition of FITC were measured as the blank. Next, the same set of magnetic beads with the addition of FITC dye was measured. Thus, the signal of bound FITC dye indicated the presence of the lectin–target–lectin complex formation on the beads. The intensity of the FITC dye was measured at an emission wavelength of 519 nm.

It was predicted that as the invertase concentration increased up to a certain level, the RFU would increase with it–indicating that the glycoprotein invertase had in fact bound to the WGA lectins modified on the magnetic beads as illustrated in Fig. 1. Fig. 2A displays the raw data of representative measurements taken with WGA and PNA modified lectins in the presence of 10 μg/mL invertase. Fig. 2B is the plot showing the dependence of the fluorescence signals on the concentration of invertase. It was noted that the highest fluorescence intensity was observed, when the concentration of invertase was 50 μg/mL. Increasing the concentration beyond this point caused the intensity of the signal to even out indicating that the surface of the beads was saturated with the bound WGA/invertase/WGA complexes. Such a crowded surface on the beads inhibited the binding of increasing amount of FITC molecules. It was also found that there was a negligible signal, indicating that a very small amount of Extravidin®-conjugated FITC dye could bind with the WGA-conjugated magnetic beads, when no invertase was present in the solution. With the presence of

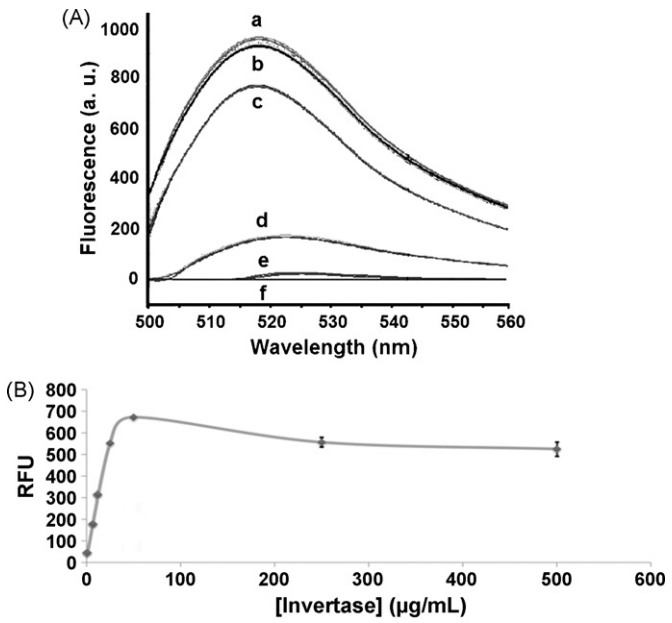

Fig. 2. (A) Fluorescence spectra of magnetic beads with the primary lectin-invertase-secondary lectin sandwich complex conjugated with Extravidin®-FITC in the presence of 10 μg/mL invertase before magnetic separation using (a) the wheat germ agglutinin (WGA) and (b) peanut lectin (PNA); (c) the same fluorescence signal was obtained from the supernatant after magnetic separation using both of the lectins; (d) the high fluorescence signal obtained after the magnetic separation and dispersion of the WGA-modified magnetic beads in blank PBS indicated the strong affinity between invertase and WGA; (e) the low fluorescence signal obtained after the magnetic separation and dispersion of the PNA-modified magnetic beads in blank PBS indicated the low affinity between invertase and PNA; (f) no fluorescence signal was obtained after following the same set of preparations in the presence of the magnetic separation and dispersion of the magnetic beads with no lectin modification. (B) Plot for the dependence of fluorescence signals on invertase concentration using wheat germ agglutinin (WGA)-modified magnetic beads. The FITC fluorescence measurements were collected at 519 nm as described in Section 2. Data points represent the average, and error bars indicate the standard deviation of the repetitive measurements ($n = 4$).

invertase, however, the WGA/invertase/WGA complex was completed and the RFU signal intensity significantly increased. As for control experiments, the same set of modifications on the beads was made in the absence of the WGA (Fig. 2A-f). As expected, no fluorescence signals were observed indicating the successful suppression of non-specific adsorption using the magnetic separation and stringent washing of the beads as described in Section 2.

The concentration of the WGA was varied, while the invertase (25 μg/mL) and Extravidin®-conjugated FITC dye (1:200) concentrations kept constant (Fig. 3). It was expected that no signals

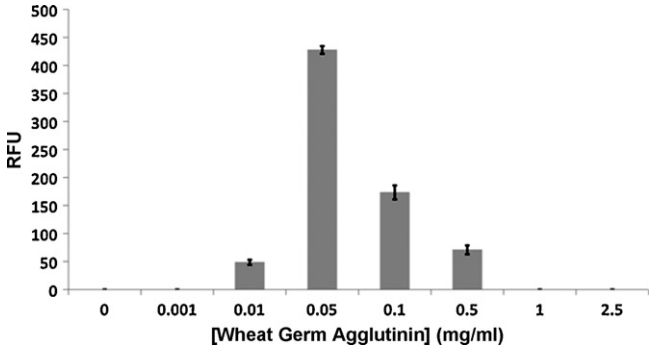

Fig. 3. Plot for the dependence of fluorescence signals on wheat germ agglutinin (WGA) concentration in the presence of 25 μg/mL invertase. Error bars indicate the standard deviation of the repetitive measurements ($n = 4$).

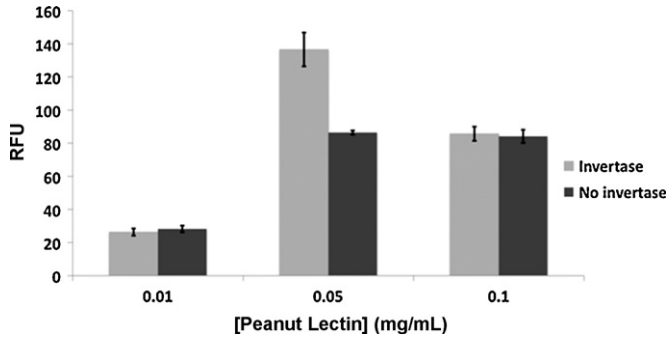

Fig. 4. Plot for the dependence of fluorescence signals on peanut lectin (PNA) concentration in the presence (gray) and absence (black) of 25 μg/mL invertase. Error bars indicate the standard deviation of the repetitive measurements ($n = 4$).

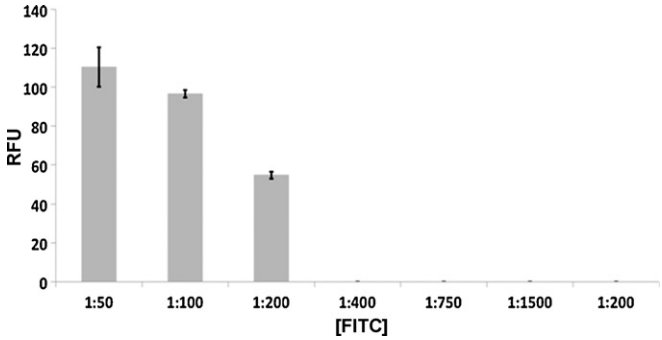

Fig. 5. Plot for the dependence of fluorescence signals on Extravidin®-conjugated FITC concentration. Error bars indicate the standard deviation of the repetitive measurements ($n = 4$).

would be observed at very low concentrations, because the FITC dye should not be able to bind without the binding of the secondary biotinylated WGA. Also, it was expected that the signal should increase as the WGA concentration increased up until the point, at which all the streptavidin binding sites on the magnetic beads were occupied. The results showed that at low concentrations, the RFU intensity was indeed low as was expected. However, increasing the concentration of the biotinylated WGA beyond the peak at 50 μg/mL caused the signal to decrease. We can deduce that at 50 μg/mL of lectin, the binding sites on the streptavidin-coated magnetic beads were saturated with the biotinylated lectins. Increasing the concentration of lectins beyond this value, however, caused the signal to decrease because of the competitive nature of the interaction: the binding affinity between biotin (in our case conjugated to the lectin) and avidin (in our case streptavidin) is the strongest affinity reaction known in nature. Because the biotinylated portion of the lectins had a strong natural affinity to the streptavidin-coated magnetic beads, increasing their concentration caused the lectins to effectively block each other while trying to reach and bind the magnetic beads, thus, the bulk of the lectin remained unbound and was removed during the repeated magnetic isolations and wash with the PBS for three times after each modification on the beads.

As shown in Fig. 4, the dependence of the fluorescence signals on the concentration of the FITC, while the concentration of the WGA and the glycoprotein invertase remained the same (WGA at 50 μg/mL and invertase at 25 μg/mL). It was expected that as the concentration of the FITC decreased, the RFU intensity would also decrease, as less of the FITC dye could bind the biotinylated lectin. It was observed that as the FITC dye concentration decreased, the RFU intensity decreased. Beyond FITC dye at 1:200, the signal disappeared, indicating that the amount of FITC dye present to bind was below threshold detection limit of the instrument used.

Furthermore, control experiments were performed in which the WGA was substituted with PNA to determine the effect of different lectins on binding affinity. Trials also compared the effect of PNA with and without the presence of the glycoprotein invertase. In these control measurements, the FITC and invertase (1:200 and 25 μg/mL, respectively) were kept constant. With invertase, in comparison with the results from the WGA, the fluorescence intensity was significantly lower at all measured concentrations, indicating that the biotinylated PNA had a low affinity for invertase (Fig. 5). It was noted that the highest signal for both the WGA and PNA was observed at 0.05 mg/mL. Without invertase, a substantial drop in the signal was observed with the PNA. Without the presence of invertase, the biotinylated PNA could only interact with the streptavidin conjugated magnetic beads directly.

The stability of the lectin-modified magnetic beads was tested using WGA. The sandwich assays were performed using 25 μg/mL invertase and 50 μg/mL WGA with 1:200 dilution of Extravidin-FITC conjugate. The WGA-modified magnetic beads were prepared and stored at 2–8 °C for 2 weeks. The measurements were collected daily using aliquots of magnetic beads from the same preparation. A significant decrease in the fluorescence signals was observed after 5 days indicating that fresh preparations of WGA-modified magnetic beads should be preferred for the technique described here.

4. Conclusions

A versatile magnetic bead-based fluorometric bioassay with high selectivity and sensitivity for carbohydrate–protein binding has been developed. Optimization of experimental conditions allowed low detection limits of invertase using WGA-modified magnetic beads. Well-described lectins (WGA and PNA) and glycoprotein (invertase) were utilized to develop this new sandwich-type format in a proof-of-principal format. These preliminary results are readily transferable to the discovery of specific carbohydrate residues in complex matrices (cell or tissue extracts and food samples).

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