Biochemical and Biophysical Research Communications 414 (2011) 84–89

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Development of recombinant *Aleuria aurantia* lectins with altered binding specificities to fucosylated glycans

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ARTICLE INFO

Article history:
Received 18 August 2011
Available online 14 September 2011

Keywords:
Lectin
Cancer
Glycobiology
Fucosylation
Hepatitis

ABSTRACT

Changes in glycosylation have long been associated with disease. While there are many methods to detect changes in glycosylation, plant derived lectins are often used to determine changes on specific proteins or molecules of interest. One change in glycosylation that has been observed by us and by others is a disease or antigen associated increase in fucosylation on N-linked glycans. To measure this change, the fucose binding *Aleuria aurantia* lectin (AAL) is often utilized in plate and solution based assays. AAL is a mushroom derived lectin that contains five fucose binding sites that preferentially bind fucose linked (α-1,3, α-1,2, α-,4, and α-1,6) to *N*-acetyllactosamine related structures. Recently, several reports by us and by others have indicated that specific fucose linkages found on certain serum biomarker glycoprotein's are more associated with disease than others. Taking a site-directed mutagenesis approach, we have created a set of recombinant AAL proteins that display altered binding affinities to different analytes containing various fucose linkages.

1. Introduction

The glycosylation of proteins is cell specific and the N-linked glycan a protein carries reflects modifications occurring in the cell from which it came [1]. Sugar (glycan) structures on the same protein secreted from malignant or diseased tissue and normal cells may, and often do, differ [2–7]. Indeed, we and others, have observed changes in N-linked glycosylation with the development of cirrhosis and hepatocellular carcinoma (HCC) [8–16]. Specifically, the amount of fucosylated N-linked glycan derived from total protein preparations isolated from the serum of individuals chronically infected with HCV and a diagnosis of HCC was consistently greater than healthy subjects or those with HCV and "inactive" disease [11].

Plant lectins are often utilized to measure changes in glycosylation. The *Aleuria aurantia* lectin (AAL) detects various fucose linkages (α-1,2; α-1,3; α-1,4; α-1,6) attached to either a *N*-acetylglucosamine (GlcNAc) or a galactose residue. The crystal structure for AAL has been solved by several groups and reveals that AAL monomers display a six-bladed β-propeller fold with five conserved fucose binding sites located between each blade [17–19]. The sixth site does not contain the conserved amino acids residues required to form the fucose binding pocket and it is proposed that the sixth site forms closure contacts between blades 1 and 6 to help stabilize the overall β-propeller structure [18]. The multivalent nature of AAL gives it an unusually high binding affinity (μM) for carbohydrate ligands compared to other lectins. In this study, we have modified amino acids residues within the fucose binding sites and also created domain swap mutations to determine whether individual fucose binding domains play a role in recognizing specific fucose linkages. Our studies indicate that the while sites 2 and 4 are primarily involved in binding to α-1,2; α-1,3; α-1,4 linked fucosylated glycans, site five may be more important in binding to α-1,6 linked core fucose.

2. Methods

2.1. Glycoconjugates and lectins

Monosaccharide and oligosaccharide bovine serum albumin (BSA) conjugates; L-fucose-BSA, D-galactose-BSA, α-1,2-fucose-BSA, α-1,3-fucose-BSA, and α-1,4-fucose-BSA were all purchased from V-Labs, Inc. (Covington, LA). Native AAL was purchased from Vector Laboratories (Burlingame, CA).

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doi:10.1016/j.bbrc.2011.09.027

2.2. rAAL plasmid construction

Briefly, the wild-type AAL sequence was engineered and synthesized to include unique *Nde I* and *Xho I* restriction sites at the 5′ and 3′ ends (respectively) of the AAL gene sequence and blunt-end cloned into shuttle vector pUC57 (GenScript, Inc. Piscataway, NJ). The synthesized gene product was subsequently subcloned as an *NdeI-XhoI* fragment from the pUC57 cloning vector into the T7 expression vectors pET 29-b (Novagen) and pQE-T7 (Qiagen) which results in the addition of the sequence for an in frame histidine tag at the C-terminal end of the AAL gene sequence.

2.3. Expression and purification

The rAAL his-tagged construct was transformed into bacterial strain BL21 Star (DE3) that carries the gene for the T7 RNA polymerase under control of the *lac*UV5 promoter and allows for high level isopropyl-beta-D-thiogalactopyranoside (IPTG) inducible expression of gene products from T7 expression vectors such as pET and pQE. Bacterial transformation and cell culture growth conditions were done following manufacturers protocol (Invitrogen). Cells were harvested by centrifugation and cell pellets were washed with 1× phosphate buffered saline (PBS) containing protease inhibitors (Sigma*Fast* EDTA-free). Total cellular protein lysates were made using an Avestin C5 high pressure homogenizer. FPLC purification methods for the rAAL histidine tagged protein used Ni–NTA (Qiagen) and IMAC HisTrap HP (GE Healthcare) columns. Bacterial cell lysate from IPTG induced cultures was loaded onto to the column in binding buffer; 50 mM $NaHPO_4$, 1 M NaCl (pH 7.0) and bound histidine tagged rAAL was washed and eluted using an imidazole step gradient (250–800 mM imidazole) in binding buffer.

2.4. Mutagenesis of AAL

The rAAL construct containing the asparagine (N) 224 to glutamine (Q) point mutation (N224Q) was synthesized as a 526 bp *Sac I–Xho I* fragment and blunt-end cloned into shuttle vector pUC57 (GenScript Inc.). A 526 bp *Sac I-Xho I* fragment containing N224Q mutation was gel isolated from the pUC57 construct and ligated into the *Sac I-Xho I* digested rAAL pQE T7 plasmid.

The X-2, 4 domain swap mutant contains the first five AAL amino acids and an intact β-propeller blade 1 (amino acids L5–A57) followed by a reiteration of blade 2 (K58–V109) and blade 4 (V163–A208). The construct was designed such that there is an intact blade six following the second blade 4. The sequence was synthesized (GenScript) as a 699 bp *Nco I-Nhe I* fragment which includes the *Nco I* site in blade 1 located at codons 44 and 45 and the rest of the blade 1 sequence to A57 followed by the blade 2–4-2–4 reiteration and the blade 6 sequence from L261 to the *NheI* site located at codons 281 and 282. The 699 bp X-2-4 fragment was subcloned from the pUC57 cloning vector as a 699 bp *Nco I-Nhe I* fragment and ligated into *Nco I-Nhe I* restricted rAAL-pQE T7.

2.5. Glycan analysis

Glycan analysis was performed on AFP (alpha-fetoprotein), as previously described [10,11,20–22].

2.6. Fluorophore and enzyme-linked lectin assays

rAAL proteins were biotinylated using NHS-PEG_{12}-biotin (ThermoScientific) according to the manufacturer's instruction. The biotin/protein ratio was determined to be ~1:1 biotin moieties per molecule of rAAL using a Pierce Biotin Quantitation kit (ThermoScientific).

Microtiter plates (Nunc Maxisorp) were coated with 0.05 μg/mL of L-fucose-BSA, D-galactose-BSA, α1–2-fucose-BSA, α1–3-fucose-BSA, and α1–4-fucose-BSA in 100 μL of coating buffer 1× Tris Buffered Saline (TBS) (50 mM Tris–HCl, 150 mM NaCl (pH 7.6)) and Fluorophore-linked immunosorbent lectin assays (FLISA) analysis was performed as described previously [23–25].

To test α-1,6 linked core fucose lectin binding either AFP, which was shown to contain only core fucose or modified human IgG was utilized. AFP purified from human cord blood (Lee Biosolutions) was directly coated at 5.0 μg/mL in 100 μL of 1× TBS, For IgG 1.0 mg of human IgG (Sigma Aldrich, St. Louis, Missouri, MO) was incubated with 1000 units of IgGZERO endoglycosidase (Bulldog Bio, Rochester, New York) in 1× PBS at 37 °C for 1 h to degalactosylate the IgG glycan [26,27]. Removal of this portion of the glycan exposes the core linked fucose and makes it available for recognition by core fucose binding lectins [28,29]. The IgG/IgGZERO reaction mixture was then run over a protein A/G Sepharose column to remove the IgGZERO enzyme. FLISA assays were run as described above using 1.25 μg/mL of IgG0 to coat the wells.

2.7. Thickness shear mode (TSM) sensors

The use of TSM has been described previously [30]. Briefly, the 100 MHz Thickness shear mode sensor used had a geometry of a double sided mesa structure. The sensor's outer diameter, diameter of the mesa, diameter of the gold electrodes and thickness of the membrane was 5, 3, 1.5 mm, and 16 μm, respectively. The sensors were a 100 MHz AT-cut quartz crystal sandwiched between two gold electrodes. Initial readings were taken in air for 5 min to detect any faulty sensors then 5 μL of PBS was dispensed on top of the sensor for another set of readings lasting 15 min to measure consistency. The PBS was removed and AFP (5 μL at 0.1 mg/mL) or control ligand (0.1 mg/mL) was adsorbed on the surface for 1 h while recording the frequency then washed 3× with 5 μL of PBS. PBS was removed and 5 μL of BSA (0.1 mg/mL) was applied and incubated while recording for 1 h. The sensor was washed, recorded and dried. Lectin (5 μL at 0.1 mg/mL) was applied for 1 h. Measurements were made on a personal computer using a program written in Labview interfaced to a HP 4395A Network Analyzer and the microwave switch. A wide span of 2000 kHz about a center frequency was made use of to identify the resonant peak, followed by a narrow span of 100 kHz. The bandwidth of the two scans was set at 1 and 100 Hz, respectively. The above settings resulted in a noise level of 35 Hz in air and 350 Hz in liquids.

3. Results and discussion

3.1. Development of A. aurantia lectins (AAL) with altered binding specificities

In our previous plate based work we utilized native (n)AAL (Vector Labs, Inc., Burlingame, CA), as it is the only commercially available fucose binding lectin [7,23–25,29,31]. In an effort to create fucose binding lectins with increased specificity and sensitivity, we constructed recombinant (r)AAL molecules containing specific point mutations and domain changes. Previous surface plasmon resonance binding affinity (Biacore) studies have indicated that there is one high affinity nanomolar (nM) fucose binding site present in rAAL that is not detected in the native commercially available lectin [17,32]. Based on structural analysis it has been proposed that the five genetically distinct fucose binding sites in AAL display varying binding affinities towards fucose and different fucose linked oligosaccharides, with the 2 and 4 sites having higher

binding affinities than the 3 and the 5 sites [17,32]. Interestingly, the high affinity (nM) site bound to α-1,2; α-1,3 and α-1,4 linked fucosylated oligosaccharides but not to α-1,6 fucose linked oligosaccharides [17,32].

To test the hypothesis that the 2 and 4 sites have higher avidity towards α-1,2; α-1,3 and α-1,4 linked fucosylated oligosaccharides, we have created a rAAL molecule with re-iterated 2 and 4 sites (X-2,4, see Fig. 1A). The X-2,4 domain swap mutant contains a 1–2-4–2-4–6 β-propeller arrangement (Fig. 1A). While the first fucose binding domain has been implicated as being a potential high affinity binding site we have found that making alterations in either propeller domain 1 or 6 has a detrimental effect on protein expression and/or purification (data not shown) so we have maintained these two domains in our constructs [17,32].

Additionally, crystal structure analysis of AAL [18,19] has shown that the 2 and 4 binding sites have glutamine (Q) residues in their fucose binding pockets that coordinate with other amino acids within the pocket to maintain a secondary structure required for high affinity fucose binding. In binding sites 3 and 5, an asparagine (N) is found in this position. As the side chain on N is one carbon shorter than Q, the 3 and 5 binding sites lack critical hydrogen bond contacts required for high affinity fucose binding [18]. Thus, we changed the 224 asparagine in binding site 5 to glutamine to create rAAL N224Q (Fig. 1A). This mutation is predicted to increase the hydrogen bonding potential of the 5 site for fucose containing targets.

Using a bacterial T7 inducible expression system we have developed a two column FPLC IMAC procedure which yields >90% pure fucose free recombinant AAL. Fig. 1B shows an SDS–PAGE coomassie stain analysis of purified wild-type rAAL, the X-2,4 AAL and N224Q mutant proteins. Bovine serum albumin (BSA) loading controls are also shown for reference and for use in quantitation.

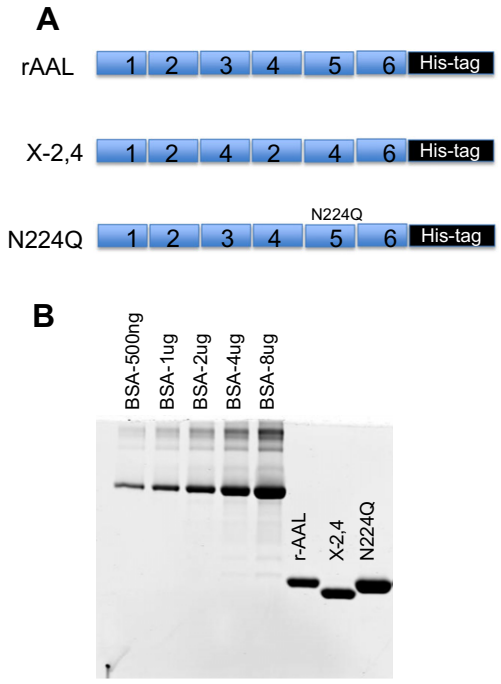

Fig. 1. (A) Arrangement of the β-propeller motifs in the recombinant AAL proteins with the location of the N224Q point mutation in motif 5 and the C-terminal histidine tag. The five fucose binding sites are located between each blade of the propeller. (B) SDS–PAGE coomassie stained gel showing purity of the Immobilized Metal Affinity Chromatography (IMAC) nickel column isolated recombinant AAL proteins. Bovine serum albumin (BSA) standards were loaded on the gel for quantitative comparison. The 320 amino acid X,2–4 domain swap mutant runs at a slightly lower molecular weight compared to the 325 amino acid rAAL and N224Q proteins.

3.2. Fluorophore-Linked Immunosorbent Lectin Assay (FLISA) analysis

Using a lectin-FLISA based approach, we compared binding affinities of the rAAL proteins and the native commercial (n) AAL to α-1,2; α-1,3 and α-1,4 fucose linked BSA glycoconjugates and also to human IgG0 which serves as a α-1,6 fucose glycoprotein target. FLISA analysis shows that the recombinant lectins all bind the fucose-linked BSA conjugates with approximately the same affinity. As shown previously [17], the amount of commercial native lectin bound to the glycoconjugates was typically 3- to 5-fold less than the rAAL proteins (Fig. 2 panels A, B, and C). Interestingly, there was a significant difference seen in the binding of the rAAL mutant lectins to α-1,6 fucose. As shown in Fig. 2D the amount of bound N224Q was more than 2-fold higher the wild-type rAAL and 8- to 10-fold higher than the X-2,4 mutant and nAAL. While the nAAL binding to the α-1,6 fucose ligand is similar to its binding affinities for the α-1,2; α-1,3 and α-1,4 fucose linked BSA glycoconjugates, the differential binding of the rAAL mutants indicates that, relative to the wild-type rAAL, the N224Q protein has an increased binding specificity for α-1,6 fucose linked analytes, while removal of AAL binding sites 3 and 5 in the X-2,4 mutant leads to a decreased affinity for α-1,6 fucose linked targets while still retaining wild-type levels of binding to the α-1,2; α-1,3 and α-1,4 fucose glycoconjugates. This binding is fucose specific, as lectin binding can be abrogated after washing with TNT containing 250 mM fucose (data not shown). Additionally, all lectins bound to an L-fucose BSA glycoconjugate with approximately the same binding affinity (data not shown) but did not bind to a galactose-BSA glycoconjugate (Fig. 2E).

3.3. Enzyme linked lectin assay (ELLA) analysis

We have employed a number of different fucosylated targets to further define the ligand binding specificity and sensitivity of the recombinant AAL proteins. One concern in lectin based detection assays is the sensitivity of the system. The diagnostic changes in glycosylation in a number of biomarker proteins are small and require very sensitive methods of detection. To assess the binding sensitivity of the rAAL proteins for low levels of target analyte we used AFP purified from human cord blood as a α-1,6 fucose glycoprotein target. AFP has a single site of glycosylation [33] and glycan analysis shows that this commercial preparation has ~3.0% core (α-1,6-linked) fucosylation (Fig. 3A). The ability of nAAL and the recombinant lectins to detect this level of fucosylation was determined by enzyme linked lectin assay (Fig. 3B and C) and by a thickness sheer mode (TSM) sensor analysis (Fig. 4) [34]. Fig. 3B shows the detection of several concentrations of AFP using biotinylated rAAL and nAAL. The results indicate that, when using equivalent amounts of lectin, the rAAL gives greater signal at all AFP concentrations tested. Fig. 3C shows the results of all lectins in the detection of 0.5 μg of cord blood AFP bound to the plate. Results shown in Fig. 3C corroborate our previous results using human IgG0 as a α-1,6 fucose glycoprotein target (Fig. 2D), and indicate that the N224Q protein has an increased sensitivity for detecting low levels of α-1,6 fucose analyte, with more than a 3-fold higher binding than the wild-type rAAL and >10-fold higher binding than the X-2,4 mutant or the nAAL.

In Fig. 4 we utilized non biotinylated lectin in a thickness sheer mode sensor (TSM) (similar to Biacore detection) to detect binding of the wild-type rAAL, N224Q, and the native nAAL to the cord blood AFP. In this system, AFP is adsorbed onto a gold electrode of the TSM sensor and lectin applied. Binding is determined by a change in resonant frequency of the sensor [34]. As this Figure shows, there is over a 6-fold increase in binding of the N224Q lectin as compared to the wild type recombinant lectin or the

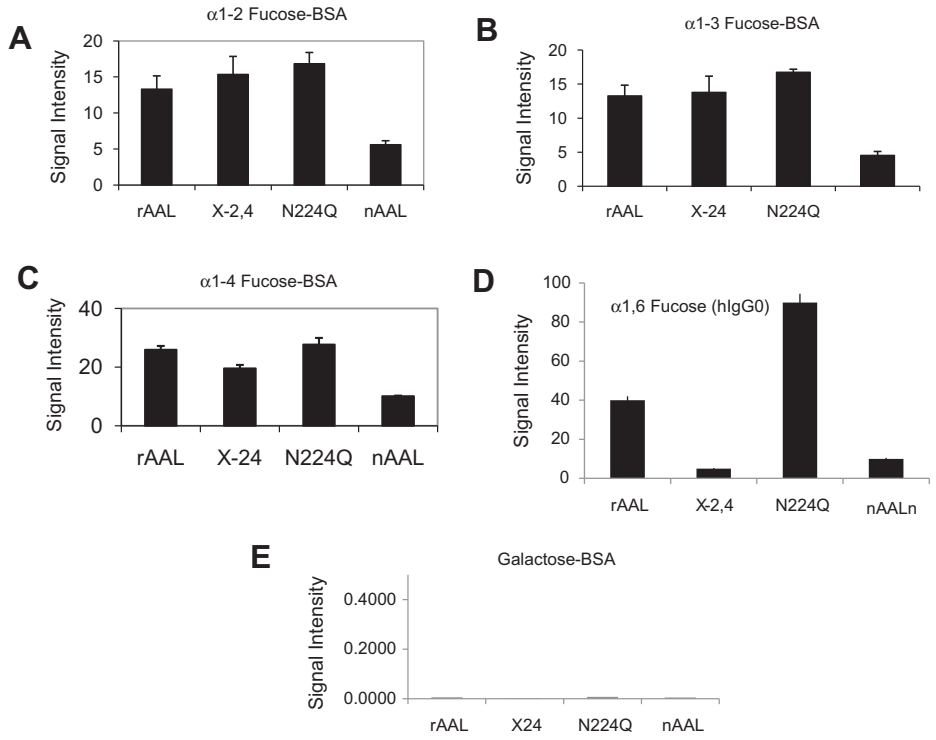

Fig. 2. FLISA analysis showing binding of recombinant AAL proteins and commercially purchased native nAAL (Vector Labs) to different fucosylated-BSA glycoconjugates (Panels, A, B, and C), and to the degalactosylated human (h)IgG0 (Panel D), which serves as a α-1,6 fucose glycoprotein analyte. All lectins were biotinylated to the same biotin/protein molar ratio's and were used at the same concentrations in all experiments. Similar results were obtained doing the same experiments in an ELLA format.

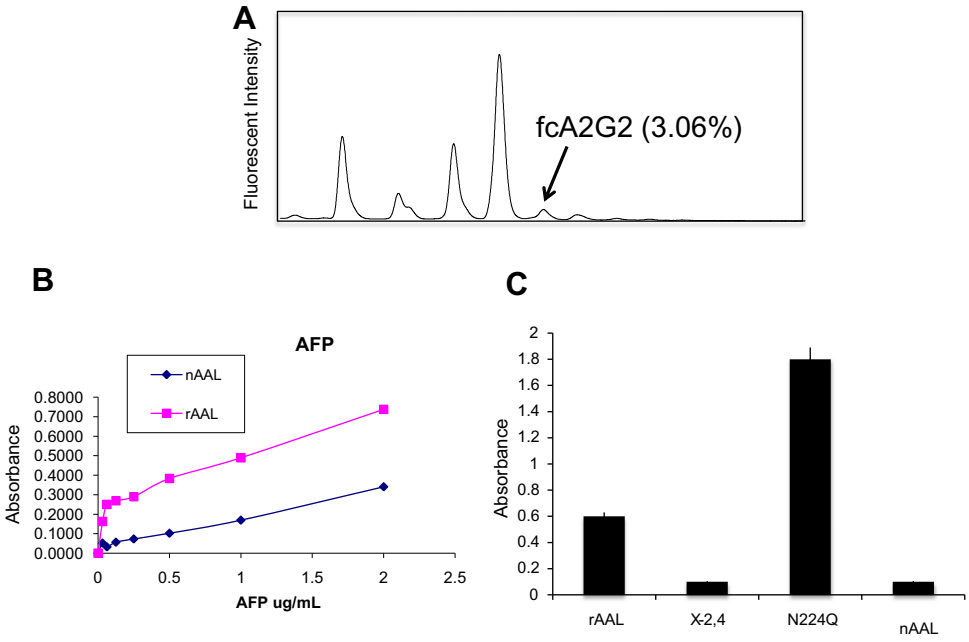

Fig. 3. ELLA analysis showing binding of recombinant AAL proteins and commercially purchased native nAAL (Vector Labs) to AFP purified from human cord blood. (A) HPLC-based glycan sequencing of purchased AFP from cord blood. The arrow indicates the peak identified as FcA2G2, which contains the α-1,6 core fucosylated biantennary N-glycan. This peak represents ~3.0% of the total AFP glycoforms. (B) ELLA analysis comparing binding of wild-type rAAL and nAAL to varying amounts of AFP containing 3.0% α-1,6 core fucosylated biantennary N-glycan. Both lectins were used at the same concentration and contained similar levels of biotinylation. (C) ELLA analysis showing binding of rAAL proteins and nAAL to plates coated with 5.0 μg/mL cord blood purified AFP.

commercially purchased nAAL in line with the results presented in Figs. 2 and 3.

It is noted that the difference in the ability of the rAAL or nAAL to detect α-1,6 fucose analytes; AFP (Figs. 3C and 4B); and human IgG0 (Fig. 2D) is related to the amount of fucosylation on the proteins examined. In the case of IgG0 (Fig. 2D), there is a single site of glycosylation and >90% of the IgG0 molecules contain this core (α-1,6) fucosylation [29]. In contrast, the AFP used in Figs. 3A–D

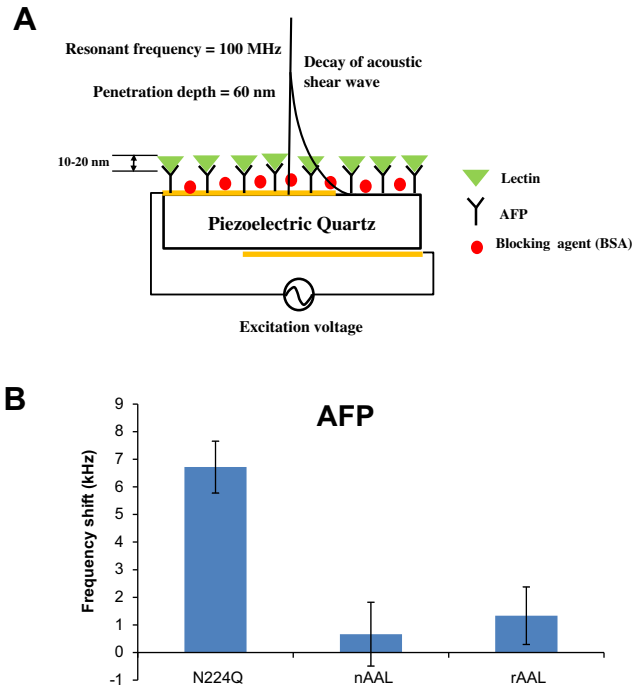

Fig. 4. (A) A conceptual model for TSM sensor operating at the fundamental resonance frequency of 100 MHz. Loading the sensor surface with liquid and biological media causes a change in the electromechanical impedance on the surface of the sensor due to the changes in mechanical properties of the medium. These changes are monitored as a function of time. (B) TSM analysis showing binding of recombinant AAL proteins and commercially purchased native nAAL (Vector Labs) to AFP purified from human cord blood.

and 4B also has a single site but only ~3.0% of the total protein is fucosylated. Treatment of either human IgG or AFP with sodium periodate (periodate oxidation) or with bovine kidney fucosidase abolished the signal confirming that lectin binding was fucose specific (data not shown).

In conclusion, these studies indicate that mutagenesis based changes of recombinant lectins has the potential to create reagents with the increased specificity and sensitivity required for robust diagnostic assay development.

Acknowledgments

This work was supported by Grants CA120206, CA120206-04S1 and CA136607 from the National Cancer Institute (NCI), the Hepatitis B Foundation, and an appropriation from The Commonwealth of Pennsylvania.

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