Overcoming the aggregation problem: a new type of fluorescent ligand for ConA-based glucose sensing

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Abstract

Competitive binding assays based on the lectin Concanavalin A (ConA) have displayed significant potential to serve in continuous glucose monitoring applications. However, to date, this type of fluorescent, affinity-based assay has yet to show the stable, glucose predictive capabilities that are required for such an application. This instability has been associated with the extensive crosslinking between traditionally-used fluorescent ligands (presenting multiple low-affinity moieties) and ConA (presenting multiple binding sites) in free solution. The work herein introduces the design and synthesis of a new type of fluorescent ligand that can avoid this aggregation and allow the assay to be sensitive across the physiologically relevant glucose concentration range. This fluorescent ligand (APTS-MT) presents a single high-affinity trimannose moiety that is recognized by ConA’s full binding site and a fluorophore that can effectively track the ligand’s equilibrium binding via fluorescent anisotropy. This is confirmed by comparing its measured fluorescent lifetime to experimentally-determined rotational correlation lifetimes of the free and bound populations. Using an assay comprised of 200 nM APTS-MT and 1 µM ConA, the fluorescence anisotropy capably tracks the concentration of monosaccharides that are known to bind to ConA’s primary binding site, and the assay displays a MARD of 6.5% across physiologically relevant glucose concentrations. Ultimately, this rationally-designed fluorescent ligand can facilitate the realization of the full potential of ConA-based glucose sensing assays and provide the basis for a new set of competing ligands to be paired with ConA.

Introduction

Concanavalin A (ConA) is a tetrameric lectin at physiological pH (pH 7.4) with four identical and independent subunits that each have a single, primary binding site for glucose or mannose (Bittiger and Schnebli 1976). In 1982, Schulz et al. introduced a fluorescence-based glucose sensing strategy using
ConA as the receptor (Schultz et al., 1982). By pairing the lectin with a fluorescent ligand to which it would bind, they generated a homogeneous, competitive binding assay that was sensitive to physiologically relevant glucose concentrations. This non-consuming fluorescence assay has the potential to be used in continuous glucose monitoring devices to track the glucose concentrations of the interstitial fluid and offers advantages over traditional enzyme-based sensors when properly tuned (Pickup et al., 2005). This significant potential has led to many variations in the design of the fluorescent competing ligands (Ibey et al., 2005; Liao et al., 2008; Tolosa et al., 1997b), optical transduction mechanisms (Barone and Strano 2009; Cummins and Coté 2012; Tang et al., 2008), and encapsulation techniques used with this assay (Ballerstadt and Schultz 2000; Cummins et al., 2011; Russell et al., 1999). However, groups have continued to report aggregate formation caused by extensive crosslinking between the traditionally-used fluorescent ligands (presenting multiple low-affinity moieties) and ConA (presenting multiple binding sites), resulting in a lack of reversibility of the assay in free solution (Ehwald et al., 1996; McCartney et al., 2001; Tolosa et al., 1997a). This aggregation problem has limited the utility of ConA-based assays and has proved difficult to overcome with traditional approaches.

The use of fluorescence anisotropy as a tool to track the glucose-dependent equilibrium binding of a ConA-based competitive binding assay and its complete mathematical description was recently introduced by our group (Cummins et al., 2013b). Briefly, the fluorescence anisotropy of a sample describes the relationship between the average rate at which fluorescence occurs upon excitation (fluorescence lifetime) and the average rate at which the fluorescent molecules are tumbling (rotational correlation lifetime). It can be useful in competitive assay development because it only requires a single component to be fluorescently labeled and the anisotropy is independent of the intensity. Therefore, such an approach can avoid problems with spectral bleed-through and photo-bleaching, respectively. Ultimately, the sensitivity of a competitive binding assay that only allows the fluorescent ligand to be in two populations (bound and free) can be described using Equation 1.

$$\frac{\Delta<r>}{\Delta G} = (r_b - r_f) \frac{\Delta%CLB}{\Delta G}$$

Equation 1

In this equation $\Delta<r>/\Delta G$ term is the change in the measured anisotropy for a given change in the glucose concentration. The anisotropies of the bound and free fluorescent ligand are $r_b$ and $r_f$, respectively, and their difference is related to the transduction mechanism. The %CLB is the amount of the fluorescent competing ligand that is bound to ConA, which is equivalent to the fractional fluorescence intensity coming from the bound population if the quantum yield of the fluorescent ligand does not change upon binding to the receptor. The $(\Delta%CLB)/\Delta G$ term is the change in the percent competing ligand that is bound for a given change in glucose concentrations and is related to the recognition mechanism. Equation 2 shows that the recognition mechanism and the transduction mechanism must both be optimized to generate a sensitive assay. This work identified the assay characteristics that maximized the change in the measured anisotropy in response to a change in glucose concentration from 0 mg/dL to 300 mg/dL.
Our previous work used the exact solution to the governing competitive binding equations to identify the concentrations that maximize the recognition mechanism of the ConA competitive binding assay for a given fluorescent competing ligand (Cummins et al., 2013b). That model showed that the affinity of ConA to 4 kDa dextran ($K_a = 5,000 \text{ M}^{-1}$) requires a ConA concentration that is higher than its solubility limit to maximize the recognition mechanism of the assay. Therefore, this ligand does not allow the maximum change in equilibrium binding to occur over physiological glucose concentrations. To, instead, identify competing ligands that fully realize the sensitivity potential of the assay, the concentration of the competing ligand can be fixed (here, it is fixed at 200 nM) and the ConA concentration as well as the affinity to the competing ligand can be varied to generate a 2D sensitivity map for the recognition mechanism. Figure 1A displays the interplay between the competing ligand chosen and the ConA concentration required for physiological glucose concentrations to be recognized by the assay.

A similar sensitivity map can be generated for the transduction mechanism by modeling the expected change in the steady-state fluorescence anisotropy of fluorescent competing ligands upon their binding to ConA. By assuming that the free competing ligand and bound competing ligand are both spherical and rigid, the anisotropy can be predicted as a function of its fluorescent lifetime and molecular weight. The differences in these predicted anisotropy values are shown in Figure 1B as a function of these variables, where it is assumed that the intrinsic anisotropy ($r_0$) of the fluorophore is the theoretical maximum, 0.4. Lower intrinsic anisotropies would change the scale of the color map but display the same profile. Together, these 2D sensitivity maps identify the assay characteristics required for a ConA-based competitive binding assay to track physiological glucose concentrations.

Figure 1: 2D sensitivity maps of the (A) recognition mechanism and the (B) transduction mechanism, where the red regions display the highest sensitivity.
Figure 1A shows that the use of higher-affinity competing ligands requires lower ConA concentrations to achieve the maximum possible recognition sensitivity. In fact, the competing ligand’s affinity to ConA needs to be above ~20,000 M$^{-1}$ to allow the required ConA concentration to be under its solubility limit (~100 µM) and still achieve maximum sensitivity. Ideally, the affinity would be somewhere between 10$^5$ M$^{-1}$ to 10$^7$ M$^{-1}$. Because ConA’s affinity to glucose is only ~400 M$^{-1}$, groups have traditionally used fluorescent competing ligands that present many monosaccharides that can bind to the primary binding site of ConA to achieve the higher apparent affinities that are required for sensitive assay responses (Schultz et al., 1982). These multivalent competing ligands (dextrans, glycosylated dendrimers, etc) increase the apparent affinity to ConA by employing proximity effects. However, the pairing of multivalent ConA with multivalent competing ligands in these assays consistently leads to extensive aggregation and assay irreversibility in free solution (Cummins et al., 2013a; Dam and Brewer 2010; Gestwicki et al., 2002; Goldstein et al., 1965). This has been the primary barrier to the realization of a stable ConA-based glucose sensor in free solution. Using traditional approaches, there is a limiting tradeoff between needing to achieve higher affinities for the assay to display sensitivity and needing to avoid aggregation to maintain reversibility.

Even if aggregation was somehow avoided, the traditional type of fluorescent competing ligand is not ideal for the transduction mechanism of an anisotropy-based assay because higher MWs are typically required to achieve these higher-affinities. This is exemplified by dextran – the most commonly used competing ligand in these assays – which is a branching polymer built of glucose subunits. As the MW of dextran increases, it presents a higher number of termini that ConA can bind which increases the apparent affinity. One commonly used fluorescent ligand in ConA-based assays is 70 kDa FITC-dextran, but this ligand still only displays an apparent affinity of ~15,000 M$^{-1}$ to ConA (Schultz et al., 1982). According to the 2D sensitivity map for the transduction mechanism of a fluorescence anisotropy assay (Figure 1B), the competing ligand’s molecular weight needs to be closer to 1 kDa than 100 kDa. Ultimately, the ideal characteristics of the fluorescent ligand with regard to the transduction mechanism would be a MW of ~1 kDa and a fluorescence lifetime of ~5 ns.

Adjacent to ConA’s primary binding site is an extended pocket that facilitates the formation of additional hydrogen bonds to the core trimannose of N-linked glycans. Based on previously reported crystallographic data, Figure 2A shows a comparison of ConA’s binding to the monosaccharide, methyl-α-mannose, with its binding to trimannose (Naismith et al., 1994; Naismith and Field 1996). The binding site for glucose is expected to be the same as for methyl-α-mannose. These 2D projections show the amino acids that form hydrogen bonds with the respective sugars, demonstrating that the full binding site of ConA forms hydrogen bonds with each mannose group of trimannose, which should increase the affinity without leaving moieties for additional ConAs to bind. It also shows that trimannose could compete with the monosaccharide for binding to a ConA subunit because they both bind to the primary binding site.
Figure 2: (A) Comparison between carbohydrate binding to the primary binding site of ConA and the full binding site of ConA, using the crystal structures from (Naismith et al., 1994; Naismith and Field 1996). The black dots represent the amino acids capable of forming hydrogen bonds to the hydroxyl groups on the sugar (within 3.5 Å of each other). (B) Schematic of the new type of fluorescent ligand that displays (a) a single core trimannose that ConA can recognize and (b) a single fluorophore to transduce the equilibrium binding.

Herein, we describe a new type of fluorescent ligand that is rationally-designed to achieve the aforementioned, desirable characteristics for the ConA-based glucose sensing assay. Instead of only using the primary binding site of ConA, it is engineered to utilize the full binding site of ConA as depicted in Figure 2B. This rationally-designed fluorescent ligand presents a single high-affinity trimannose moiety to achieve the desirable affinity in a manner that avoids the aggregation that has plagued ConA-based assays. In addition, the fluorescent ligand is designed to have a molecular weight and fluorescent lifetime that can allow the sensitivity of the transduction mechanism to be maximized. Overall, this work has been performed in an attempt to realize the full potential of ConA-based glucose sensing assays by offering a new approach to the fluorescent ligand to be paired with ConA.

Materials and Methods

Materials

Mannotetraose and trimannose were purchased from V-Labs, Inc (Covington, LA). Methyl-α-mannose (MaM), and 2 MDa dextran were purchased from Sigma (St. Louis, MO). 8-Aminopyrene-1,3,6-trisulfonic acid, sodium salt (APTS) was provided by Dr. Gyula Vigh (TAMU). A generation-three glycosylated dendrimer (24 terminal amines, 24 terminal glucose residues) was used as provided by Dr. Eric Simanek (Texas Christian University, Fort Worth, TX). All other chemicals were purchased from Sigma and used as received. TRIS buffer was constituted of 10 mM TRIS, 1 M NaCl, 1 mM CaCl$_2$, 1 mM MnCl$_2$, at pH 7.4.
**Dynamic Light Scattering**

To explore whether the interaction of ConA with a single presentation of the core trimannose leads to aggregation in free solution, dynamic light scattering (DLS) measurements were performed. This interaction was compared to the interactions between ConA and other high-affinity ligands that are known to aggregate in ConA-based assays. Dextran, dendrimer and trimannose were added to separate cuvettes of ConA solutions and allowed to interact for 12 hours at 22 deg C. Unlabeled ligands were used to compare specific carbohydrate binding achieved by the different approaches. The final concentration was 3 µM for the competing ligand and 3 µM for ConA. Negative controls were run for each competing ligand (3 µM) without ConA present. ConA was also independently added to a separate control cuvette (3 µM). All solutions were made in TRIS buffer and filtered to remove any particulate material before combining the solutions. After 12 hours, the solutions were mixed with a pipette to allow for the re-suspension of any settled material and dynamic light scattering studies were performed. The reported data is the average particle size as determined by percent-volume.

**Synthesis and Purification**

To synthesize the rationally-designed fluorescent ligand, the following protocol was used. Mannotetraose (0.9 mg, 1.35x10⁻⁶ mol) was mixed with 13.5 µL of 1 M APTS (1.35x10⁻⁵ mol) prepared in 15% acetic acid aqueous solution. Then, 54µL of 15% acetic acid aqueous solution was added into the mixture. The acidic environment promotes the ring-opening of the reducing terminus of mannotetraose. The reaction mixture was stirred for 5 minutes at room temperature. Then, 13.5μL of 1M sodium cyanoborohydride (NaBH₃CN, 1.35x10⁻⁵ mol) in tetrahydrofuran was added into the reaction mixture and stirred for 14 hours at room temperature. Sodium cyanoborohydride is a reducing agent that converts the Schiff base into the stable APTS-mannotetraose (APTS-MT) conjugate. The schematic is shown in Figure 3A.

Following reductive amination, the crude product was purified by hydrophilic interaction liquid chromatography (HILIC). The chromatogram is shown in Figure 3B. The solid line shows the elution profile for the reaction mixture of APTS and mannotetraose after reductive amination. The peak labeled A is from unconjugated APTS. The peak labeled B is from the APTS-MT. Peak B was collected and the collected fraction was again analyzed on the column to show its purity. The dotted line is the elution profile of the purified conjugate. The inset shows the absorbance profiles of the unconjugated APTS and the conjugated APTS. These, along with the ESI-MS of the purified fraction were used to confirm conjugation.
**Fluorescent Characterization**

Steady state fluorescence and intrinsic anisotropy measurements were performed on a Fluorolog-3 from Horiba Jobin Yvon. Excitation and emission scans were performed to characterize the fluorescence of 100 nM APTS-MT in TRIS buffer. Intrinsic anisotropy was measured by adding APTS-MT in a 50% glycerol solution at a concentration of 100 nM and setting the temperature to 5 deg C. This effectively slowed the rotation of the fluorescent molecules in the excited state to negligible amounts, which insures that the steady-state anisotropy value is essentially the intrinsic anisotropy. Ten measurements were taken for each sample; the recorded anisotropy value is the average of those measurements.

The fluorescence lifetime and dynamic anisotropy data were collected using a DeltaFlex time correlated single photon counting system from Horiba that was equipped with a 482 nm DeltaDiode pulsed source. The effect that ConA binding has on the fluorescence lifetime and rotational correlation lifetime of APTS-MT was studied using a solution of 200 nM APTS-MT with and without 1 µM ConA present. The fluorescence intensity decays were analyzed with the Decay Analysis Software (v. 6.6) from Horiba, and fits were used according to the expected distribution in the solution. The dynamic anisotropy decays were analyzed by using a reconvolution algorithm to determine the best-fit rotational correlation lifetimes.

**Binding Studies**

Binding studies were carried out with a filter-based fluorescence microplate reader that was equipped with polarizers and the appropriate fluorescence filters for conjugated APTS. A microplate was loaded with serial dilutions of ConA and the same concentration of APTS-MT (200 nM). Solutions in the plate
were allowed to reach equilibrium at room temperature (22 deg C), and scans were performed in the perpendicular and parallel directions. Background values were subtracted from each measurement. The G-factor was determined by setting the fluorescence anisotropy of free APTS-trimannose at a value of 0.03, and this G-factor was used to calculate the anisotropy of each well.

Using the results from the modeled assay, the concentrations for the binding assay were chosen to be 200 nM APTS-MT and 1 µM unlabeled ConA. Following a similar strategy as the affinity-binding studies between APTS-MT and ConA, microplate wells were loaded with this assay for varying concentrations of methyl-α-mannose, glucose, and galactose from ~0.2 mg/dL to ~10,000 mg/dL. The assay was given an appropriate time to reach equilibrium at room temperature (22 deg C) and steady state anisotropy was measured using the filter-based fluorescence microplate reader. This assay configuration was exposed to additional glucose concentrations in the physiological range. To compare the assay’s performance with the ‘gold standard’, the actual glucose concentrations were determined on a YSI biochemistry analyzer. These anisotropy values were used to generate a calibration fit via the typical competitive binding equation, and this equation was used to predict the glucose concentrations.

**Results and Discussion**

**Dynamic Light Scattering**

The primary problem that has plagued ConA-based glucose sensing assays is the tradeoff between needing a high-affinity competing ligand for sensitivity and needing to avoid the extensive crosslinking between the high-affinity competing ligand and ConA in free solution. This aggregation changes the binding kinetics in an assay and results in a different response to physiologically relevant glucose concentrations over time. Figure 4A compares ConA’s interaction with traditional competing ligands that achieve the higher affinity by presenting many low-affinity moieties to ConA with the proposed competing ligand that presents a single high-affinity moiety. The particle-size data from the DLS measurements highlights the potential utility of trimannose to overcome the aforementioned tradeoff. It can be seen that the average size of the particles is much larger for the traditional ligands, indicating that ConA and these ligands have already formed extensive aggregates after only 12 hours. In comparison, the average size of particles in the solution that contains trimannose and ConA has not increased, indicating that, if binding occurred, trimannose did not lead to significant aggregation.

**Synthesis and Purification**

To achieve the ideal characteristics as identified by the 2D sensitivity maps, the goal was to engineer a fluorescent ligand in a manner that displayed a fully-preserved trimannose group and a single fluorophore. Therefore, the commonly used periodate oxidation method to generate carbonyl groups on a polysaccharide for fluorophore attachment was not appropriate (Bobbitt 1956). This would destroy the mannose moieties required for binding to ConA’s full binding site and could introduce several
fluorophores to a single glycan. Reductive amination, on the other hand, is a more controlled method that has been used to label glycans for subsequent chromatographic or electrophoretic separation and identification of the glycans (Bigge et al., 1995; Guttman et al., 1996; Harvey 2000; Sato et al., 1998). By using an amine-bearing fluorophore, this method introduces a single label at the reducing terminus of the glycan. This causes the reducing sugar of the glycan to be acyclic but leaves the remaining sugars of the glycan in their cyclic, unaltered form to be recognized by receptors (Leteux et al., 1998). Thus, the trimannose moiety-bearing mannotetraose was used to maintain the trimannose structure after conjugation. 8-Aminopyrene-1,3,6-trisulfonic acid (APTS) was chosen as the amine-bearing fluorophore to attach to mannotetraose. APTS is a water-soluble fluorophore whose fluorescence is independent of pH over a wide range. It has been used to label populations of glycans enzymatically cleaved from glycoproteins to facilitate their separation via capillary electrophoresis due to its three negative charges per fluorophore at neutral pH (Guttman et al., 1996). Because ConA’s isoelectric point is around 5, these negative charges also minimize non-specific electrostatic interactions between the fluorescent ligand and ConA at physiological pH.

![Figure 4](image-url)

**Figure 4:** (A) Average particle size in solutions of ConA and various ligands where the error bars indicate the standard deviation between different samples. (B) Fluorescence characterization of the purified APTS-MT in TRIS buffer.

**Fluorescent Characterization**

The APTS-MT fluorescent ligand that was synthesized is a stable conjugate that presents a single trimannose moiety and a single water-soluble fluorophore (MW = 1.1 kDa). Fluorescence characterization of the purified APTS-MT conjugate shows an excitation maximum at ~455 nm and an emission maximum at ~518 nm. This is a red-shift in the excitation and emission spectra compared to those of the unconjugated APTS. The fluorophore shows a relatively high Stokes shift of approximately 60 nm, which allows for a large band of fluorescence wavelengths to be collected to improve detection.
The intrinsic anisotropy of the APTS-MT conjugate is relatively constant near the excitation maximum with a value of ~0.30. The maximum possible intrinsic anisotropy is 0.4, and occurs only when the excitation and emission dipoles are in the exact orientation. For comparison, fluorescein’s intrinsic anisotropy is ~0.37 (Jameson and Ross 2010).

The fluorescence lifetime decay of 200 nM APTS-MT displayed a single exponential decay with a lifetime of ~5.3 ns and a $\chi^2$ value of 1.29. To determine the effect of ConA binding on the fluorescence lifetime of APTS-MT, the fluorescence lifetime decay of 200 nM APTS-MT in the presence of 1 µM ConA was measured. This displayed a single exponential decay with a lifetime of ~5.4 ns and a $\chi^2$ value of 1.38. Since the addition of 1 µM ConA should result in a majority of the APTS-MT being bound to ConA, the results indicate that the lifetime does not significantly change upon binding to ConA. Therefore, the assumption that the percent of the fluorescent competing ligand bound is equal to the fluorescence intensity from the bound population holds true. Compared with the 2D sensitivity map of the transduction mechanism in Figure 1B, the combination of the experimentally determined fluorescent lifetime (5.3 ns) and molecular weight (1.1 kDa) is well-suited to track the ConA binding.

This change in anisotropy could potentially be increased even further by decreasing the molecular weight of the fluorescent ligand or using a fluorophore with a higher intrinsic anisotropy. However, decreasing the molecular weight has practical limitations. The fluorescent competing ligand must have a specific carbohydrate moiety for ConA to bind and a fluorophore to transduce the binding events. An alternative way to increase the sensitivity of the transduction mechanism would be to increase the molecular weight of the receptor and use a longer-lifetime fluorophore on the competing ligand. Nevertheless, APTS-MT is a significant improvement over previous fluorescent competing ligands and can be considered an appropriate fluorescent ligand to maximize the sensitivity of a fluorescence anisotropy glucose sensing assay based on native ConA.

Dynamic anisotropy measurements were performed to determine the actual rate of tumbling for the free APTS-MT and the bound APTS-MT. The reconvolution algorithm for the dynamic anisotropy was used to fit the rotational correlation lifetime and the relative amounts in each population. Without ConA present, the fit showed a single rotational correlation lifetime of ~0.6 ns. This is expected to be solely due to APTS-MT. The fit for the solution with 1 µM ConA present showed two rotational correlation lifetimes (0.6 ns and 20 ns) and relative populations that agree with the steady-state titration studies in Figure 5A. This indicates that the rotational correlation lifetime of the bound APTS-MT is 20 ns. The modeled anisotropy of the free and the bound APTS-MT were 0.5 ns and 48 ns. The disparity between the modeled and experimental values for the bound APTS-MT can be explained by the slight motion between APTS-MT and ConA and the non-spherical shape of the bound complex.

These experimentally-determined rotational correlation lifetimes can be used to further validate the suitability of the experimentally-determined fluorescence lifetime of APTS-MT to the actual change in tumbling upon binding. The steady-state anisotropy for each rotational correlation lifetime was predicted for the full range of possible fluorescence lifetimes. The difference between these two curves is the expected change in steady-state anisotropy upon binding for any given fluorescence lifetime. Using an intrinsic anisotropy of 0.3, the maximum possible change upon binding was 0.19, requiring a
fluorescence lifetime of 4.5 ns. The fluorescence lifetimes that displayed greater than 99% of this maximum possible change ranged from 3.5 ns to 5.7 ns. Therefore, the fluorescence lifetime of APTS-MT (5.3 ns) is shown to be ideal to track this binding event and optimize the transduction mechanism of the fluorescence assay.

**Binding Studies**

Fluorescence anisotropy was used to determine the binding affinity between APTS-MT and native ConA in TRIS buffer at 22 deg C. The higher ConA concentrations lead to a greater percentage of APTS-MT bound to ConA. This population should tumble at a slower rate, and higher percentages of bound APTS-MT should increase the overall steady-state anisotropy from the solution. Assuming the change in fluorescence lifetime upon binding to ConA to be negligible, the ConA-dependent anisotropy was calculated and shown in Figure 5A. These results were fit with a Boltzmann curve to determine the association constant to be $5.61 \times 10^6$ M$^{-1}$. This value is significantly higher than what is seen for monosaccharides, like glucose, that only exploit the primary binding site ($400$ M$^{-1}$). Compared with the 2D sensitivity map of the recognition mechanism in Figure 1A, this affinity would allow the maximum sensitivity of the assay to be reached at ConA concentrations that are well below its solubility limit.

![Figure 5](image-url)  
*Figure 5: (A) Steady-state anisotropy of 200 nM APTS-MT with increasing ConA concentrations on a semi-log plot and the calibration fit that shows an affinity of $5.61 \times 10^6$ M$^{-1}$. (B) Steady-state anisotropy of an assay containing 200 nM-MT and 1 µM ConA for a range of concentrations of various monosaccharides on a semi-log plot using the corresponding calibration fits.*
To test APTS-MT in the ConA-based assay, the assay concentrations were chosen to be 200 nM APTS-MT and 1 µM ConA according to the map in Figure 1A. A range of concentrations of various monosaccharides were added to microplate wells with these final assay concentrations, and sufficient time was given to reach equilibrium prior to fluorescence anisotropy measurements. The assay reached steady state in less than one minute. The fluorescence anisotropy responses are shown in the semi-log plot of Figure 5B. It is important to note that each data point is an independent assay in a unique microplate well. The anisotropy responses show that the binding of the APTS-MT to ConA is effectively inhibited by monosaccharides that are known to only bind to the primary binding site of ConA (methyl-α-mannose and glucose). In addition, high levels of the monosaccharides allow for the full reversal of the equilibrium binding, as indicated by anisotropy values that are equal to those if ConA wasn’t present (Figure 5A). Methyl-α-mannose is known to have a binding affinity that is ~20-40 times higher than glucose, and galactose shows no affinity to ConA (Mandal et al., 1994). As a result, the relative responses to the monosaccharides were expected as described by the Cheng-Prusoff equation for competitive binding (Cheng and Prusoff 1973). This set of results is highly encouraging and suggests that the APTS-MT that binds to ConA’s full binding site is truly undergoing competitive binding with a monosaccharide that binds to the primary binding site alone.

![Figure 6](image)

**Figure 6**: (A) Steady-state anisotropy of the 200 nM APTS-MT and 1 µM ConA assay to physiologically relevant glucose concentrations shown on a linear plot. (B) The predicted glucose vs. actual glucose from the best fit of the 200 nM APTS-MT and 1 µM ConA assay.
The assay was exposed to additional, physiologically-relevant glucose concentrations and displayed on the linear plot in Figure 6A. This shows that the majority of the response occurs over the physiologically relevant glucose concentration range and is a direct result of the assay being designed to maximize the recognition mechanism. While this response is fairly linear, a traditional competitive binding equation was used to fit the data that can be used to predict the glucose concentrations from the measured fluorescence anisotropy.

The glucose concentrations were predicted with this fit from the anisotropy data and compared to the actual glucose data in Figure 6B. This shows a standard error of calibration of 8.5 mg/dL and a mean absolute relative difference (MARD) of 6.5% across the physiologically relevant glucose concentrations. In comparison to the predicted vs. actual glucose concentration plot of the assay based on the 4 kDa FITC-dextran, the points using APTS-MT are much closer to the line depicting an ideal sensing assay. The slight fluctuations are expected to be caused by pipetting error rather than error with the assay.

Conclusions

In summary, a rationally-designed fluorescent ligand was introduced for use in a ConA-based glucose sensing assay that used a new approach intended to avoid the aggregation problems that have traditionally plagued such assays. By engineering the ligand to present a single high-affinity trimannose moiety that binds to the full binding site of ConA, the required high affinity can be reached without requiring multiple moieties that can lead to crosslinking with ConA. A desirable fluorophore was introduced in a manner that left the entire trimannose moiety unaltered, and the glucose-dependent equilibrium binding of the rationally-designed fluorescent ligand was effectively tracked via fluorescence anisotropy. This new approach to the design of the fluorescent ligand can potentially serve as a solution to the long-standing problems of ConA-based assays, and variants of this fluorescent ligand can be engineered for use in continuous glucose monitoring devices.

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