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FRET-Based Luminescence Sensors for Carbohydrates and Glycoproteins Analysis

Zeev Rosenzweig*, Nitsa Rosenzweig and Gabriela Blagoi
Department of Chemistry and the Advanced Material Research institute (AMRI), University of New Orleans, New Orleans, LA 70148
e-mail of corresponding author Zeev Rosenzweig: zrosenzw@uno.edu

ABSTRACT

This paper describes the development of novel particle-based fluorescence resonance energy transfer (FRET) biosensors. It describes the fundamentals of FRET in heterogeneous systems and the application of the new sensors in monitoring the binding affinity of carbohydrates and glycoproteins to lectins, which are carbohydrate binding proteins. The sensing approach is based on FRET between fluorescein (donor) labeled lectin molecules, adsorbed on the surface of micrometric polymeric beads, and polymeric dextran molecules labeled with Texas Red (acceptor). The FRET signal of the sensor decreases in the presence of carbohydrates or glycoproteins that inhibit the binding of Texas Red-labeled dextran molecules to the lectinic binding sites. The new FRET sensors could discriminate between carbohydrates and glycoproteins based on their binding affinity to the FRET sensing particles. Thery were also used for quantitative analysis of carbohydrates and glycoproteins in aqueous samples.

Keywords – Fluorescence resonance energy transfer, biosensors, carbohydrates, glycoproteins

1. INTRODUCTION

Biosensors have emerged in the last two decades as useful tools for the analysis of biological samples. Glucose sensing in vivo and in vitro has been the most impressive success story of biosensor technology (1,2). Luminescence biosensors demonstrate significant advantages over commonly used electrochemical sensing techniques since they do not consume their targeted analyte. Most currently used luminescence sensors are based on a direct interaction of the sensing molecules with their targeted analyte. This approach is restricted to a limited number of analytes that include pH, ions and molecular oxygen. The incorporation of enzymes, antibodies and cells for analyte recognition has largely expanded the scope of analytes that could be determined with fluorescence based sensors. For example, luminescence biosensors were used to detect and quantify DNA-carcinogen adducts (3), organomercury compounds (4), tributyltin using recombinant bioluminescent Escherichia coli strains (5), heavy metals (6,7), and β-lactams (8). Nevertheless, there is still a need to develop new signal transduction mechanisms that could be coupled to biorecognition components like enzymes and antibodies in miniaturized biosensors.

Glyconjugates have been the target of sensor development since they play a major role in many biological processes (9,10). For example, abnormalities in glycoconjugate content and level are indicative of pathological conditions (11). Fluorescence sensors were developed to measure the level of carbohydrates in solution (12). These sensors show significant advantages over other measurement techniques due to the inherently high sensitivity of fluorescence techniques and the simple instrumentation needed for analysis. Several research groups developed and employed fluorescence resonance energy transfer (FRET) based sensors for carbohydrate detection (13-15). FRET sensors were also developed and employed to study protein-protein interactions and protein folding (16, 17), and for DNA and RNA hybridization studies. Recent work in this area focused on the optimization of the donor and acceptor pairs in order to reduce false-positive signals (18, 19). Another interesting direction has been the utilization of lanthanide atoms as donors in FRET sensors for DNA and RNA analysis (20).

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Since the FRET efficiency depends largely on the distance between a donor and acceptor molecules, FRET based sensors provide valuable information in systems involving ligand-receptor binding. The strength and kinetics of ligand binding to receptors localized on the sensor surface can be measured directly from the FRET efficiency. This paper focuses on the fundamentals of FRET and summarizes recent studies in our laboratory in which FRET-based microsensors were used to monitor binding interactions between carbohydrate, glycoproteins and particles labeled with carbohydrate binding protein molecules.

2. FUNDAMENTALS OF FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

Fluorescence resonance energy transfer (FRET) is an energy transfer from the excited state of a fluorescent donor (D) to an acceptor (A). The energy transfer does not involve the emission and re-absorption of photons. FRET occurs when the emission spectrum of the fluorescent donor overlaps with the absorption spectrum of an acceptor (figure 1). When the donor molecules are excited in the presence of acceptor molecules the donor fluorescence intensity decreases and the acceptor fluorescence increases (21). FRET only occurs over distances up to 100Å (22). The donor and acceptor molecules must be separated to prevent interactions between their electronic clouds that could alter their electronic spectra.

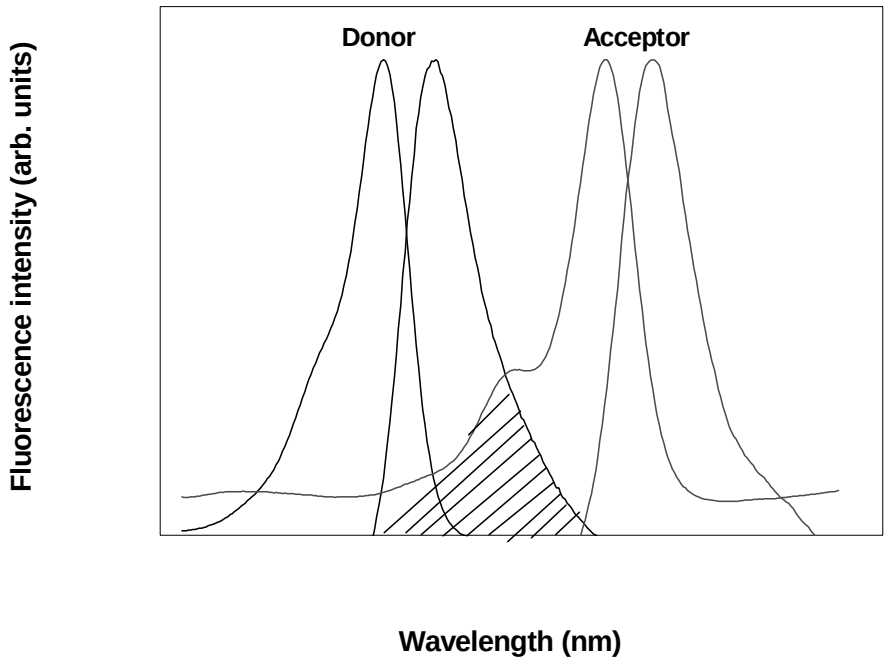

Figure 1. A spectral overlap (marked with diagonal lines) between the donor emission and the acceptor absorption is necessary for fluorescence resonance energy transfer (FRET).

The rate constant of FRET is derived from the Forester inductive-resonance theory (21). It is expressed as:

$$k_t = \frac{9000(\ln 10)Q_D K^2}{128\pi^5 N n^4 \tau_D R^6} \int_0^\infty F_D(\nu)\varepsilon_a(\nu)\frac{d\nu}{\nu^4} \quad (1)$$

where $\mathbf{K^2}$ is an orientation factor, $\mathbf{F_D(\nu)}$ is the spectral distribution of the donor fluorescence (normalized to the wave numbers), $\boldsymbol{\varepsilon_a(\nu)}$ is the extinction molar coefficient of the acceptor, **N** is Avogadro's number, **n** is the refractive index of the solvent, $\boldsymbol{\tau_D}$ is the intrinsic lifetime of the donor in the absence of quenchers, $\mathbf{Q_D}$ is the quantum yield of the donor in the presence of the acceptor is the and **R** is the distance between the donor and acceptor molecules.

The orientation factor $\mathbf{K^2}$ is calculated from the relative orientation of the emission transition dipole of the donor and the absorption transition dipole of the acceptor. Values of $\mathbf{K^2}$ range between 0 for perpendicular dipole moments and 4 for parallel dipole moments. For randomly oriented donors and acceptors $\mathbf{K^2}$ is equal to 2/3. The effect of specific orientation geometries of the donor and acceptor dipoles on the FRET efficiency has been studied extensively (21-24). Considering the effect of the orientation factor ($\mathbf{K^2}$) on the FRET efficiency is needed when the interacting molecules are well defined geometrically, like when the donor and acceptor molecules are attached to specific amino acids in a protein. In most applications of FRET, including in our experiments, the procedure to label biomolecules with donor and acceptor molecules introduces significant heterogeneity. Under these conditions an average value of 2/3 is used to describe $\mathbf{K^2}$ (25).

Equation (1) was simplified by Forster to take the following reduced form:

$$k_t = \tau_D^{-1} \left(\frac{R_0}{R} \right)^6 \quad (2)$$

Where $\boldsymbol{\tau_D}$ is the lifetime of the donor in the absence of the acceptor, $\mathbf{R_0}$ is the critical radius of the transfer or the Forster distance, which is the distance at which the energy transfer efficiency is 50%. As can be seen from equation (2) the rate of energy transfer is highly dependent on the sixth power of the distance between donor and acceptor. For a donor and acceptor pair that is covalently bound, the energy transfer efficiency, **E**, is expressed as:

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad (3)$$

It can be seen that the FRET efficiency depends on the 6th power of the distance between the donor and acceptor molecules. The decrease in donor fluorescence due to FRET also depends on the dimensionality of the acceptor distribution (21). Maximal FRET efficiency is obtained when the donor and acceptor are bound to each other through a linker.

In our laboratory we synthesized FRET sensing particles and used them to detect carbohydrates and glycoproteins in solutions. The results of this study are summarized in the following sections.

3. FRET-BASED BIOSENSORS FOR CARBOHYDRATES AND GLYCOPROTEINS

Sensitive and selective FRET based sensors for mono-carbohydrate quantification have been recently developed. Specific lectins were used as biorecognition elements in these sensors to provide the specificity for carbohydrates (1, 14). These sensors were applied for high-throughput drug screening (26). In developing our FRET sensors we focused on minimizing the analyte volume, time of analysis and interferences while maximizing the FRET efficiency. The donor fluorophores were immobilized to micrometric-sized particles along with Concanavalin A (ConA). ConA is a mannose and glucose biding protein. It was used as the biorecognition component in our biosensors (27). The Con A was labeled with the donor fluorophore Fluorescein isothiocyanate (FITC). FRET signal was obtained when Texas Red labeled dextran (dextran-TR) bound to the particles.

3.1. Synthesis of FITC-ConA coated particles

FITC-ConA coated particles were synthesized by suspending 1.6-μm polystyrene particles (40 mg/mL, 10^7 particles/ml) in a 200μg/ml FITC-Con A solution (in HEPES 100mM, 150 mM NaCl, with 1mM Ca^{2+} and Mn^{2+}, pH 7.2) for 3 hours at room temperature. The excess protein and uncoated particles were separated from coated particles by repeated cycles of precipitation by slow speed centrifugation (3000rpm for 15 min) and

resuspension in a phosphate buffer solution at pH 7.2. Then, for imaging the FITC-ConA coated particles were deposited on the surface of microscope glass slides coated with 1% poly-L-lysine.

3.2. Choice of the donor and acceptor pair

The optical signal resulted from FRET between the FITC (donor) labeled ConA and the Texas Red (acceptor) labeled dextran (dextran-TR). We found that the signal to noise ratio of our FRET sensing particles was higher than the signal noise ratio obtained when Rhodamine molecules were used as acceptor molecules rather than Texas Red. This was explained by minimizing direct excitation of acceptor molecules by the excitation light when Texas Red was used as an acceptor (28). To simplify the sensor fabrication, we adsorbed fluorescein labeled ConA directly on the polymeric particles surface. Our carbohydrate and glycoprotein assays were based on their inhibitory effect on the binding of dextran-TR to the FITC-ConA labeled particles. The efficiency of FRET between FITC-ConA coated particles and dextran-TR was measured using an inverted fluorescence microscope in the absence and presence of carbohydrates and glycoproteins.

3.3. Binding of dextran-TR to the FITC-ConA coated particles

Fluorescence images of the FITC-ConA sensing particles prior and following binding of dextran-TR to the particles are shown in figure 2. We found that dextran-TR bound effectively to the FITC-ConA labeled particles. Using 0.12 µM Dextran-TR (MW=10000) the FITC-donor label fluorescence decreased by ~40% due to FRET between the bound dextran-TR and the FITC-ConA coated particles (figure 2-A). The acceptor fluorescence increased by the same order of magnitude as the donor fluorescence was quenched by FRET. As shown in figure 2-B, the particles emit green light when imaged through a donor+acceptor filter cube (λ_{ex}=470nm, λ_{em}>515 nm). Utilizing the same microscope channel, the particles emitted orange light following dextran-TR binding to the particles (figure 2-C). The characteristic fluorescence of FITC (green) and Texas Red (red) reached the CCD detector to give an orange color. Figure 2-D shows the fluorescence of the same particles following binding to dextran-TR taken through the FRET channel (λ_{ex} =470 nm, λ_{em}>590nm). The red emitted light was a clear indication that dextran-TR bounded to the particles. In later experiments carbohydrates and glycoproteins were used as inhibitors of dextran-TR binding to FITC-ConA coated particles.

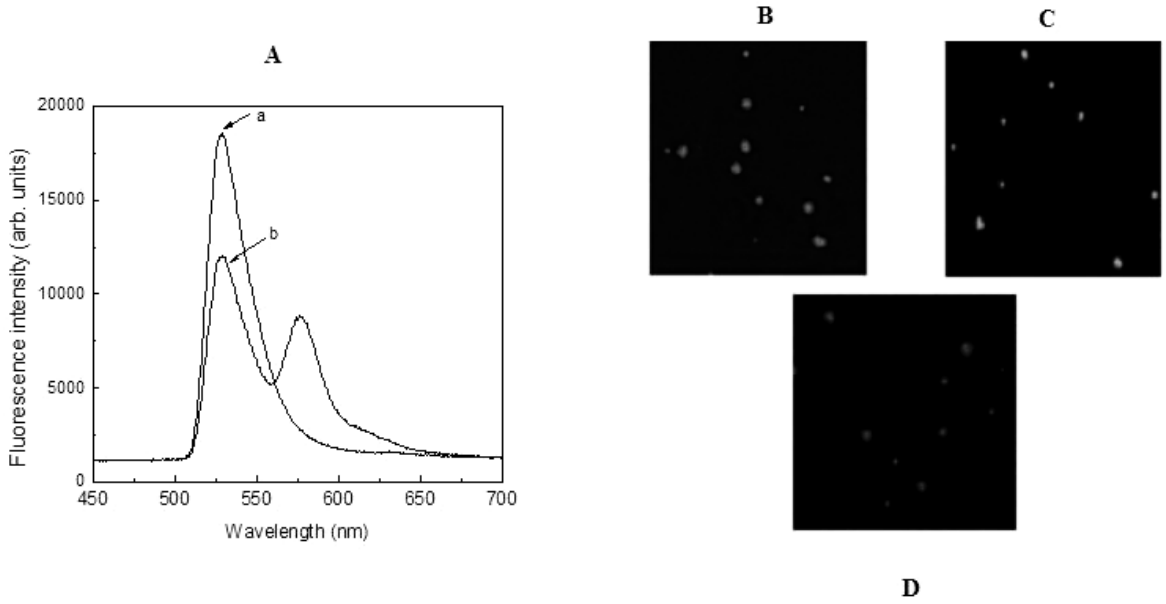

Figure 2. A) Digital fluorescence spectra of FRET sensing particles (λ_{ex}=470nm, λ_{em}>520nm) in a) the absence of Dextran-TR, b) 15 minutes following the addition of 0.12 µM Dextran-TR, B) A digital fluorescence image of FITC-ConA coated particles through the donor+acceptor channel (λ_{ex}=470nm, λ_{em}>520nm) C) A digital fluorescence image of particles through

the donor+acceptor channel (λ_{ex}=470nm, λ_{em}>520nm) when dextran-TR bound to the FITC-ConA. D) A digital fluorescence image of the particles through the FRET channel (λ_{ex}=470nm, λ_{em}>590nm) when dextran-TR bound to the FRET sensing particles.

The dextran-TR binding efficiency to the FITC-ConA coated particles was measured as the donor quenching efficiency. The fluorescence intensity of the particles (F_d) following the addition of dextran-TR was measured through a donor cube (λ_{ex}=470nm, 520nm<λ_{em}<560nm) and was normalized to the initial donor fluorescence (F_{d0}). The same sample analysis procedure was followed when glycoproteins or monomeric carbohydrates were used as inhibitors. The temporal dependence of the fluorescence spectra of the FRET sensing particles is shown in figure 3 for 0.12 µM dextran-TR (λ_{ex} = 470 nm). F_{d0} represents the fluorescence of FITC-ConA coated particles prior to dextran-TR addition. F_d is the fluorescence of the particles in the presence of dextran-TR. As seen in figure 3, dextran-TR bound to the FITC-ConA labeled particles and the ConA-dextran recognition reaction reached equilibrium in less than 15 minutes.

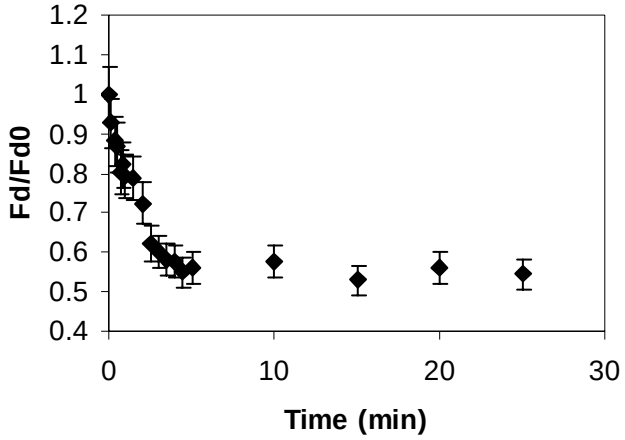

Figure 3. Dynamic fluorescence response of FITC-ConA coated particles to 0.12µM dextran-TR. The fluorescence intensity of the particles (F_d) was measured through donor cube (λ_{ex}=470nm, 520nm<λ_{em}<560nm) and was normalized to the initial donor fluorescence (F_{d0}).

3.4. FRET Inhibition Assays

The FRET sensing particles were used to quantify the inhibition efficiency of several carbohydrates and glycoproteins based on their affinity to the ConA absorbed on the particles. Mannose, Galactose, Ovalbumin and Glucose Oxidase were allowed to compete against dextran-TR for the ConA binding sites on the surface of the particles. The FITC-ConA labeled particles were incubated for one hour with different inhibitor concentrations prior to adding dextran-TR (0.12 µM Dextran-TR, MW=10 000) to the analyte solution. Figures 4 A-C show the binding isotherms for Mannose, Ovalbumin and Glucose Oxidase, that were used as model inhibitors. The inhibition percentage was calculated as follows:

$$\text{Inhibition\%} = ((F_i - F_{ni}) / \Delta F) \times 100 \quad (4)$$

Inhibition% is the percent inhibition of the dextran-TR binding to the FITC-ConA coated particles by the screened substances. $\mathbf{F_i}$ is the fluorescence intensity of the particles 15 minutes following the addition of dextran-TR in the presence of inhibitor, and $\mathbf{F_{ni}}$ is the fluorescence intensity of the particles 15 minutes following the addition of dextran-TR in the absence of inhibitor. ΔF is defined as:

$$\Delta F = F_{Inh100\%} - F_{ni} \quad (5)$$

Where, $\mathbf{F_{Inh100\%}}$ is the fluorescence intensity of the particles at 100% inhibition. Mannose displaced dextran-TR from the FITC-ConA coated particles at the milimolar concentration (figure 4 A), while Ovalbumin a glycoprotein that shows a larger number of mannose and glucose residues proved to be a better inhibitor in the micromolar range (figure 4B). Galactose was found to be an infective FRET inhibitor (results not shown).

Glucose Oxidase, which contains a large number of mannose residues reduced more effectively the FRET efficiency between the FITC labeled particles and dextran-TR (figure 4 C). In the case of Glucose Oxidase, low micromolar concentration was sufficient to largely inhibit the binding of dextran-TR to the FITC-ConA coated particles (figure 4 C). Under our experimental conditions we could measure limits of detection from ~5mM Mannose to ~100nM Glucose Oxidase. This detection levels are comparable with recently developed competitive binding assays (1,2,13,14). The limit of detection depends mainly on the strength of interaction between the inhibitor and lectinic protein absorbed on the sensing particles. The large number of glucose and mannose residues in glycoproteins enhances the binding affinity to the FRET sensing particles through multivalent interactions. More importantly, our assay shows great specificity towards carbohystrates and glycoproteins due to the use of highly selective biorecognition components in ythe sensor's design.

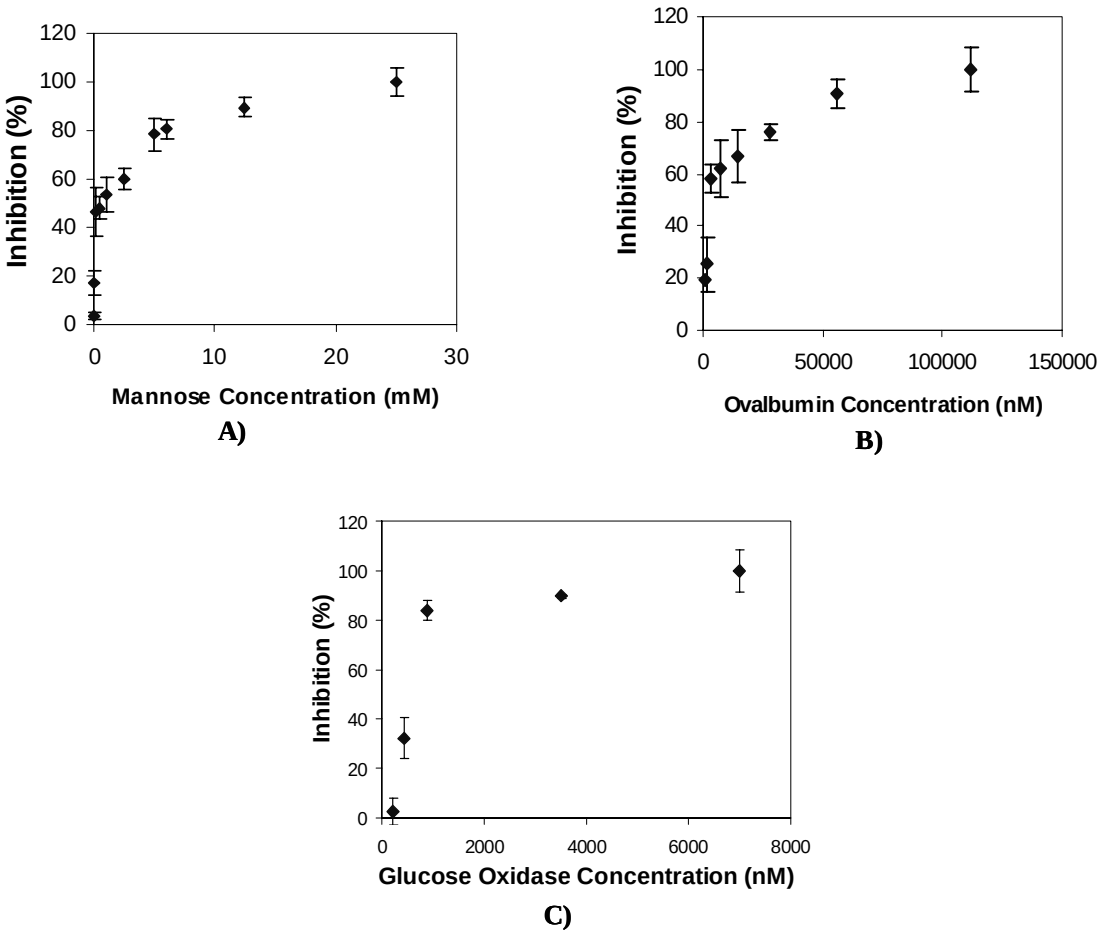

Figure 4. The inhibition of dextran-TR binding to FITC-ConA labeled particles by: a) Mannose b) Ovalbumin and d) Glucose Oxidase. The fluorescence intensity of the FITC-ConA coated particles was measured through donor cube (λ_{ex}=470nm, 520nm<λ_{em}<560nm) in the presence of different concentrations of inhibitors before and after the addition of dextran-TR.

4. SUMMARY AND CONCLUSIONS

FRET based luminescence biosensors are unique since they combine the sensitivity and selectivity of luminescence with the strong dependence of FRET on the distance between the donor and acceptor molecules. The newly developed FITC-ConA coated particles were able to discriminate between monomeric carbohydrates and glycoproteins due to their unique inhibitory effect on the FRET efficiency between Dextran-TR and the particles. The FITC-ConA sensing particles distinguished between monomeric carbohydrates like Mannose and Galactose. A millimolar level of mannose was required to effectively inhibit the FRET between dextran-TR and the FITC-ConA labeled particles. It was also possible to differentiate glycoproteins with a different number of glycosilic residues. The limit of detection for each carbohydrate or glycoprotein was found to depend mainly on the strength of the interaction between the carbohydrate or glycoprotein analyte and ConA, the recognition element of the sensor. The development of particle-based FRET sensors in this study provided a novel way to detect analytes previously inaccessible for biosensor technology. Our FITC-ConA coated could be used in the future as the building blocks of FRET sensing arrays for high throughput screening of carbohydrate and glycoprotein based drugs. However, several problems still limit the development of FRET sensing arrays. These problems include instabilities due to high photobleaching rates of sensing fluorophores and leakage of fluorophores from the sensing support. Other environmental parameters such as pH, polarity and temperature also affect the analytical properties of fluorescence sensors. Future studies in our laboratory will address these stability issues by replacing the conventionally used fluorophores and fluorescent heavy metals complexes with luminescent nanoparticles capable of fluorescence resonance energy transfer.

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