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Toward Photostable Multiplex Analyte Detection on a Single Mode Planar Optical Waveguide

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ABSTRACT: We have developed a waveguide-based optical biosensor for the sensitive and specific detection of biomarkers associated with disease. Our technology combines the superior optical properties of single-mode planar waveguides, the robust nature of functionalized self-assembled monolayer sensing films and the specificity of fluorescence sandwich immunoassays to detect biomarkers in complex biological samples such as serum, urine and sputum. We have previously reported the adaptation of our technology to the detection of biomarkers associated with breast cancer and anthrax. However, these approaches primarily used phospholipid bilayers as the functional film and organic dyes (ex: AlexaFluors) as the fluorescence reporter. Organic dyes are easily photodegraded and are not amenable to multiplexing because of their narrow Stokes' shift. Here we have developed strategies for conjugation of the detector antibodies with quantum dots for use in a multiplex detection platform. We have previously evaluated dihydroxylipoic acid quantum dots for the detection of a breast cancer biomarker. In this manuscript, we investigate the detection of the *Bacillus anthracis* protective antigen using antibodies conjugated with polymer-coated quantum dots. Kinetics of binding on the waveguide-based biosensor are reported. We compare the sensitivity of quantum dot labeled antibodies to those labeled with AlexaFluor and demonstrate the photostability of the former in our assay platform. In addition, we compare sulfydryl labeling of the antibody in the hinge region to that of nonspecific amine labeling. This is but the first step in developing a multiplex assay for such biomarkers on our waveguide platform.

KEYWORDS: anthrax, quantum dots, waveguides, multiplex, biosensor

1. INTRODUCTION: Anthrax is a deadly acute disease caused by *Bacillus anthracis*, an encapsulated spore-forming gram-positive bacterium. The bacterium can persist in the environment for elongated periods of time and hence, eradication of the disease is difficult. The disease affects both humans and animals. Humans can become infected when exposed to the bacteria by one of three routes: inhalational, cutaneous or systemic. Of these, inhalational anthrax, caused by breathing in the spores, is the most deadly form of the disease. The infectivity of the bacterium, combined with its persistence as a spore, has resulted in devolopment of anthrax as a bioweapon. The 2001 mailing and subsequent spread of *Bacillus anthracis* spores in the US, resulted in five deaths and left 17 individuals infected with the disease (Basil you might check these numbers, make sure its not just the Hart building targeted that gave these numbers)) Unfortunately, anthrax was confirmed in only one of the five individuals before death, suggesting

that current methods of detection and diagnosis of the disease are not rapid enough to detect bioagents and initiate effective therapy.

The major virulence factor of *Bacillus anthracis* is the anthrax toxin. The toxin causes septecemia and death in the infected host. The toxin is comprised of three major proteins, namely protective antigen (PA), lethal factor (LF) and edema factor (EF). Binding of PA to receptors on the host macrophages triggers an endocytotic event that results in the internalization of EF and LF into the host cell. Once internalized, LF, a matrix metalloproteinase disrupts cellular signal cascades. EF, on the other hand, is a calmodulin activated adenylate cyclase that causes an increase in intracellular cyclic AMP and hence, edema. The disruption of signal transduction cascades results in septic shock and cell death.

((Basil I don't know if this isa true statement (i.e. think of Ezzell), plus this paragraph doesn't really add much))The biosensor team at the Los Alamos National Laboratory has developed a waveguide-based biosensor for the sensitive, specific and rapid detection of biomarkers associated with disease. Our technology uses functionalized planar optical waveguides for biosensing. The waveguide is functionalized either with phospholipids bilayers (Martinez, JS et al, 2005, Mukundan H et al Bioconjugate chemistry, 2009) or self-assembled monolayers (SAMs, Anderson AS et al, Langmuir, 2008) and assembled in a flow cell. Coupling of light into gratings etched on the waveguide allows for its propagation by total internal reflection. Much of the light stays within the guided mode. However, a small portion, the evanescent field, leaks out. This field is extremely bright at the surface of the waveguide and is completely eliminated at a distance of 200-400 nm from the surface. The decay of the evanescent field allows for a sensitive biodetechtion within the evanescent field, and little background fluorescence further from the functionalized waveguide, minimizing nonspecific binding in complex biological samples (eg. serum and urine). Further reduction of nonspecific binding comes from the use of SAMs as the functional surface. These surfaces, also developed in our team, are robust, stable for long time periods, can be washed with detergents and are associated with lower non-specific binding when used with complex samples (Anderson AS et al, 2008).

Our first demonstration of the feasibility of this platform for bioassays was, in fact, an assay for the anthrax PA (Martinez JS et al, 2005). Although a sensitive assay for the antigen was developed (limit of detection of 1 pM in complex fluids), the assay used organic dyes (Alexafluors) as the fluorescence reporter. Unfortunatley, organic

fluorophores are easily photobleached and multiple organic dyes can not be excited by the same excitation source, thus limiting them for simple multiplex excitation.

Quantum dots (QDs) are fluorescent semiconductor nanocrystals that have been extensively used for bio-applications (ref). They are intrinsically more photostable than organic dyes (ref), their fluorescence emission wavelength can be tuned. QDs have broad Stokes shifts, which allows the simultaneous excitation of multiple QDs at a single waveglength. This factor makes them extremely exciting candidates for use in multiplex detection platforms. Although QDs have been extensively used in bio-imaging, immunohistochemistry and other bio-applications, their use in immunoassays is rather limited (Goldman et al). We have previously conjugated dihydroxylipoic acid QDs to antibodies and evaluated them in the biodeteciton of carcinoembryonic antigen, a breast cancer biomarker (Mukundan H et al, Bioconjugate Chem 2009). However, the dihydroxylipoic acid QDs have low quantum efficiency and hence, the resolution of the assay was poorer than that obtained with organic dyes. In this manuscript, we present for the first time, the conjugation of antibodies for the anthrax protective antigen with polymer encapsulated QDs and their successful evaluation in a sandwich immunoassay on our waveguide-based biosensor. In addition to the traditional amine labeling, we also evaluated the labeling of antibodies with QDs in the hinge region (thiol labeling)., a strategy wepreviously evaluated with organic dyes for superior signal amplification (Mukundan H et al, 2009). Finally, we have explored the kinetics of antibody-QD binding to PA. We also report a concentration response and the limit of detection of our assay in this format.

This is but the first step in the development of a multiplex assay for anthrax toxins. We are in the process of evaluating a sandwich assay for lethal factor using an antibody with a different QD. Initial evaluations are promising and we hope to transition these assays to a multiplex format for the simultaneous detection of these antigens.

2. MATERIALS AND METHODS

Materials: Purified antibodies that bind orthogonal epitopes of the anthrax PA (anti-PA antibody BAP106 for capture and BAP105 as reporter) were obtained from QED Bioscience Inc. Both are mouse monoclonal IgG1 antibodies (human adsorbed). We have previously documented the ability of these antibodies to form a effective sandwich by binding orthogonal epitopes of PA (Martinez JS et al, 2005, Anderson AS et al, 2008). Amino-functionalized QD (655 nm, QD655), Qdot®655 ITK™ carboxyl quantum dots and AF647-*N*-hydroxysuccinimide (NHS) ester labeling kits were purchased from Invitrogen. Biotinylation, protein estimation and electrophoresis reagents were obtained from Pierce.

Tri-n-octylphosphine oxide (TOPO) and tri-n-octylphosphorine (TOP) capped CdSe/ZnS core/shell QDs were a generous gift from Dr. J. Hollingsworth(Los Alamos National Laboratory) (ref).

Gel filtration columns and spin filters were from Harvard Apparatus. Buffers, chemicals, immunoblot materials and other components were purchased from Sigma Aldrich or Fisher Scientific, unless otherwise specified. Waveguides were obtained from nGimat, Atlanta, GA. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(cap biotinyl) (sodium salt) were purchased from Avanti Polar Lipids. Materials required for SAM preparation are listed in detail elsewhere (Anderson AS et al, 2008). 96 well plates were purchased from Invitrogen.

Methods:

Preparation of Antibodies: The capture antibodies were modified by biotinylation to allow their immobilization on the waveguide surface. The detailed protocols for antibody biotinylation have been described before (Martinez JS et al, 2005, Mukundan H et al, 2008). Carboxyl QD605 or QD655 stock (70 µl, 8 µM, Invitrogen), diluted with 500 µl borate buffer (10 mM at pH7.3) was mixed with 200 µg anti-PA or anti-LF antibody, respectively, in the presence of aqueous N-ethyl-N'-dimethylaminopropyl-carbodiimide (EDC) and reacted at room temperature for 1.5 hours. The reaction was quenched by addition of borate buffer (500 µl, 50 mM at pH9.0) and incubated at 4 °C overnight, then washed five cycles with 1mL borate buffer (25 mM at pH7.5) using 100 kDa MWCO spin filter. The reaction mixtures were then passed through the 1000 kDa MWCO spin filter. **Hongzhi, please reword to not duplicate Angew chemie article. 0 ******where are the details for the hinge labeling????? (really this paper doesn't have much ground breaking things, except for the difference in the hinge....which I guarnetee that is a problem with this antibody and not all...(I do thiol coupling to antibodies with QDS and those antibodies are fine...also Invitrogen sells kits for thiol labeling of antibodies with QDs.)**

Detection of the Labeled Material using Fluorescence Immunoassays: The antibodies were first tested in a standard plate-based immunoassay format. Capture antibody [50 nM] was coated on a Nunc 96 well plate for 2 hrs at room temperature (RT). The plates were then blocked for 2 hrs with 4.5% fish gelatin. Antigen was added in a series of increasing concentrations to the plate and incubated overnight at 4°C. Unbound antigen was removed by washing with PBS containing 0.01% tween 20. The plates were then washed three times with PBS and fluorescently labeled reporter antibody was added and incubated for2 hrs, at room temperature. Unbound reporter was removed by washing and the

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specific signal was measured using a TECAN Saphire fluorescence plate reader. In all experiments, non-specific binding of the reporter antibody in the absence of the antigen was measured as control.

Preparation of the Flow Cell and Assay Protocol: The detailed protocols for waveguide cleaning and functionalization with either biotinylated (0.1%) SAMs (Anderson, AS et al, 2008, Mukundan H et al, 2009) or lipid bilayers (Martinez JS et al, 2008 and Mukundan H et al, 2009) has been described before. Once functionalized, the waveguide is assembled in a flow cell. Lipid bilayers are allowed stabilization (12 hours, room temperature (RT)) on the waveguides and blocked for 2 hrs with phosphate buffered saline (PBS) containing 2% bovine serum albumin before use. Neither stabilization nor blocking is required for use of SAMs. Once assembled, the waveguide-associated parameters such as coupled power and waveguide background are measured for all experiments. Subsequently, 10 pM of streptavidin labeled with Alexa Fluor 647 is added to the flow cell. After 5 minutes of incubation, unbound streptavidin is washed off and the specific signal due to binding of the fluorescently labeled streptavidin to the biotinylated functional layer is measured. A small, but reproducible signal is measured. This signal is a measure of the system performance and the viability of the functional surface. This signal is completely photobleached by a 2 min exposure to the laser. Following saturation of the functional surface with unlabeled streptavidin, the biotinylated capture antibody is added (80 nM). Following a 5 minute incubation, unbound antibody is washed from the surface. A non-specific binding measurement (NSB) is performed when the reporter antibody is added on the functional surface in the absence of the antigen. This is typically < 500 RFU (Figure XX). The antigen is then added and incubated for 5 minutes, followed again by the reporter antibody and the specific signal is measured after a wash step to remove unbound components.

3. RESULTS:

The comination of quantum dots and optical waveguides, creates an assay format that is sensitive and amenale to simple multiplex antigen analysis. Toward this goal we have investigated the assay parameters of sensitivity and kinetics of binding for the QD amine-labeled antibody on the waveguide biosensor and compared the sensitivity to an equivalent plate-based assay. Further, we have compared the antibody activity of the amine- versus thiol-labeled antibodies.

A) The QD amine-labeled antibodies were used for the detection of protective antigen in a plate based sandwich immunoassay. As shown in Figure 1, the sensitivity of the assay is very poor for picomolar concentrations of the antigen in this assay format. The signal over background (S/B) was 1.8 for 250 pM protective antigen. This

concentration is considered here as the limit of detection for the plate based assay. This sensitivity of detection is insufficient for most biodetection strategies. This lack of sensitivity is contrasted with thedetection of 100 ppM PA using the optical biosensor platform is indicated in Figure 2. While the plate based assay took over 5 hours, the waveguide assay is not only more sensitive, but faster (30 minutes). For the waveguide assay the specific binding measured at 803 RFU. The non-specific binding, associated with the reporter antibody, is ~40 RFU, resulting in a S/B of 20.6 for 100 pM PA. The signal is roughly 4.5 times the signal-over-ackground of the plate assay, when singals are corrected for similar concentrations ((Basil I am not certain we can really say this..my rough calculation assumes its linear from 100 to 250 pM...which it might not be)).

B) Concentration-dependent detection of PA on the optical biosensor: As indicated in Figures 3 and 4 ((Basil I didn't see this figure in the excel sheets....perhaps you can find it..perhaps make a figure that combines ¾), we measured different concentrations of PA in our assay. The data were generated on different waveguides. Different waveguides have varying refractive indices,backgrounds, and coupling efficiencies. Therefore, we do not expect a linearity in the data as is seen in the plate based assay (Basil, was this data actually "linear" doesn't look like it to me)) (Figure 1). However, it is evident from this data that we can detect 1 pM PA in our assay ((Basil, I can't see that data...must be "figure 4" so I can't evaluate if this is true)), which is farsuperior than the 250 pM detection limit obtained in the plate based assay format.

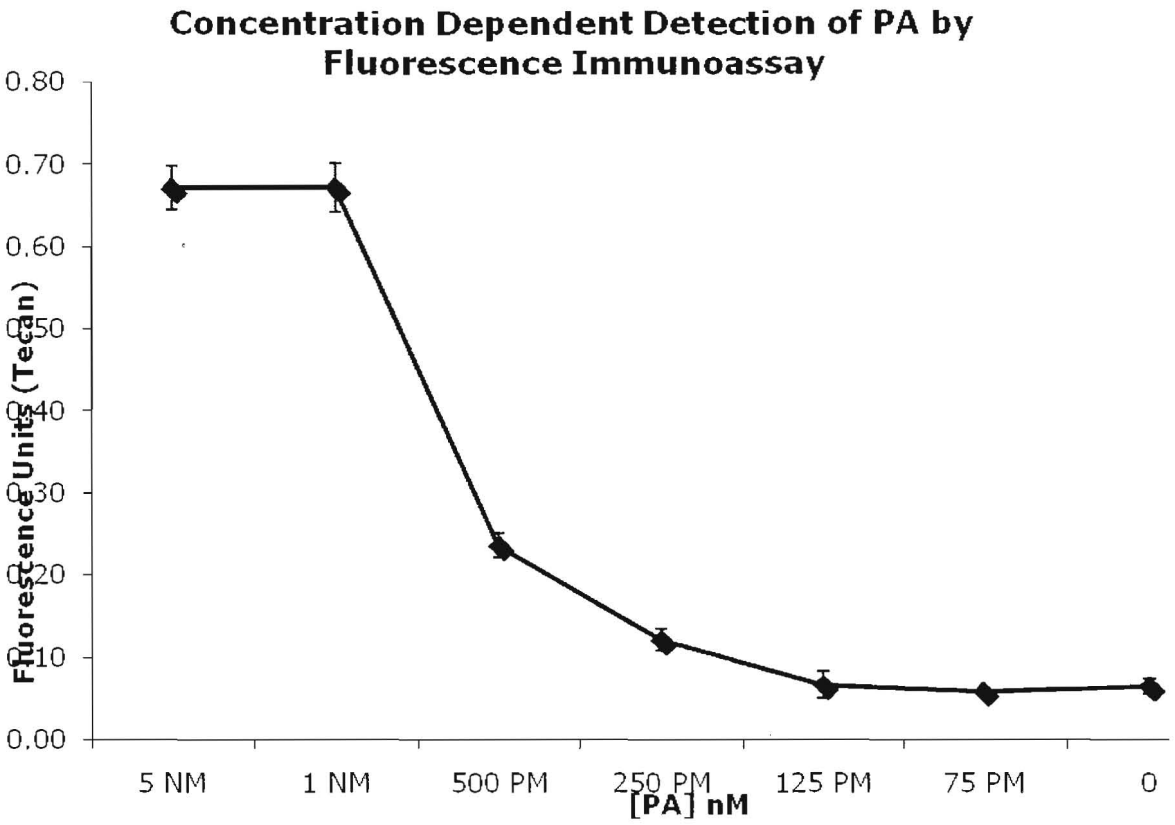
C) We determined the kinetics of amine labeled QD-antibody binding to PA by measuring the fluorescence every 30 seconds (Figure 5). As indicated, the signal steadily increased over the first 7 minutes and then began to saturate. After saturation (10 minutes) unbound antibody was washed off, resulting in a small decrease in signal. In general, we believe that it takes 10 minutes for the antigen-antibody interaction to saturate at 100 pM of antigen.

D) As mentioned elsewhere, we have evaluated hinge labeling of our antibodies with both DHLA QDs and with organic dyes. We used a similar strategy here with the polymer coated QDs. Interestingly labeling polymer-coated QDs in the hinge region of the anti(PA) antibody results in a complete loss of functionality of the antibody (Figure 6). We validated this result with a second preparation of the antibody. It is possible that this conjugation alters the conformation of the antibody and prevents its binding to PA. ((Basil, do you know if 1) fluorescence was maintained? 2) was QD really labeled with antibody? 3) ELISA reactivity of the conjugate?))

4. **DISCUSSION:** No single biomarker can accurately predict disease. Therefore, the ability to multiplex is required for efficient use of biomarkers in bio-detection and diagnosis. The biosensor team at the Los Alamos National Laboratory has developed a waveguide-based biosensor for the sensitive, specific and rapid detection of biomarkers associated with disease. We have previously adapted this technology to the detection of biomarkers associated with breast cancer (Mukundan H et al, BC, 2009), influenza (Kale RR et al, 2008), anthrax (Martinez JS et al, 2005) and tuberculosis. Most of these assays were performed using organic dyes as the fluorescent label. In this manuscript, we present the use of polymer-coated QDs for detection of the PA on our waveguide-based sensor, toward multiplex detection. Our biosensor is more sensitive, specific and rapid than standard immunoassay platforms. (no mention of lack of sensitivity...need to make a statement like....While the QD labeled antibody is not more sensitive than that of the organic fluorophore, labeled antibody, XXX and YYYY,(kinetetics, multiplex, etc) (so this kicks ass etc).

(Basil, the figures below need to be incoroporated into the text....and using the SPEI method...which typing and being a passenger on the drive of Soliders Summit...doesn't allow me to investigate (I m lucky Im not sick)). (Also below each figure I have tried to indicated what needs to be changed in the excel figure, please fix those)

Figure 1. Detection of protective antigen in a plate-based immunoassay.



((prolems with this figure:

-take the labels off of the numbers in the x axis

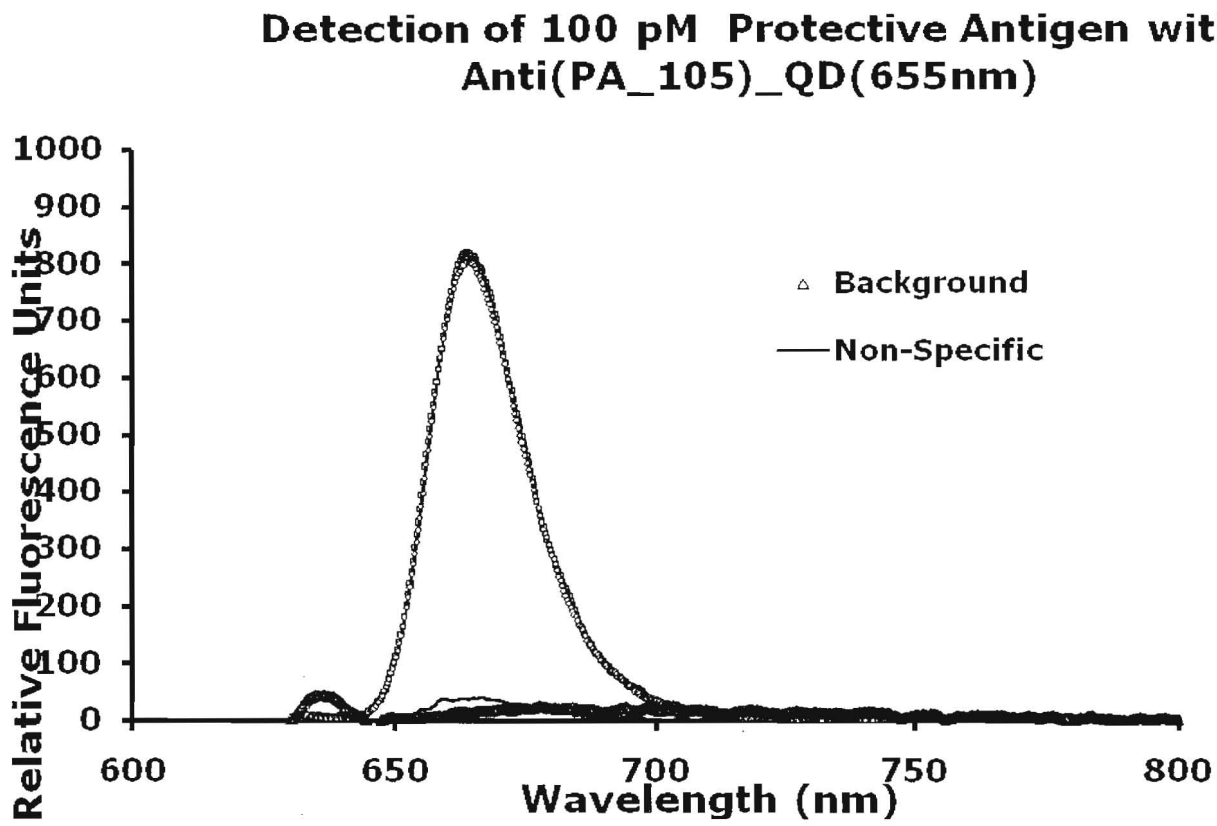
- adjust the numbers so all in nM units

-shouldn't you start the x axis as 0 and not 5?

-take the title off of the figure

-the left axis lable shift is a prolem from the import from excel to here, without a text box and can be fixed after you fix the figure itself))

Figure 2. Detection of 100 pM antigen on the waveguide-based optical biosensor platform.



((Problems you need to fix with this figure:

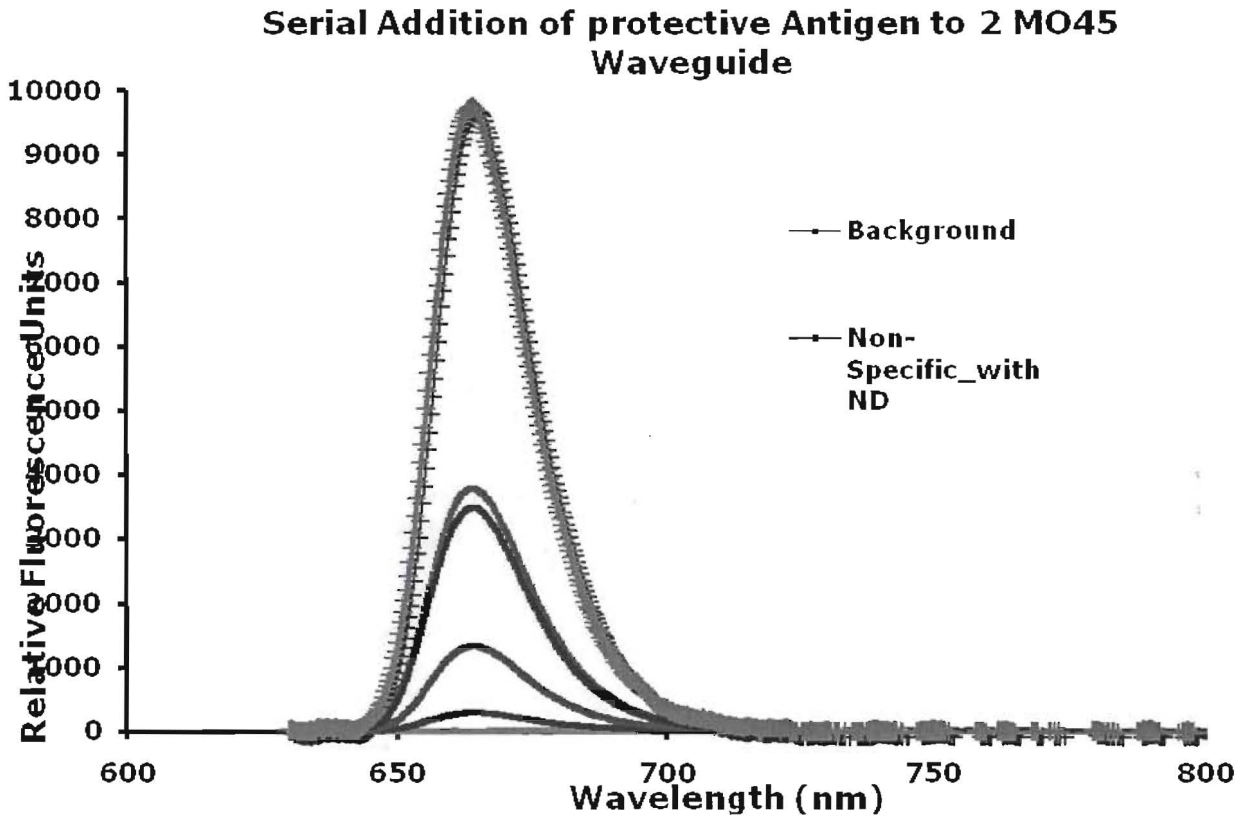
-take off the title

-the y axis x axis label size is different than the other figures, reduce

- I can't see the difference between the lines fix the data labels.

-the left axis lable shift is a prolem from the import from excel to here, without a text box and can be fixed after you fix the figure itself))

Figure 3. Concentration-dependant detection of PA on the optical biosensor.



(((Basil, please fix these problems with this figure:

-take off the figure label

- fix the damn legend (i.e. 100npM, non of those labels make sense toanyone, unless you also write a description of PB in the figure caption. What does min PB_1 nM 5 mean?

- problem with y axis label shift is import problem, fixed later

Figure 4.

(I don't see this figure)

Figure 5. Ki netics of PA detection on the waveguide-based biosensor.

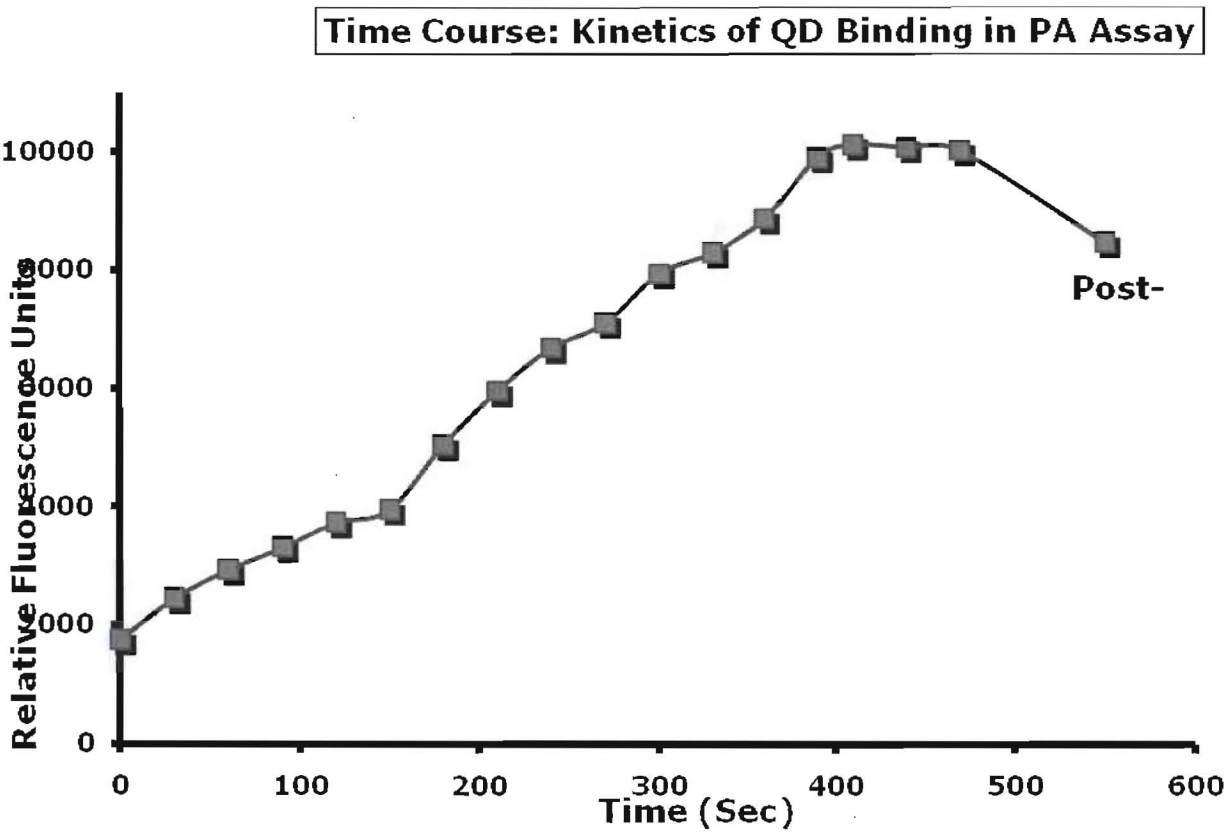
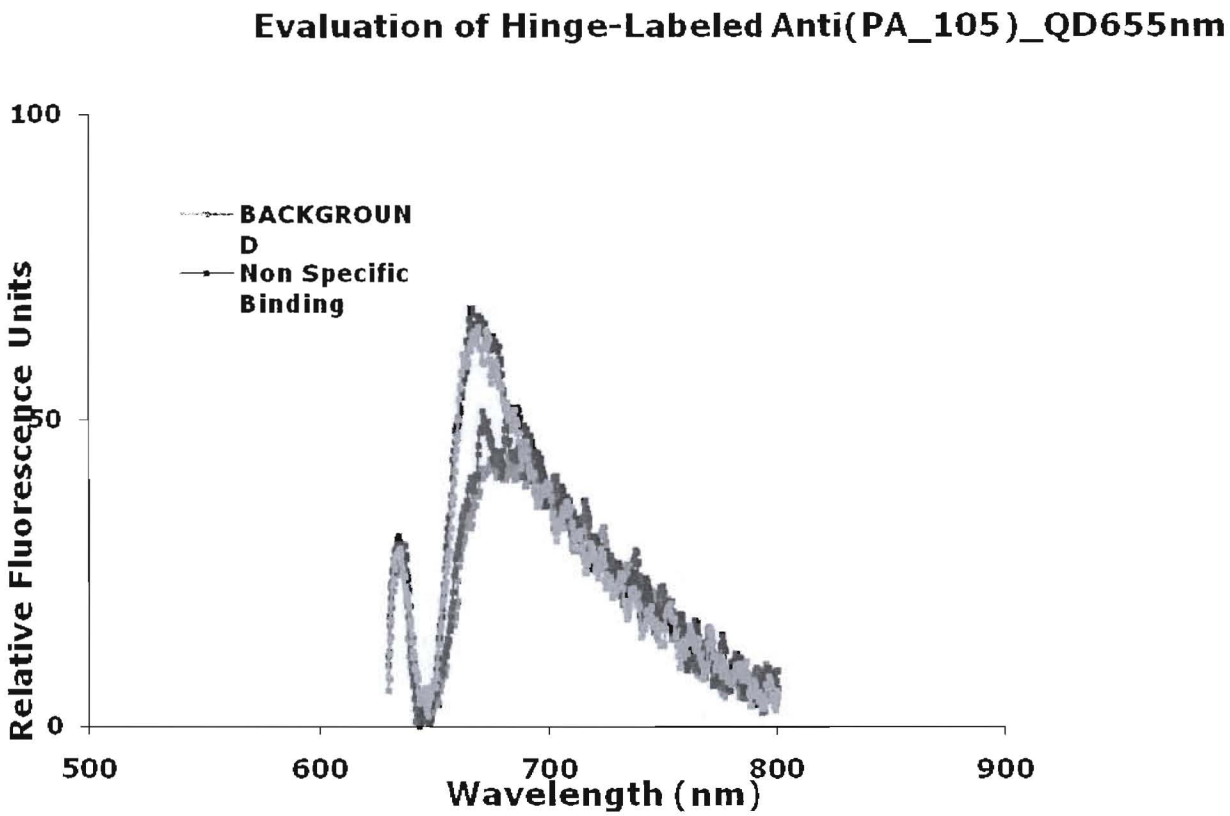


Figure 6. Optical and binding evalution of the QD hinge-labled antibody.



Basil you need to fix these problems with the figure:

-take off the figure label

-fix the legend…i.e. nM PA 10 ???

**********Basil, please add the bibliography in the SPEI template format. Please add acknowledgements

(someone seriously ows me a big bottle of petpto! Don't type on curvy drives!) ☺