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Author(s):	Hongzhi Xie (C-PCS), Harshini Mukundan(C-PCS), Aaron S. Anderson (C-PCS), W. Kevin Grace(C-PCS), Jennifer S. Martinez(MPA-CINT), Basil I. Swanson(C-PCS)
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Quantitative Multiplex Detection of Biomarkers on a Waveguide-based Biosensor using Quantum Dots.[**]

Hongzhi Xie, Harshini Mukundan, Aaron S. Anderson, W. Kevin Grace, Jennifer S. Martinez, Basil I. Swanson*

[*] Dr. H. Xie[+], Dr. H. Mukundan[+], A.S. Anderson, W.K. Grace, Dr. B. I. Swanson
Physical Chemistry and Applied Spectroscopy Group
Los Alamos National Laboratory
Los Alamos, NM 87545 (USA)
Fax: (+1) 505-667-0440
E-mail: basil@lanl.gov

Dr. J. S. Martinez
Center for Integrated Nanotechnologies
Los Alamos National Laboratory
Los Alamos, NM 87545 (USA)

[+] These authors contributed equally to this work.

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The quantitative, simultaneous detection of multiple biomarkers with high sensitivity and specificity is critical for biomedical diagnostics, drug discovery and biomarker characterization [Wilson 2006, Tok 2006, Straub 2005, Joos 2002, Jani 2000]. Detection systems relying on optical signal transduction are, in general, advantageous because they are fast, portable, inexpensive, sensitive, and have the potential for multiplex detection of analytes of interest. However, conventional immunoassays for the detection of biomarkers, such as the Enzyme Linked Immunosorbant Assays (ELISAs) are semi-quantitative, time consuming and insensitive. ELISA assays are also limited by high non-specific binding, especially when used with complex biological samples such as serum and urine (REF). Organic fluorophores that are commonly used in such applications lack photostability and possess a narrow Stoke's shift that makes simultaneous detection of multiple fluorophores with a single excitation source difficult, thereby restricting their use in multiplex assays.

The above limitations with traditional assay platforms have resulted in the increased use of nanotechnology-based tools and techniques in the fields of medical imaging [ref], targeted drug delivery [Caruthers 2007, Liu 2007], and sensing [ref]. One such area of increasing interest is the use of semiconductor quantum dots (QDs) for biomedical research and diagnostics [Gao and Cui 2004, Voura 2004, Michalet 2005, Chan 2002, Jaiswal 2004, Gao 2005, Medintz 2005, So 2006 2006, Wu 2003]. Compared to organic dyes, QDs provide several advantages for use in immunoassay platforms, including broad absorption bands with high extinction coefficients, narrow and symmetric emission bands with high quantum yields, high photostablility, and a large Stokes shift [Michalet 2005, Gu 2002]. These features prompted the use of QDs as probes

in biodetection [Michalet 2005, Medintz 2005]. For example, Jaiswal et al. reported long-term multiple color imaging of live cells using QD-bioconjugates [Jaiswal 2003]. Gao [Gao 2004] and So [So 2006] have used QDs as probes for *in-vivo* cancer targeting and imaging. Medintz et al. reported self-assembled QD-based biosensors for detection of analytes based on energy transfer [Medintz 2003]. Others have developed an approach for multiplex optical encoding of biomolecules using QDs [Han 2001].

Immunoassays have also benefitted from the advantages of QDs. Recently, dihydrolipoic acid (DHLA) capped-QDs have been attached to antibodies and used as fluorescence reporters in plate-based multiplex immunoassays (Goodman 2004]. However, DHLA-QDs are associated with low quantum efficiency and are unstable at neutral pH. These problems limit the application of this technology to the sensitive detection of biomolecules, especially in complex biological samples. Thus, the development of a rapid, sensitive, quantitative, and specific multiplex platform for the detection of biomarkers in difficult samples remains an elusive target.

The goal stated above has applications in many fields including medical diagnostics, biological research, and threat reduction. The current decade alone has seen the development of a need to rapidly and accurately detect potential biological warfare agents. For example, current methods for the detection of anthrax are grossly inadequate for a variety of reasons including long incubation time (5 days from time of exposure to onset of symptoms) and non-specific ("flu-like") symptoms. When five employees of the United State Senate were exposed to *B. anthracis* in the mail (2001), only one patient had a confirmed diagnosis before death. Since then, sandwich immunoassays using both colorimetric and fluorescence detectors have been developed for key components of the

anthrax lethal toxin, namely protective antigen (PA), lethal factor (LF), and the edema factor [Mourez 2001]. While these platforms were successful in assays against anthrax toxins, the sensitivity was poor. Furthermore, no single platform exists for the simultaneous and quantitative detection of multiple components of the *B. anthracis* toxin. Addressing multiple biomarkers at the same time will increase confidence in a positive result, and may lead to application in the simultaneous detection of anthrax and other bio-warfare agents.

To this end, the sensor team at the Los Alamos National Laboratory has applied a waveguide-based biosensor to the problem of multiplex detection. Using this platform, we have successfully developed assays for the sensitive detection of single biomarkers associated with anthrax (Martinez, 2005), breast cancer (Mukundan H et al, 2008; BC) and influenza (Kale R et al, 2008). This detection occurred in complex biological samples using either lipid bilayers (Mukundan H et al, Sensors and Actuators, 2009) or self assembled monolayers (SAMs, Anderson AS et al, 2008; Mukundan H et al, Bioconj Chem 2009) for waveguide-functionalization. However, no single biomarker can accurately predict disease. For medical diagnostics, the rapid, quantitative, sensitive and simultaneous detection of multiple biomarkers is desired. To this effect, we have developed a multiplex assay on our waveguide-based biosensor platform using photostable QDs as the fluorescence reporter. In this manuscript, we present for the first time, a rapid (15 minutes), sensitive (1 pM) and quantitative assay for the simultaneous detection of PA and LF in serum using photostable QDs as the fluorescence reporter.

The assay design is illustrated in Figure 1. A 532 nm laser was used to excite different colored, antibody conjugated quantum dots, taking advantage of their large

Stokes shifts.. The fluorescence emission was collected by a fiber optic spectrophotometer, and the data was collected and analyzed on a standard laptop computer. The surface of the waveguides were functionalized with a biotinylated SAM (as has been previously described, Anderson AS et al, 2008), and assembled into a flow cell [Anderson2008, Mukundan H, 2008, Martinez2005]. Labeled streptavidin (QD-565; 565 nm emission) was used as an internal standard to analyze system parameters during the course of the experiment. The internal standard was necessary for two reasons. First, waveguides differ intrinsically from each other with respect to the efficiency with which they couple light. Second, performance of the waveguides depends on which functional surface coating is used—phospholipid bilayers or covalently attached SAMs. Use of the QD-streptavidin conjugate as an internal standard allowed for normalization of this inter-assay variability and comparison of data generated in different waveguides. Addition of the targeted antigens (PA and LF) in serum followed by differential QD-labeled antibodies (anti-PA and anti-LF) allows for the detection of the two antigens and the determination of their concentrations.

A variety of core/shell CdSe/ZnS QDs coated with polyacryl acid polymers were evaluated for use with our assay. These QDs were chemically attached to anti-PA and anti-LF antibodies by a standard 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) condensation reaction. QDs with emission at 605 nm (QD605) and 655 nm (QD655) were used at a molar ratio of 3:1 (QD: antibody) for antibody conjugations. Unfortunately, it is not possible to control either the ratio of biomolecules per QD or the subsequent orientation of the bioconjugates [Liu 2007]. Also, tools to characterize such protein-QD conjugates are limited [Pathak2007]. We determined that quenching of the

reaction is necessary to avoid formation of larger protein-QD aggregates. To achieve this, the conjugate was incubated at pH 9 for three hours at room temperature, then...how was the reaction quenched?. The QD-antibody conjugates were characterized by gel electrophoresis (Sup Inf. Fig. S1), and were purified by filtration (1000 kDa molecular cut-off film filter) to remove any large aggregates. The conjugates were stored at 4 °C in a neutral buffer (pH 7.4) for later use, and were found to be stable for at least a month under these conditions.

Activity of the conjugated antibodies was assessed by both indirect and sandwich ELSIA. We noted that the chemical modification of antibodies with QDs does not affect their activities (Figure 2S in Supporting information), suggesting that the variable domains of the antibody remain unaffected by our modification strategy. The limit of detection using the fluorescence-based sandwich ELISA approach is 120 pM (Figure 2S). It is also worth noting that the QD conjugated antibodies resist photobleaching under our assay conditions, a significant advantage over organic dyes (Figure 3S). These experiments confirmed the suitability of our conjugates for use as reporters in a waveguide-based assay platform.

Assay development and optimization for each antigen was done in single biomarker assays before testing them simultaneously in a multiplex format. Non-specific binding was evaluated by flowing bovine serum in the flow cell, then adding each reporter antibody. Repeat additions were done to confirm saturation of non-specific binding. The signal associated with non-specific interactions was subtracted from the specific signal during data processing. As shown in Figure 2a, both the waveguide background (55 RFU), and the non-specific signal (164 RFU) are low. To perform

assays, the internal standard (streptavidin-QD conjugate) was added, followed by an aliquot of unlabeled streptavidin. A biotin-labeled capture antibody was immobilized on the surface using the familiar biotin-streptavidin interaction. Non-specific binding was evaluated by adding bovine serum and an aliquot of the reporter antibody. Next, the antigen of interest was added, followed by another aliquot of the appropriate reporter antibody. The specific signal associated with antigen binding was 9030 RFU (signal-to-noise ratio of 161) for 100 pM PA. A kinetic measurement indicated that the signal associated with reporter binding saturates in 6 minutes for the QD-labeled antibody (Figure 2b). The diffusion of the antibody is influenced by the dimensions of the flow cell holder used in the waveguide apparatus, which is 3mm wide × 31.8 mm long, with a volume of approximately 50 μL. Therefore, incubation time was set at 10 minutes for all subsequent measurements. Other attributes of these assays that should be noted are the fact that the waveguides are functionalized with SAMs prior to performing the assays (Anderson AS et al, 2008) and that entire immunoassay requires only 30 minutes to complete.

After validation of the QD-labeled antibodies in single analyte assays, we applied the technology to a multiplex format. This step required analysis of several strategies for antibody-nanoparticle conjugation for several reasons. First, antibodies have intrinsically different sensitivities to their antigen. Also, QDs differ in quantum efficiency. Several other factors such as the size of the QDs, number of antibodies conjugated on the QD surface, antibody conformation, stability and sensitivity regulate assay performance. Our preliminary experiments determined that conjugation of the anti (LF) antibody with QD655 and the anti (PA) antibody with QD 605 offered the maximum signal resolution

in a multiplex format (Figure 3a). Secondly, quantitation of results from a multiplex platform with several inherent differences (e.g, differences among waveguides and in the functional surfaces) demands an internal standard to be incorporated within each experiment, The multiplex assay using QD565-labeled streptavidin (10 pM) as an internal standard is shown in Figure 3b.

A linear, concentration-dependent increase in signal was measured for both PA an LF (1-100 pM range, Figure 4a), and the limit of detection (LoD) was determined for each antigen in serum to be 1 pM. Using the optical waveguide platform to detect these antigens, we were able achieve superior sensitivity when compared to plate-based ELISA. Figure 2S shows 120 pM detection of PA, while the lowest published LoD for PA is 70 pM (Quin et al 2002). For LF, do you have a value for this?. These results demonstrate our ability to rapidly and quantitatively detect very low concentrations of PA and LF in serum, with better sensitivity that achieved by conventional immunoassay methods. The ratio between the fluorescence intensities of the specific binding of antigen and that of the internal standard is also linear with respect to the concentration of the antigen, validating the use of streptavidin-565 QD for normalization of inter- and intra-assay variability (Figure 4b).

In summary, we have applied our waveguide-based, optical biosensor to the development of an assay for the quantitative, sensitive (LoD = 1 pM), rapid (30 minutes), and simultaneous detection of PA and LF in serum. Furthermore, this assay employs photostable QDs as the fluorescence reporter, which allows the use of an internal standard, in this case streptavidin-565 QD, to normalize for inter- and intra- assay variability. This research represents the first demonstration of a multiplex assay platform

that can theoretically be adapted to all biomarkers for which antibodies that bind orthogonal epitopes (and form an effective sandwich) are available.

Experimental Section

Synthesis of QD-antibody conjugates

Pairs of monoclonal antibodies (QED Bioscience Inc.) specific for *B. anthracis* including anti-PA antibody pair (BAP106 for capture and BAP105 as reporter) and anti-LF antibody pair (BA17-0106 as capture and BA17-105 as reporter) were modified for use in the waveguide-assay. The capture antibodies were biotinylated with sulfo-NHS biotin, purified, and quantitated as has been described elsewhere (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, and washed five cycles with 1mL borate buffer (25 mM at pH7.5) using 100 kDa MWCO spin filter. The reaction mixtures were passed through the 1000 kDa MWCO spin filter. The detailed procedure for synthesis and characterization of antibody-QD conjugates are described in Supp Information section of the manuscript.

Waveguide-Functionalization and Flow Cell Assembly: These protocols have been described in detail elsewhere (Martinez JS et al, Mukundan H et al). Briefly, SAM functionalized waveguides are assembled in a flow cell. After each reagent addition, the flow cell is allowed to incubate at room temperature for 10 minutes, followed by a wash

with Phosphate buffered saline containing 0.5% bovine serum albumin. All measurements were made in RFU.

System parameters such as power coupled and waveguide background are measured. Standard Streptavidin-565 QD signal (10 pM) is then measured. Due to the inherent photostability of QDs, this signal remains stable during the course of the experiment. It is thus an indirect measure of the power coupled and instrumentation settings. After addition of labeled streptavidin, an aliquot of unlabeled streptavidin was added, followed by biotin-labeled capture antibody. Non-specific binding associated with control serum (bovine serum, Hyclone Laboratories) was measured, followed by non-specific binding of the reporter antibodies to the functional surface. In separate experiments, repeat measurements of both serum and reporter antibodies were performed to ensure saturation of non-specific binding. At the concentrations used (100 nM), non-specific binding is saturated with single addition of the reporter antibodies. No increase is measured with subsequent measurements (data not shown).

Following the non-specific measurements, antigens (in bovine serum) are added to the flow cell. Following wash to remove any unbound antigen, the reporter antibodies are added to the flow cell and signal associated with specific binding is measured. Concentration dependent responses were performed on separate waveguides in different experiments (Figure 3). The streptavidin-565 QD standard was used to normalize waveguide and functional surface associated differences (Figure 3 b).

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Figure Captions:

Figure 1. Schematic representation of a multiplex sandwich immunoassay for PA and LF on the waveguide-based biosensor using antibodies conjugated to QDs as the fluorescence reporter. The waveguide surface is functionalized with Biotinylated SAMs. Capture antibodies are immobilized on the functional surface using standard biotin-avidn chemistry. The capture and detector antibodies bind orthogonal epitopes on the antigen forming an effective sandwich. Excitation with the laser generates the evanescent field that extends into the medium close to the surface of the waveguide and excites the reporter QDs. Streptavidin labeled with QD 565 serves as the internal standard. A fiber optic spectrometer is positioned to the waveguide to collect fluorescent signal from the waveguide.

Figure 2. Detection of PA on a single optical waveguide sensor. a) Fluorescent spectral response of the excited waveguide at 532 nm laser beam using anti-PA-QD655 as reporter following exposure to 100 pM PA (red curve), in the absence of PA (olive curve), and waveguide background (blue curve). b) Time-dependent fluorescence changes of anti-PA-QD655 as reporter in the presence of 100 pM PA.

Figure 3. Detection of multiplexed antigens of PA and LF on a single optical waveguide sensor. a) Fluorescent spectral response of the excited waveguide by 532 nm laser beam using anti-LF-QD605 and anti-PA-QD655 as reporters following exposure to a mixed sample of 1nM LF and PA (red curve) and waveguide background (blue curve). b) Spectral response of the excited waveguide by 532 nm laser beam using anti-PA-QD605 and anti-LF-QD655 as reporters following exposure to a mixed sample of 100 pM LF and PA and a internal standard using streptavidin-QD565 (red line). The olive curve is the nonspecific response of the waveguide applied the reporter antibodies without containing antigens and the internal standard. The orange and blue curves are the response of the internal standard and waveguide background, respectively.

Figure 4. The standard curves that were obtained in the presence of multiplexed analytes of PA and LF antigens on a single optical waveguide sensor. a) The signal-to-noise ratio (S/N) as a function of the concentration of antigens. b) The signal-to-standard ratio (I/I_S) as a function of the concentration of antigens.

Keywords:

Optical waveguide sensor, Quantum dots, Antibodies, Immunoassays, Anthrax toxins, Multilplexed assays, Diagnostic tools

Figure 1 Xie et al. *Angew Chem*

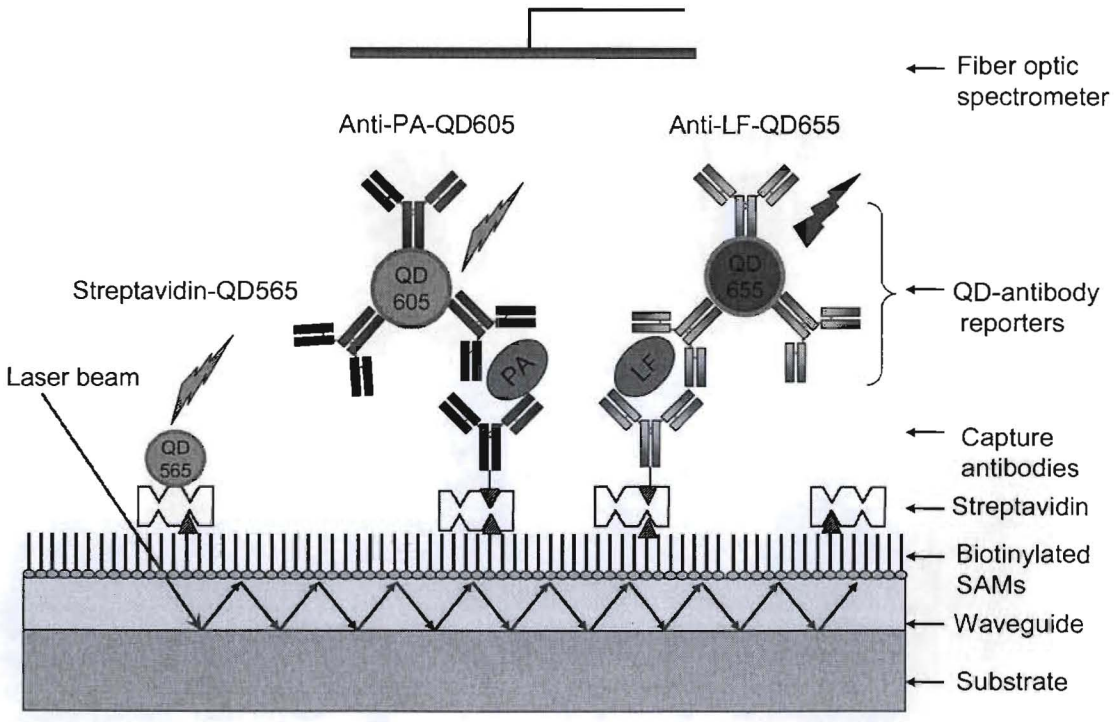

Figure 2

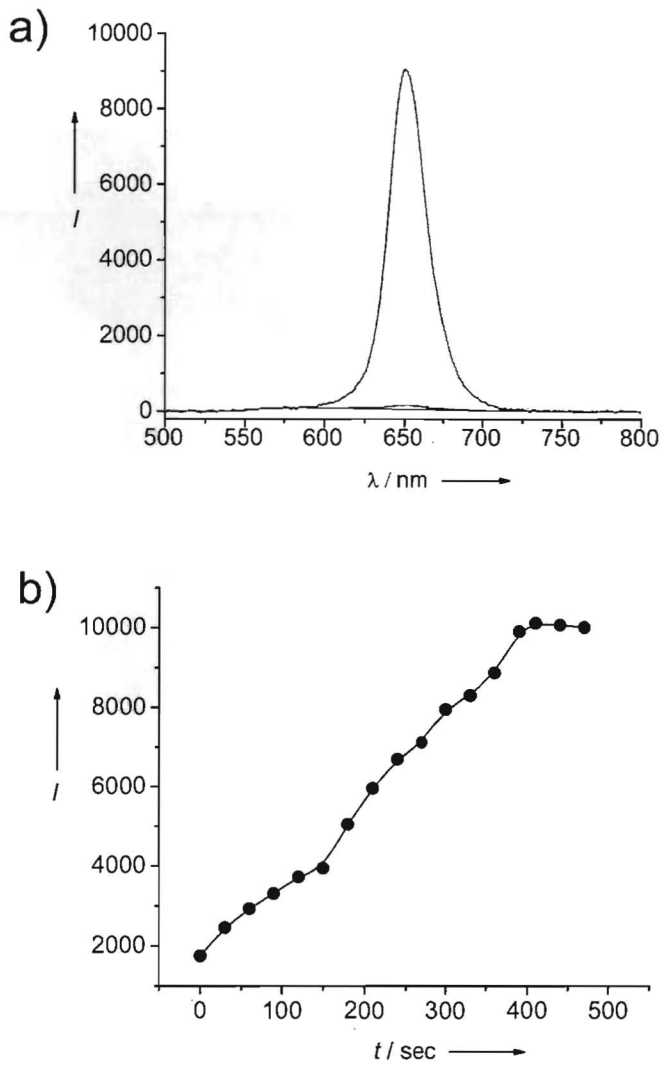

Figure 3

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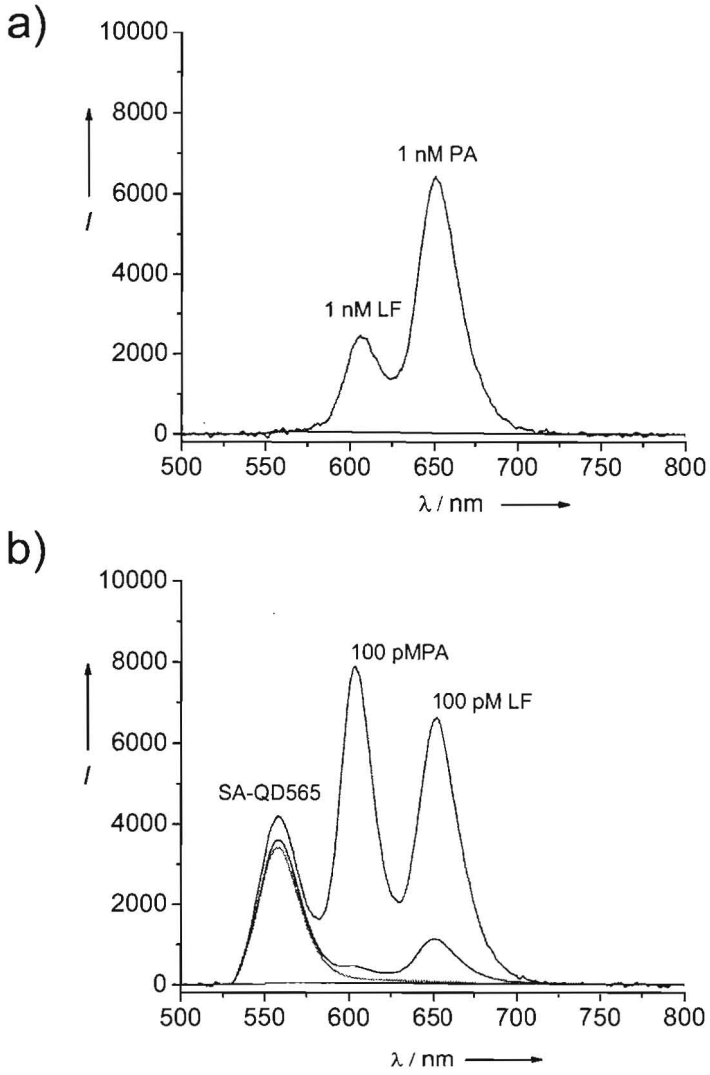

Figure 4

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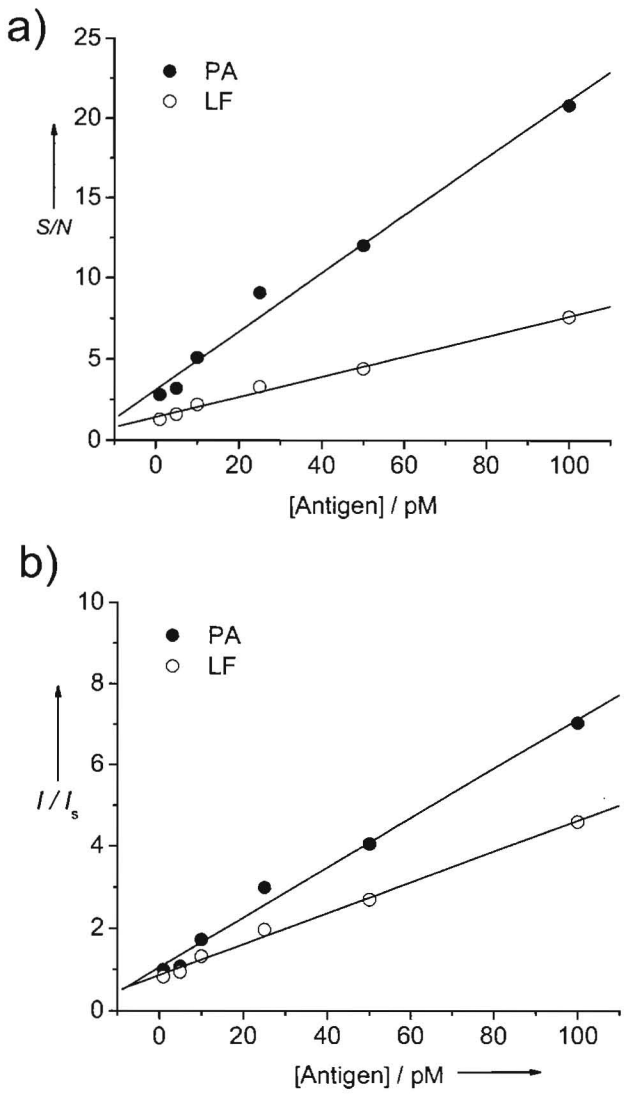
a)
PA
LF
S/N
[Antigen] / pM
b)
PA
LF
I / I_s
[Antigen] / pM

Supporting Information

Multiplexed Pathogen Detection Biosensor Based on Optical Waveguide and Quantum Dots[**]

Hongzhi Xie, Harshini Mukundan, Aaron S. Anderson, W. Kevin Grace, Jennifer S. Martinez, Basil I. Swanson*
[*] Dr. H. Xie[+], Dr. H. Mukundan[+], A.S. Anderson, W.K. Grace, Dr. B. I. Swanson
Physical Chemistry and Applied Spectroscopy Group
Los Alamos National Laboratory
Los Alamos, NM 87545 (USA)
Fax: (+1) 505-667-0440
E-mail: basil@lanl.gov

Dr. J. S. Martinez
Center for Integrated Nanotechnologies
Los Alamos National Laboratory
Los Alamos, NM 87545 (USA)

[+] These authors contributed equally to this work.

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Synthesis of QD-antibody conjugates

Pairs of monoclonal antibodies (all purchased from QED Bioscience Inc.) specific for *B. anthracis* including a pair of Anti-PA antibodies (BAP106 modified with biotinylation for use in capture antibody and BAP105 conjugated with quantum dots for use in reporter antibody) and a pair of anti-LF antibodies (BA17-0106 with biotinylation as capture antibody and BA17-105 by conjugation of QDs as reporter). Generally, prior to mix with QD solution, antibodies were buffer-exchanged from PBS to borate buffer (10 mM pH7.3) by using 10 kDa molecular weight cut-off (MWCO) spin filters (Millspore). Carboxyl QD605 or QD655 stock (70 µl, 8 µM in borate buffer at pH9.0, Invitrogen) was diluted with 500 µl borate buffer (10 mM at pH7.3), then mixed with 200 µg anti-PA or anti-LF antibody solution (BAP105 or BA17105), respectively. 8.4 µl of N-ethyl-N'-dimethylaminopropyl-carbodiimide (EDC) in aqueous solution (100 mM) was added and the reaction mixture was stirred gently 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 at least five cycles with 1mL borate buffer (25 mM at pH7.5) using 100 kDa molecular weight cut-off (MWCO) membrane spin filter. The reaction mixture was then passed through the 1000 kDa MWCO spin filter to remove any larger aggregates, and then the filtrate was concentrated using 10 kDa MWCO, and final solution buffer exchange to pH7.5. The concentration of QDs were measured based on the absorbance of QD605 at 532 nm with ε_{532}= 810,000 M^{-1} cm^{-1} and QD655 with ε_{532}= 2100,000 M^{-1} cm^{-1}. The concentration of antibodies in conjugates were measured by using as Bovine serum albumin as standard and BCA™ working reagent (PIERCE).The molar ratio of antibody to QD in the conjugate was measured and is about 3:1.

The formation and purity of antibody-QD bioconjugates were identified by a 4-15% gradient non-denaturing polyacrylamide gel eletrophoresis (PAGE, Tris-HCl, pH8.8, BIO-RAD) at Tris/glycine buffer (pH8.9), and 60 mV for 5h at room temperature. SDS PAGE (12.5%) and Agarose gel (1%) made with Tris-borate EDTA (TBE) buffer at pH8.0 was also used for the characterization of the conjugates by running the gels at 50 mV for 1h. Proteins were visualized by staining with GelCode Blue Stain Reagent (PIERCE) and the free QDs and QD-labeled conjugates were visualized of the unstained gels using UV-illumination (366nm excitation) and digital camera imaging.

The capture antibody in PBS buffer was mixed with EZ-link sulfo-NHS-biotin (PIERCE) in a 20 molar ratio excess to antibody and reacted at room temperature for 50 min. Un-reacted biotin reagents were removed by passing the reaction solution through three well-balanced spin filtration columns (Sephadex G25, BioSciences).

Enzyme-linked immunosorbent assays (ELISAs)

For anti-PA-QD605 and anti-LF-QD655 enzyme-linked immunosorbant assays (ELISAs), we coated 96-well Nunc immunosorp plate wells (Nalgene Nunc, Milwaukee, WI) with protective antigen (PA) and lethal factor antigen (LF) at a concentration of 2 µg/ml. We incubated wells with anti-PA-QD606 or anti-LF-QD655 diluted 1:20 (100 nM) in 4.5 % fish gelatin PBST, followed by incubation with anti-mouse HRP-conjugated species specific secondary antibody (Jackson ImmunoResearch, West Grove, PA) at a dilution of

1:2000. We then added tetramethylbenzidine substrate (Pierce, Rockford, IL), and determined optical density values at filter at 405 nm with a plate reader. We also completed all of positive and negative controls for ELISA.

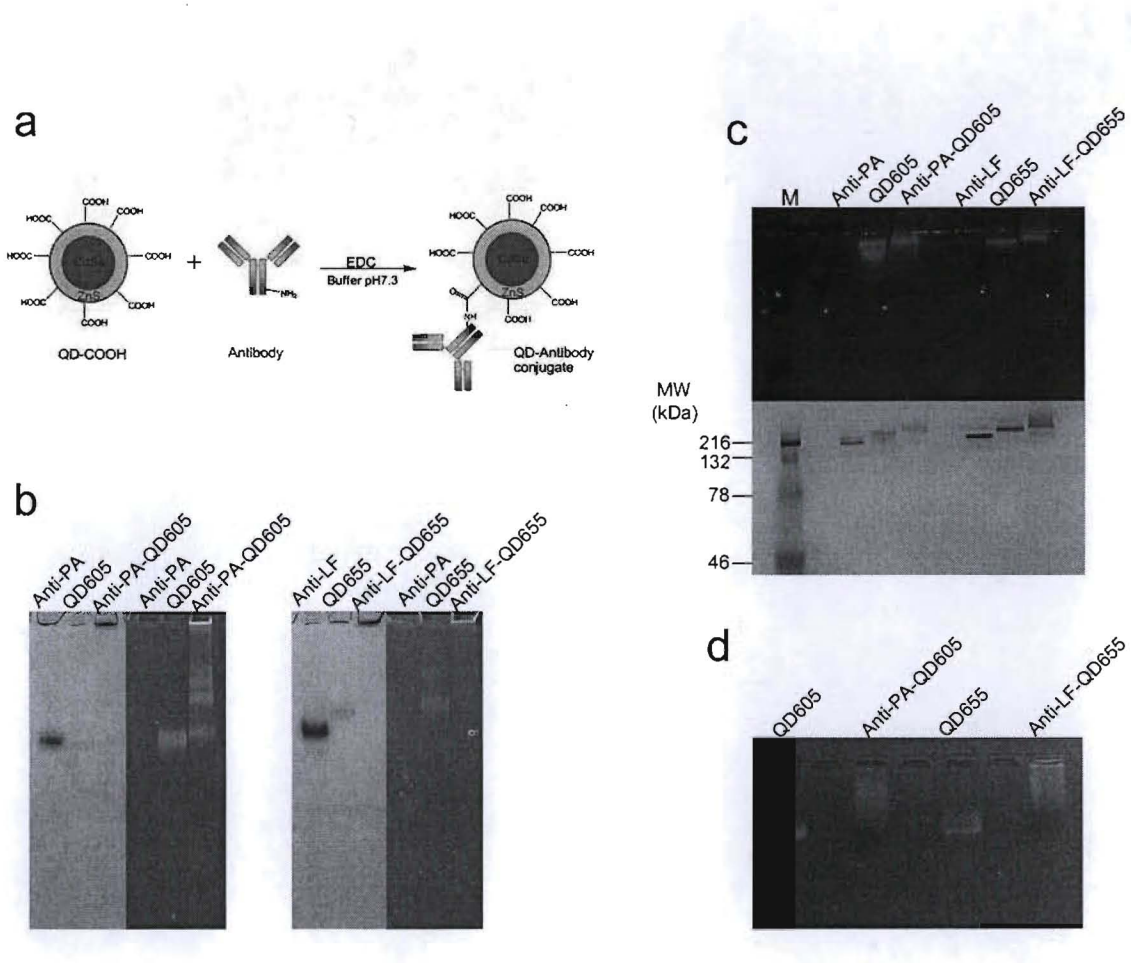

Figure 1S. Synthesis and purification of reporter quantum dot-antibody conjugates. (a) Structure a carboxylic-polymer coated QD chemically modified with a full chain antibody through EDC condensation reaction in borate buffer (10 mM at pH7.3). The reaction mixture was washed five cycles with a 100 kDa cut-off spin filter against borate buffer (50 mM at pH9.0) and passed through a 1000 kDa cut-off spin filter before applied to electrophoresis analytic gels. (b) Native gradient polyacrylamide gel electrophoresis(4 ~15 %) showing formation of the antibody-QD conjugates with the stained gels (left in each panel) and fluorescence-imaged gels(right in each panel). (c) SDS-polyacryamide gel elctrophoresis (12.5%) showing formation of the antibody-QD conjugates with the stained gel (bottom) and fluorescence-imaged gel (top). (d) Agarose gel electrophoresis (1%) showing formation of the antibody-QD conjugates with the fluorescence-imaged gel.

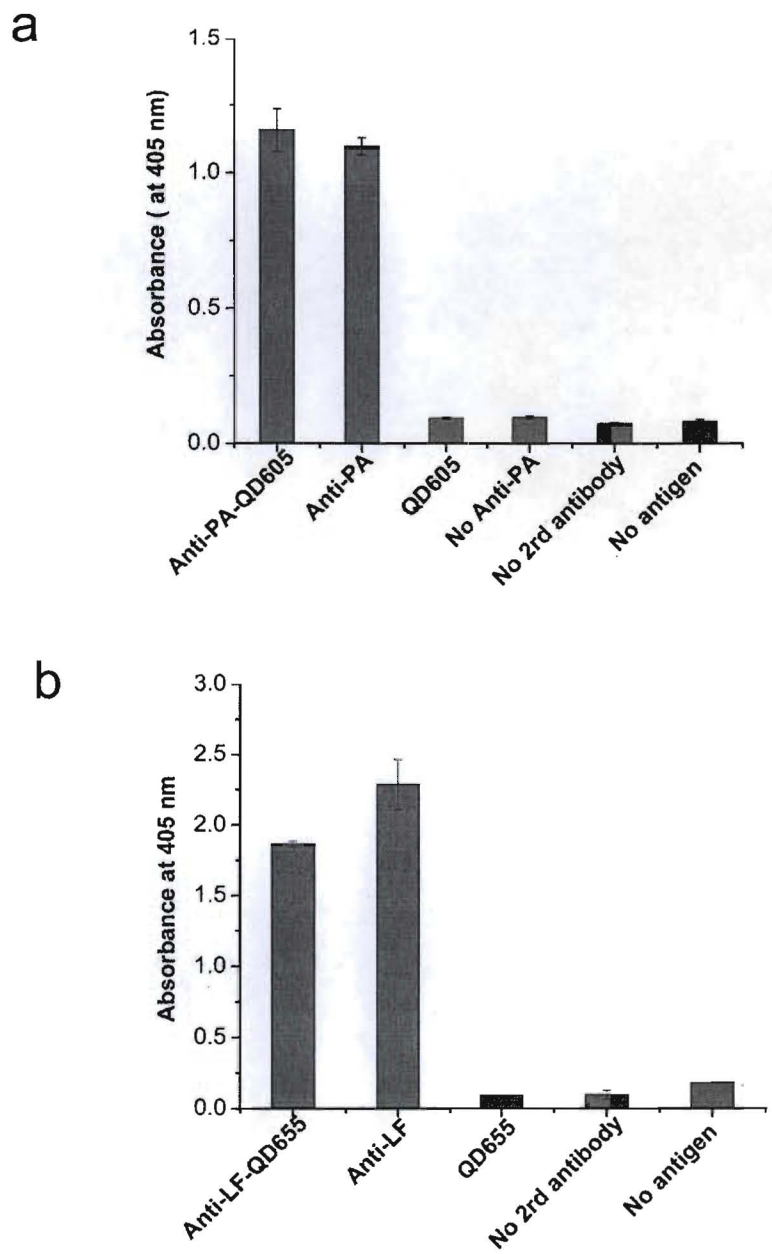

Figure 2S Activity assays of QD-labeled antibodies by ELISA. Anti-mouse-HRP is used as second antibody and TMB as the substrate. (a) Activity of Anti-PA-QD605 conjugate in comparison with free anti-PA antibody and controls, QD605: QD605+antigen PA+ second antibody; No Anti-PA: antigen PA+ second antibody; No 2ed antibody: QD605-anti-PA + antigen PA and No antigen: QD605-anti-PA + second antibody. (b) Activity of Anti-LF-QD655 conjugate in comparison with free anti-LF antibody and controls: QD655: QD655+antigen LF+ second antibody; No 2ed antibody: QD655-anti-PA + antigen LF and No antigen: QD605-anti-PA + second antibody. The data are shown as mean±error of three independent measurements.

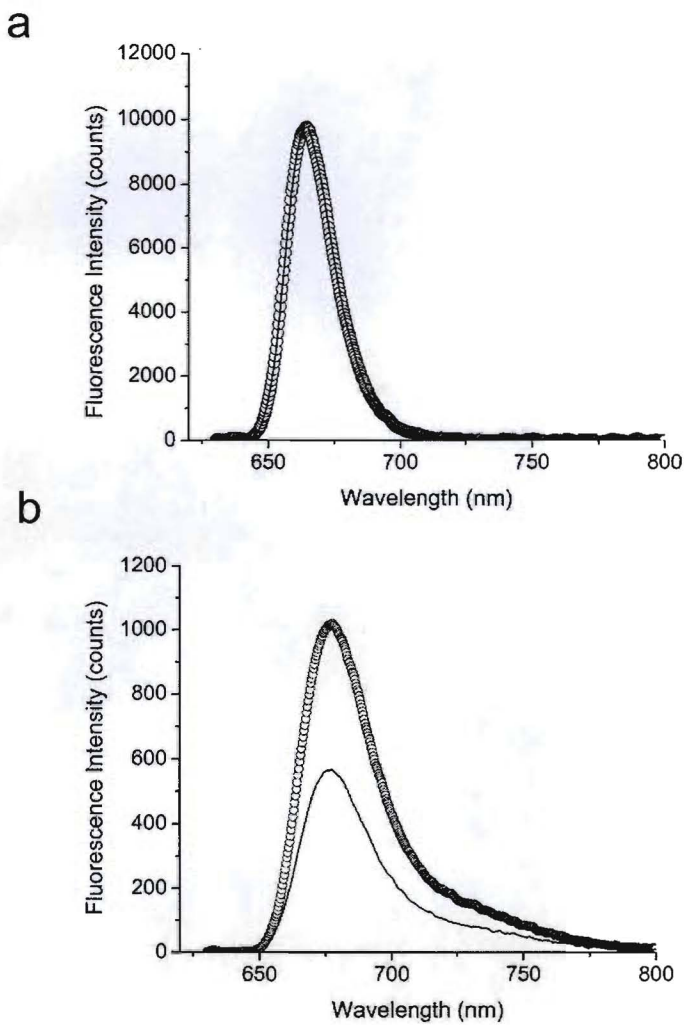

Figure 3S. Photostability of QD in comparison with organic dye as signal reporter on the optical waveguide. (a) Fluorescent spectra of streptavidin-QD655 before (○) and after (—) exposed to laser beam for 5 min. (b) Fluorescent spectra of streptavidin-Alexa Fluro647 before (○) and after (—) exposed to laser beam for 12 sec.

Full list Reference [2]: C. P. Quinn, V. A. Semenova, C. M. Elie, S. Romero-Steiner, C. Greene, H. Li, K. Stamey, E. Steward-Clark, D. S. Schmidt, E. Mothershed, J. Pruckler, S. Schwartz, R. F. Benson, L. O. Helsel, P. F. Holder, S. E. Johnson, M. Kellum, T. Messmer, W. L. Thacker, L. Besser, B. D. Plikaytis, T. H. Taylor, Jr., A. E. Freeman, K. J. Wallace, P. Dull, J. Sejvar, E. Bruce, R. Moreno, A. Schuchat, J. R. Lingappa, N. Marano, S. K. Martin, J. Walls, M. Bronsdon, G. M. Carlone, M. Bajani-Ari, D. A. Ashford, D.S. Stephens, B. A. Perkins, *Emerg. Infect. Dis.* **2002**, 8 (10), 1103–1110.