NANOSENSORS FOR DETECTION OF BIOLOGICAL THREAT CONTAMINANTS IN CRITICAL BUILDINGS

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ABSTRACT

A nanoscale sensing modality for detection of bacterial contaminants was developed. The modality uses fluorescent nanoparticles, also known as "Quantum dots" (QDs) conjugated to fragmented antibodies of targeted contaminants, and a quenching dye that labels an antigen surrogate. Coupling of the QD-labeled fragmented antibody and the quencher-labeled antigen allows sufficient fluorescent resonance energy transfer (FRET) to quench QD emissions. The subsequent addition of the targeted antigen displaces the dye-labeled bacteria, eliminating FRET and resulting in a concentration-dependent increase in QD photoluminescence. The novelty of this procedure lies in the applications for detection of a broad range of biological contaminants including viral and bacterial contaminants with a high degree of specificity and sensitivity. Furthermore, through the use of QDs of varying emission wavelengths the system can easily be adapted into a multiplexing immuno-assay

1. INTRODUCTION

Army mission critical fixed facilities and installations currently lack capabilities to "detect-to warn" and identify, in real time, airborne biological threat contaminants, so that countermeasures can be taken to protect lives and mission. Emerging sensors for airborne biological contaminants, currently being evaluated by researchers at the Defense Threat Reduction Agency (DTRA), the Department of Homeland Security (DHS), and Edgewood Chemical and Biological Center (ECBC) still require approximately 15-20 minutes for the biological contaminants detection and identification. Within a mission – critical building's heating ventilation and air-conditioning (HVAC) system, airborne biological threat contaminants would be rapidly disseminated throughout the building unless corrective action is taken. Therefore, S&T is needed to reduce the time for detection of threat biological contaminants in Army mission critical facilities to cue the response assets for countermeasures to protect lives and mission, in case of a release.

Lack of understanding of the photophysics of nanoparticles conjugated with antibodies, bound to quenchers and exposed to target biological contaminant limits their use for real time detection and identification of specific contaminants. The barriers to solving the problem are:

- Limited knowledge of spectral interactions among nanoparticles, bacterial contaminants, and quenchers.
- Steady state fluorescence properties nanoparticle-antibody conjugates are not quantified.
- Changes in fluorescence properties with respect to time are unknown.

The goals of this research are to obtain fundamental knowledge on the fluorescence spectrum of nanoparticles and quenching phenomena by analyzing steady state fluorescence, lifetime fluorescence and the effect of environmental variables such as concentration, pH, temperature and time. This will be accomplished by:

- Analysis of nanoparticle-quencher distances and spectral criteria necessary for energy transfer and quenching in single quenching and dual quenching schemes.
- Understanding of quenching and energy transfer phenomena in nanoparticle-quencher biological contaminant using non-classified biological representative surrogates/antibody systems.
- Understanding of quenching mechanism necessary for consistent spectral signatures for multiple biological contaminant detection.

Biomolecules labeled with fluorescent dyes are used in a wide variety of scientific and technological applications. A number of examples can be found in the fields of molecular biology, cell biology,

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biochemistry, and medical diagnostics. These applications are based on the inherent advantages of fluorescent assays, some of which are their high sensitivity, the facile introduction of fluorescent labels on a wide variety of biomolecules (such as nucleotides, proteins, and peptides) with minimal effects in their activity, and their usefulness in microscopy applications. These characteristics make them valuable tools in several fields of science and technology. The potential for assays utilizing fluorescent dyes is further expanded through the use of fluorescent resonance energy transfer (FRET) reactions that can take place between fluorescent dyes and donor quencher molecules under controlled circumstances. The manipulation of FRET reactions in this manner allows for the development of highly specific and highly sensitive sensors for detecting biological contaminants. A model system employing FRET reactions between fluorescent nanoparticles (quantum dots) and organic quencher molecules has been developed to detect *E coli* 0571H7, which is used as a model biological contaminant. In this system quantum dots and bacteria-specific antibodies detect *E* coli in aqueous solutions. The system is based on a FRET reaction between the quantum dots and quencher that inhibits fluoresces in the absence of target bacteria in the environment. In the system as shown in Figure 1, an immobilized target surrogate is bound to a quencher, while quantum dots are tagged to an antibody specific for the target. In a dormant state the immobilized target will be bound to the tagged antibody and the proximity of quencher and quantum dots will allow for the inhibition of fluorescence via FRET. This distance is also called the Forster distance and is representative of the span at which 50% of emissions from the quantum dots will be absorbed by the neighboring quencher. When a test sample containing unlabeled *E* coli is added to the conjugate, equilibrium reactions will cause the displacement of the quencher labeled target. No longer adjacent to a quencher the quantum dots will freely fluoresce and emit energy at their characteristic wave lengths.

2. EXPERIMENTAL PROCEDURE

2.1 Materials and Methods

For this study, *E* coli antibody fragments conjugated to quantum dots with an emission at 605 nm in concert with a Black Hole Quencher (BHQ-2) were used. Quantum dots and quenchers were chosen such that emission and absorbance ranges overlapped with one another as closely as possible to insure maximal FRET. While concentrations were established

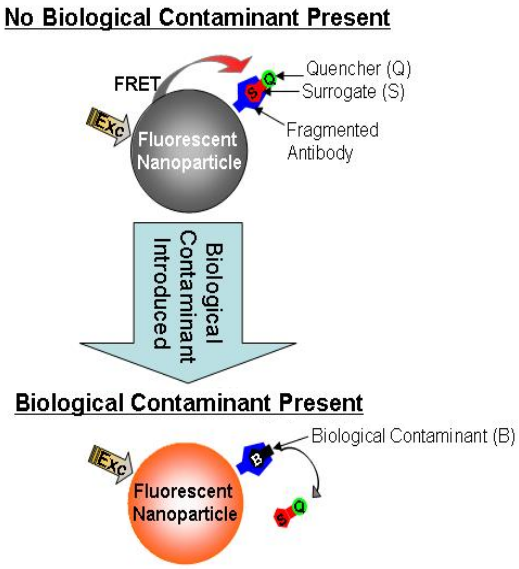

Figure 1. FRET-based Nanosensor for biological contaminants

for quantum dot labeling, there was no set concentration of quencher for use in this experiment so an ideal concentration had to be evaluated. Using the BHQ-2 quencher we explored the effects of varying the concentration to determine the necessary concentration needed to effectively quench the 605nm Quantum dots. (See Figure 2) After testing it was concluded that 25 micro-Molar concentrations would be used to maximize FRET but also avoid quenching of a positive signal. This information was also used to calculate overall FRET efficiency of the system as shown below.

2.2 Antibody Selection

IgG monoclonal antibodies were selected for use in this experiment over other possible ligands for their exceptional properties. Antibodies can be developed to meet nearly any possible sensing need. Furthermore, when selected correctly antibodies are very sensitive to their targets and a properly designed system can nearly eliminate false positives due to their strong specificity for the target. Antibodies can avoid false positives even in the case of two very closely related bacterial strains as seen in this experiment where E. coli 0571H7, a foodborne pathogen, is selected specifically over other E. coli strains.

2.3 Antibody Fragmentation

For this experiment an IgG monoclonal mouse derived antibody was used. These antibodies were fragmented in an immobilized pepsin digest developed by Pierce

Biotechnology Inc. Pepsin is an enzymatic molecule that is particularly adept at cleaving peptides at the aromatic amino acids, phenylalanine, tryptophan, and tyrosine. This protocol generates the $F(ab_2)$ fragments of IGg antibodies utilized in this experiment and numerous small Fc Fragments which are then separated by a 50,000 molecular weight cut off (MWCO) filter. The resulting solution containing the $F(ab_2)$ fragments was then concentrated to approximately 1.5mg/ml for use in the conjugation step**.**

2.4 Fluorescent Nanoparticle Selection

Luminescent colloidal semiconductor nanocrystals, or quantum dots, were chosen for this experiment over other commercially available fluorescent dyes or fluorophores due to their exceptional intensity, their resistance to bleaching, and their large Stokes shifts. These characteristics all support a highly sensitive system that allows for low concentration detection. The quantum dots used in the experiment were nanoparticles with a Zn coated CdSe core acquired from Invitrogen's Molecular Probes division. It has been established that the size of the quantum dot core proportionally affects the emission wavelength of the quantum dot. Quantum dots function similar to classically used fluorescent dyes in converting absorbed light of one frequency into emitted light of another frequency, however they do it with a much greater yield while also being more chemically stable. Furthermore, since quantum dots are available in a wide variety of emission wavelengths or "colors", separate conjugations can be developed in a single experiment. This multiplexing would allow for several targets to be tested for simultaneously each represented by a different emission wavelength.

2.5 Fluorescent Nanoparticle - $F(ab_2)$ conjugation

605nm quantum dots were activated using the hetero-bifunctional crosslinker, 4-(maleimidomethyl)-1-cyclohexanecarboxylic acid N-hydroxysuccinimide ester (SMCC), yielding a maleimide-nanocrystal surface. Concurrently DTT was used to reduce the antibody exposing free sulfhydryls. The chemical treatment of the antibody and conjugate allowed the two to covalently bond. The protocol and materials used were from the Quantum Dot Corp Antibody Labeling Kit. Individual solutions were conjugated, concentrated and purified for future experimentation. This protocol is available in a wide assortment of quantum dots which when paired with different fragments can lead to the detection of multiple contaminants in the same reaction.

2.6 BHQ-2 Quencher

Quenchers function by absorbing light within a wavelength range and converting it into heat, thus effectively dampening fluorescence. Currently most FRET detection is being accomplished either through quenching of a signal or through conversion of a signal to an alternate wavelength through pairing. In quenching quantum dots, the actual fluorescence is quenched by a neighboring particle, while in pairing of these molecules, the fluorescence of one fluorescent nanoparticle is absorbed by a neighboring fluorescent nanoparticle and reemitted at a different wavelength. Detection in a quenching protocol is based around an increase in net fluorescence at a particular wavelength while detection in a pairing protocol is based around a color or wavelength shift. While pairing methods show potential they are not a good selection for a protocol destined to develop into a multiplexing system as they are prone to cross-talk between fluorophores that will only increase as additional fluorophores of different wavelengths are added to the experiment. As such a quenching protocol was chosen for the study. The quencher chosen for the experiment was from Biosearch Technologies' Black Hole Quencher line, specifically Black Hole Quencher-2 (BHQ-2). BHQ-2 was chosen for its strong absorbance around 605nm which is the fluorescence of the quantum dots in use. Black Hole Quenchers are organic quencher compounds that offer exceptional quenching with no native fluorescence. Additionally, BHQs can quench throughout the visible and near IR spectra making them suitable for future multiplexing where multiple emission wavelength quantum dots will be in use.

2.7 Experimental Protocol

Solid *E coli* was obtained from KPL Inc. in the amount of 1mg containing ~3.0×10^8 colony forming units (cfu's). This was then resuspended in 2ml of 1x PBS. Quencher BHQ-2 was then added to 1ml of this E coli solution at a final concentration of 25ug/ml as suggested by the manufacturer and incubated for 2 hours in darkness. The rest of the E coli solution was then used for dilutions ranging from 10^8 to 10^2 cfu's for measurement with the Qdot - $F(ab_2)$ fragment conjugate system.

After the 2 hour incubation of the *E coli* with BHQ-2, the bacteria were washed of unbound quencher by two washes with 1ml of 1x PBS and centrifugation for 2 minutes at 13,000 rpm. This mixture was then resuspended in 75ul of 1x PBS and added to 75uL of Qdot - $F(ab_2)$ conjugate and reacted for 30 minutes in the dark. After 30 minutes this solution was washed with 1x PBS as before and resuspended in 500ul of 1x PBS. This step completed assembly of the nanosensor.

Each serial bacterial dilution (10^8 down to 10^2) of heat killed *E coli*, in this case, contained a volume of 450ul to which 50ul of the quenched Qdot - F(ab_2) solution was added. The reaction was then allowed to occur for 15 minutes and the results were read on a Fluoro-Max fluorometer with Xenon lamp excitation at 400nm. Each solution was excited at 400nm and the emission was measured from 500 to 700nm with a focus on 605nm.

3. RESULTS AND DISCUSSION

3.1 FRET Based Sensing modalities

Figure 2 shows the addition of increasing amounts of Black Hole Quencher-2 to steady state 605nm Quantum Dots (Qdots) with previous 15 minute incubation. This was done to estimate the correct concentration of quencher to BHQ-2/Quantum Dot to adequately diminish the fluorescent signal but still maintain a background baseline of fluorescence. As seen in the graph, the FRET efficiency of the BHQ-2 quencher is concentration dependent

The decrease in signal is due to transfer of energy from a donor (605nm Qdot) to an acceptor (BHQ-2) in lieu of being emitted as a photon of light, which is manifested as fluorescence resonance energy transfer (FRET). Quantum dots are fluorophores capable of emitting tremendous amounts of fluorescent energy and thus interacting with multiple acceptors which in this particular case are quenchers. The rate at which these quencher can absorb the fluorescence is dependent on the concentration of both the Qdots and quenchers. This can be expressed by Equation 1.

$$E = nR_0^6/(nR_0^6 + r^6) \quad \textbf{(Eq. 1)}$$

This states that the rate of FRET (E) is dependent on the number of acceptors interacting with a donor molecule (*n),* the Förster distance (R_0), and the distance of centro-symmetric equal separation between donor molecules (r). In order to calculate the efficiency, the Förster distance must first be evaluated. The Förster distance is defined as the optimal distance at which FRET efficiency is estimated to be at about 50%. It is given by equation 2

$$R_0 = (BQ_D J)^{1/6} = \left[\frac{9000(\ln 10)\kappa_p^2 Q_D}{N_A 128\pi^5 n_D^4} J \right]^{1/6} \quad \textbf{(Eq. 2)}$$

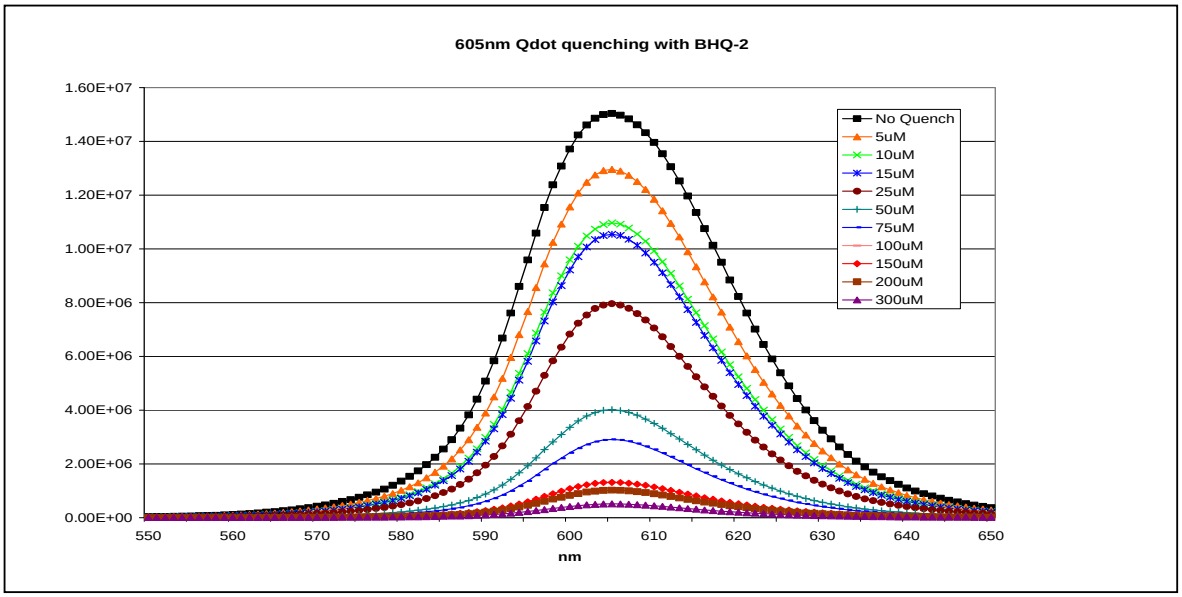

Figure 2. Varying concentrations of BHQ-2 quencher were incubated with 4uM Quantum dots for 0.5 hours in the dark. They were then measured on a fluorometer to determine what concentration of quencher to use for future experiments.

Referring now to Equation 2, Q_D is the quantum yield of Qdots, I is the integral of spectral overlap between acceptor absorption and donor emission, B is a function of Avagadro's number, N_A is the refractive index of the medium, n_D, and a parameter κ_p which depends on the dipole orientation of the individual molecules. We used 0.66 for dipole orientation as based on previous experimentation and other publications for randomly oriented dipoles. The value used for n was limited to 36 to provide an estimate of the separation distance from the Quantum dot center to the acceptor along with the size being approximately 77 Å from center to surface for 605 nm Qdots. These values along with constants and the experimentally available values (molar concentrations, medium refractive index, etc.), allow development of the graph in Figure 3, which shows relatively good agreement between experimental data and predicted intensities.

At higher concentrations of quencher, the experimental values do not attain the value described by the "ideal" FRET efficiency which assumes no loss of energy in the system. This discrepancy is due an increased molar ratio of quenchers to Quantum dots. There is a finite surface area available on each quantum dot and once that area is occupied, there is an achievement of maximum quenching. No matter what total concentration of BHQ-2 is added to the solution, the level of quenching will not rise.

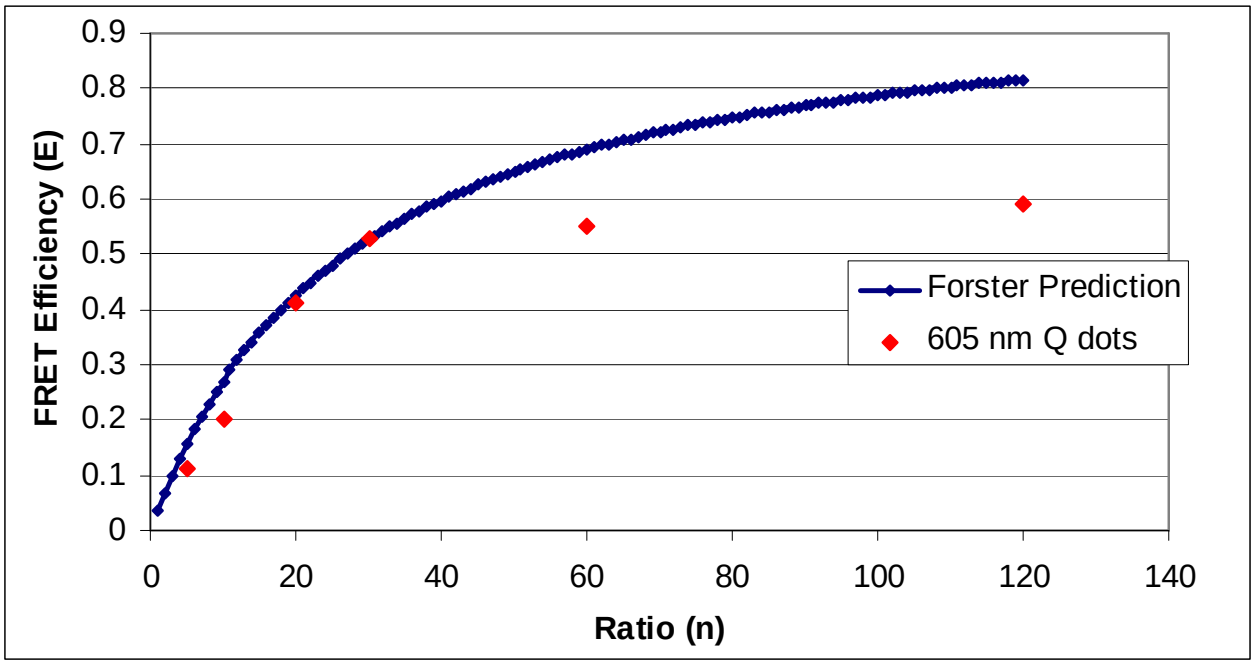

Figure 3. Forsters Radius from quenching assay experiments vs. predicted Forsters radius.

3.2 Detection Threshold

This detection protocol has resulted in a sensing modality with a detection threshold of 10^2 cfu's/mL, as shown in Fig 4. At his time this threshold is well within diagnostic needs for detection of food-borne pathogens. Furthermore, the intensity of fluorescence is dependent on the concentration of target cells present, allowing for a quantitative calculation of the amount of target present, as shown in Fig. 5. The ability to detect and identify which contaminants are present, as well as the concentration allows for a highly adaptable protocol that can be applied to any number of biological contaminants. Through the use of multiple quantum dots of varying emissions a single nanosensor device could be developed that is capable of detecting and identifying multiple biological contaminants simultaneously. Furthermore, this sensing modality is fairly rapid, as it has the ability to detect the target cells in less than 3 minutes.

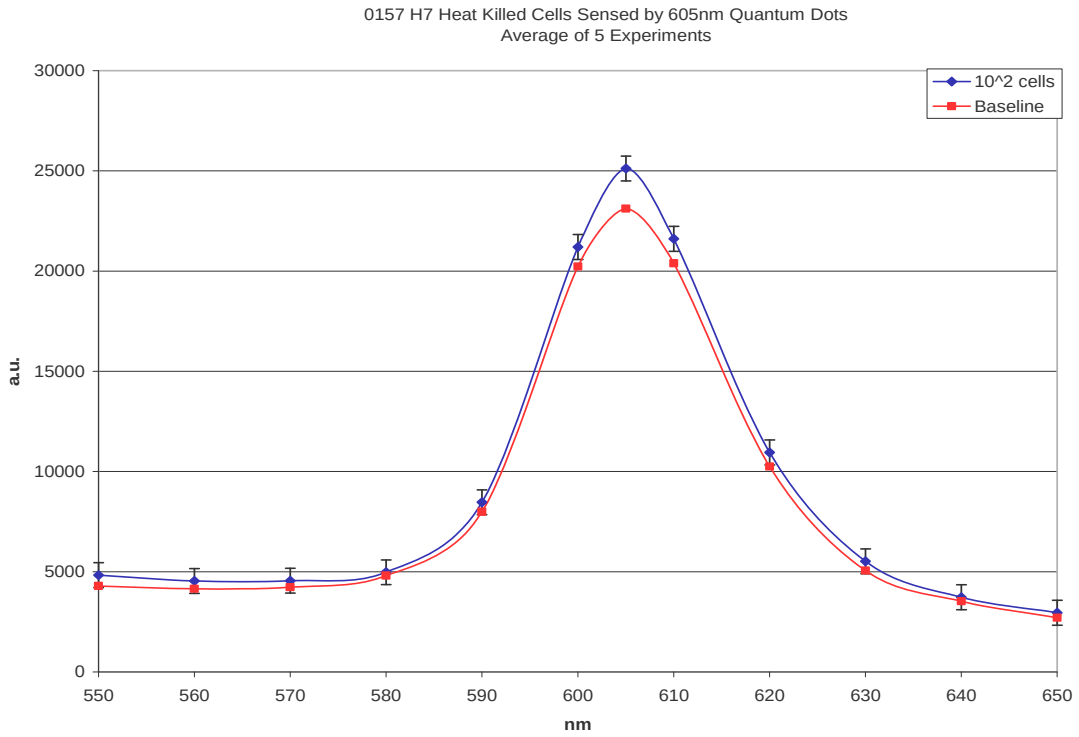

Figure 4. Consistent low-end concentrations of 10^2 cfu show that even 100 cfu's can be accurately measured by the Qdot – F(ab2) system.

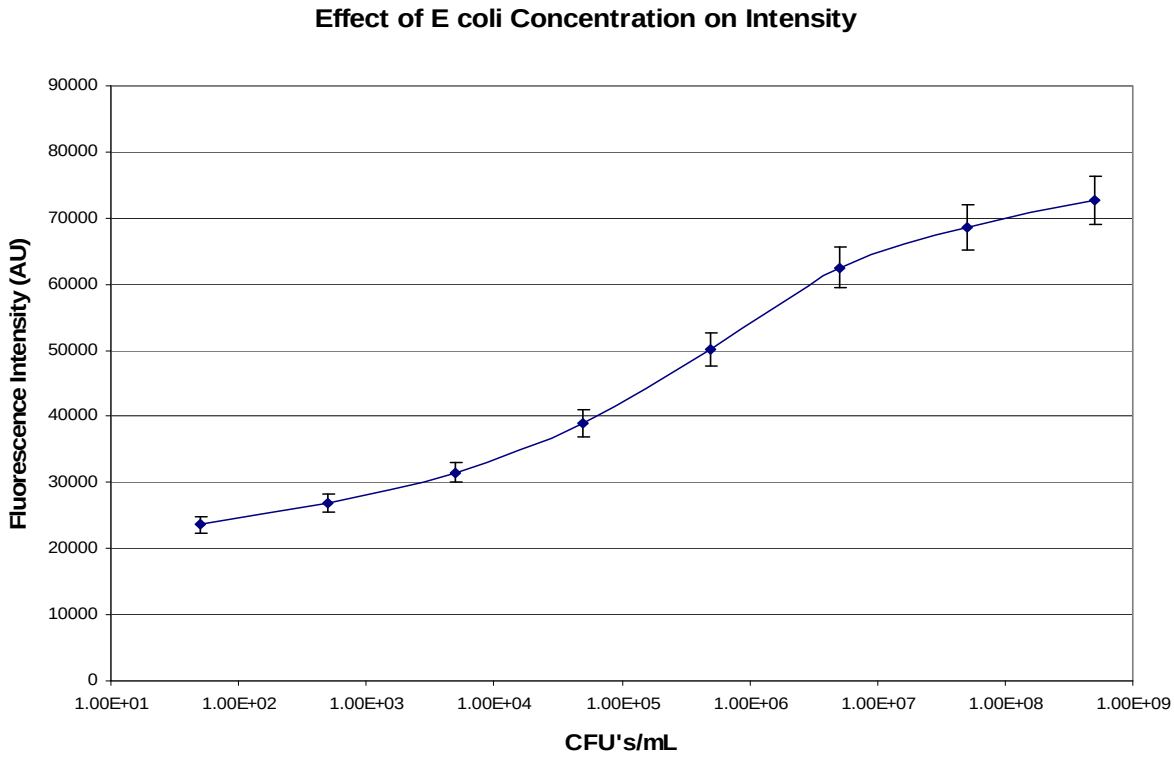

Figure 5. Nanosensor Fluorescence Intensity vs. Concentration

3.3 Specificity

Limited experiments have been conducted to study the specificity of the nanosensing modality for the targeted contaminants over non-targeted contaminants. Specific binding of the target contaminant has been achieved in 2 nanosensors thus far studied. In this case, the first nanosensor was designed to detect only E coli and the second sensor was designed to detect only ovalbumin. Fluorescent nanosensors that emit at 625 nm were conjugated with Ovalbumin antibodies, while nanoparticles that emit at 705 nm were conjugated with E coli antibodies. When both nanosensors were placed into the same solution, only the ovalbumin nanosensor responded with emissions, when challenged with ovalbumin, as shown by the red curve in Figure 6. Likewise, only the E coli nanosensor responded with emissions when challenged with E coli, as shown by the blue curve in Figure 6. The demonstrated specificity has the potential to allow for accurate identification of multiple contaminant targets simultaneously present (multiplexed solution).

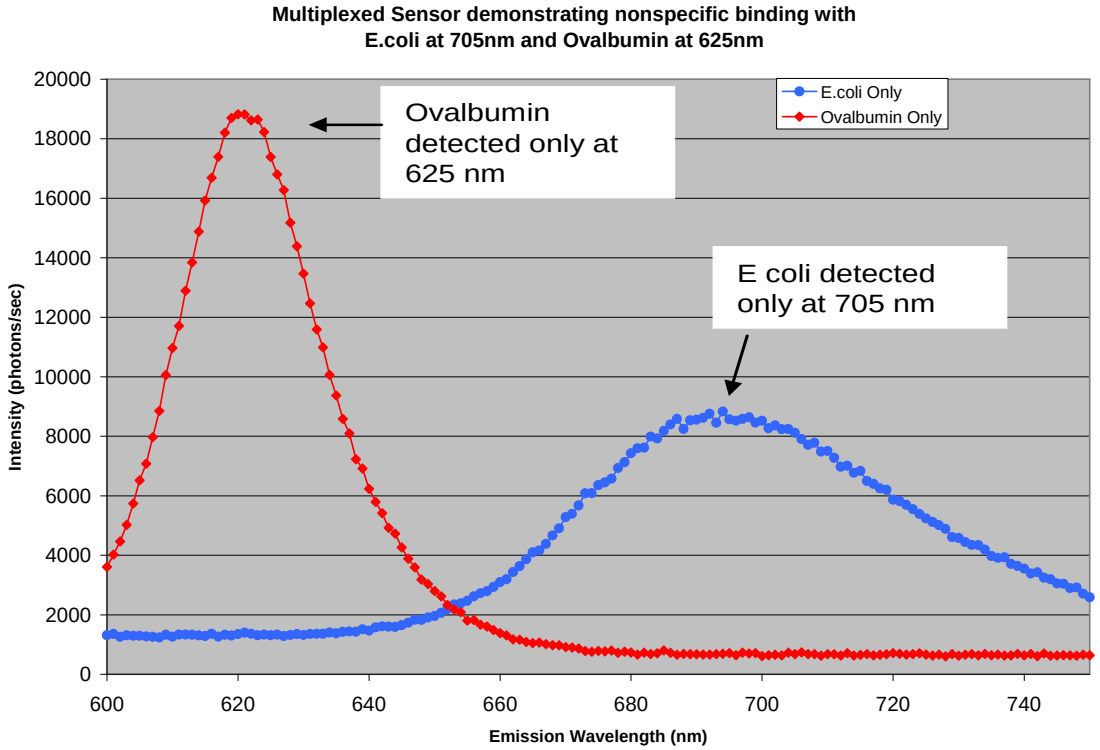

Fig. 6. Selectivity of FRET based nanosensor for E coli and Ovalbumin

4. CONCLUSIONS

A nanoscale sensing modality that allows for detection of bacterial contaminants has been developed and tested using fluorescent semiconducting nanoparticles conjugated with antibody fragments of targeted biological contaminants. The nanosensors have demonstrated high sensitivities, down to as low as 100 cfu for E coli, and excellent selectivity. The

demonstrated ability to detect not only the contaminants, but also the concentration allows for a highly adaptable protocol that can be applied to any number of biological contaminants. Through the use of multiple quantum dots of varying emissions a single device can be developed that scans for multiple threats simultaneously

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Although highly desirable in terms of speed and ease of execution, there are few examples of FRET-based immunoassays in the literature. More often studied are FRET based PCR assays involving a fluoroscein dye quenched in the presence of Tetramethyl-6-Carboxyrhodamine (TAMRA). However, as the present work demonstrates, immuno-assays utilizing FRET have great potential for the development of real-time biosensors. These facts make the present work

valuable in terms of methodology and even broader potential microbiological applications.

This sensing modality has potential for use by the warfighter in devices that can sense biological contamination in buildings n heating ventilation and air conditioning (HVAC) systems and in potable water systems.

Poor indoor air quality (IAQ) can result from biological contaminants such as airborne toxins from molds. Mold is a growing and increasingly important problem for Army installations, as it can damage buildings and affect the health of building occupants. Molds can grow on virtually any substance, as long as moisture or water, oxygen, and an organic source are present. Protection of personnel against non-BWA/non-CWA biological contaminants that cause IAQ and other toxic hazards is an evolving requirement. Many bio-contaminants have been identified in HVAC systems; they affect Indoor Air Quality (IAQ) and pose adverse health risks, such as: (1) allergic reactions. (2) respiratory irritations. Usually there is a mix of mold growth and bacteria together in wet HVAC environments Filtration helps, but in some cases can make matters worse, as fungi can grow on HEPA filters. Other airborne toxic hazards and disease-producing organism can be transmitted in facilities HVAC systems as well.

Hygienic water is the single most important aspect to soldier survivability. Insuring waters are free of disease causing microorganisms is of the utmost importance in maintaining a viable fighting force in order to win the Nation's wars. Additionally, as environmental regulations become increasingly stringent and contaminants released via Army related activities need to be closely monitored to insure compliance.

Failure to do so will inevitably lead to training restrictions and/or range closures, directly impacting the Army's mission to prepare soldiers for battle.
Currently used methods for detecting and identifying contaminants in both air and water are relatively slow, and often require laboratory analysis. Also, they do not allow detection of many contaminants using a single sensor., in contrast to the present nanosensing modality.

Although originally developed for sensing airborne contaminants such as mold spores and viruses in heating ventilation, and air conditioning (HVAC) systems, this nanosensor has great potential for detecting and identifying biowarfare agents injected into air intakes of HVAC systems, or internally released, or introduced intentionally into potable water systems. Furthermore, the nanosensor has potential for use as a portable system that may be conveniently carried by the warfighter in the field.

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