

RECEPTOR-CONJUGATED NANOPARTICLES TO DETECT BIOLOGICAL WARFARE AGENTS

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ABSTRACT

Recent research has examined the feasibility of detecting biological warfare agents by conjugating their antibodies (receptors) with taggant nanoparticles (also known as “quantum dots”), which subsequently fluoresce upon excitation, when they are bound to a specific biowarfare agent, or its simulant. Furthermore, when they react with their target bacteria, optically excited nanoparticle-receptor conjugates generate spectra in which the intensities of primary emission peaks are diminished, while the secondary emission peaks increase in intensities, i.e., energy is transferred from major peaks to minor peaks. These optical emission spectral signatures, with emission wavelength shifts of 140 nm in some cases, strongly suggest the possibility of homogeneous (one step) assays, leading to positive detection of bacterial agents, without wash steps using nanoparticle-receptor conjugates.

1. INTRODUCTION

Army Installations need the capability to rapidly detect and identify biowarfare agents (BWAs) to determine the appropriate countermeasures required to protect personnel. Recent research has focused on

determining the feasibility of detection of biowarfare agent simulants representing single small-scale release of BWAs, such as anthrax or smallpox, dispersed as non-explosive point sources, external or internal to buildings at Army installations. One means of detecting BWAs on surfaces, such as walls and floors is by the use of an aerosol, which could be sprayed into the surfaces where the nanoparticle-antibodies conjugates would bind to their target BWA. As shown in Figure 1, a handheld fluorometer could be used in the field to analyze the emissions from the bound BWAs, or wipe samples taken from suspected surfaces could be analyzed more accurately in the laboratory by fluorometry.

These detection systems to be used in such an aerosol are based on innovations that capitalize on the ability of antibody receptor-nanoparticle conjugates to change the intensities of their optical emission wavelengths upon binding to BWA or their simulants. In recent years, nanoparticles have received considerable attention for their high fluorescence efficiency, lack of photobleaching, and long fluorescence (decay) lifetimes, properties which allow nanoparticles to compete with

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conventional fluorescent dyes for many applications [1,2].

The taggant nanoparticles being used are biologically inert, conjugation ready, nano-scale particles for assay developers. They are based on the unique characteristics of nanocrystal quantum dots, composed of CdSe/ZnS and offer the optical and chemical characteristics, ideal for high stability, color multiplexing, single excitation assays, and they are available with carboxyl or amine terminal groups for conjugation, and in sizes ranging from 30 to 50 nanometers and have multiple reactive functional groups per particle. “Adirondack Green” and “Fort Orange” nanoparticles both have excitation maxima near 400 nm and emission peaks of 520 nm and 600 nm, respectively.

Nanoparticles (NP) composed of CdSe/ZnS appear to down shift or “blue” shift their fluorescence emission by 60-140 nm when they are conjugated to antibodies and bound to bacterial surfaces. The shift appears to occur by adding energy from the fluorescence emission to a minor peak near 440-460 nm that exists for the unconjugated and unbound NPs themselves. This minor peak near 440-465 nm appears to increase in intensity with concentration of analytes in the two systems studied with various species of bacteria as the target analytes. It is not known at present if other quantum dot compositions beside CdSe/ZnS exhibit similar shifts.

NPs essentially “confine” electrons and photons and their size dictates fluorescence emission wavelength. Other investigators have observed changes in NP size and emission wavelength allegedly due to oxidation and ionic strength or other environmental effects that were thought to affect the size and shape of the NP [3, 4]. It is possible that NP-antibody conjugates, which bind bacterial or other cell surfaces, might experience a different chemical interface, which might alter the NPs’ size or deform their shape, thereby altering their emission wavelength. Results from the current investigation suggests that these observations of blue shifting upon binding of NP conjugates to bacteria may be useful in the development of one step (homogeneous) assays without wash steps.

2. MATERIALS AND METHODS

2.1 NPs, Antibodies, and Bacteria.

“Adirondack Green” and “Fort Orange EviTags™” (NPs) were purchased from Evident Technologies Inc. (Troy, NY). These classes of NPs both have excitation maxima near 400 nm and emission peaks of 520 nm and 600 nm, respectively. Both amine and carboxyl derivatives of these NPs were used on

separate occasions and conjugated to antibodies as described below. Murine anti-*Escherichia coli* O111:B4 monoclonal IgM was purchased from Novus Biologicals, Inc. (Littleton, CO). A simulant for anthrax, known as *Bacillus globigii* (BG) and its antibody were used in some of these experiments. BG spores were obtained from Dugway Proving Ground, while BG polyclonal antiserum was the kind gift of Dr. Richard Karalus at the Calspan Research Center in Buffalo, NY.

2.2 NP Conjugation to Antibodies

In general, 6.25 µg (25 µL) of EviTag NPs (amino or carboxyl terminated) were added to 4.3 mL of sterile deionized water and 0.5 mL of sterile 10X PBS (0.1 M Phosphate Buffered Saline, pH 7.2 to 7.4) with 200 µl (10 mg) of sterile EDC (1-ethyl-3-(3-dimethylamino propyl) carbodiimide). The solution was allowed to incubate at room temperature (RT) for 1 h with occasional mixing. Then 0.2 mg of IgM antibodies or 1 mg of IgG antibodies was added and the solution was further incubated at RT for 2 h. The reaction was stopped with 5.5 ml of sterile 1 M Tris (pH 7.4). The liquid was transferred to a spin filter centrifuge and spun at 3,000 x G for 30 to 60 min. The retentate containing the antibody-NP conjugate was stored at 4°C until used.

2.3 Spectrofluorometry

Samples were diluted up to 4 ml in 1X phosphate buffered saline (PBS) or 1XBB as appropriate and analyzed in plastic cuvettes on a Jobin-Yvon Fluoromax 3 spectrofluorometer with 400 V PMT setting, 0.05 second integration time, sensitivity and threshold settings of 1. Excitation was always at 400 nm with 5 nm excitation and emission slits. Bacteria were carefully resuspended immediately prior to emission spectra acquisition.

2.4 Fluorescence Microscopy

To confirm that NP-antibody conjugates were bound to bacteria, 100 µl of undiluted antibody-Adirondack Green and Fort Orange NP conjugates were added to heat-fixed bacterial smears on microscope slides. Antibody-NP conjugates were allowed to bind for 1 hour at room temperature and were then rinsed with 1X PBS for several minutes. Coverslips were added to 1X PBS-wetted slides and they were examined on an Olympus BH-2 fluorescence microscope with a standard fluorescein filter cube (blue excitation for both the Adirondack Green and Fort Orange NPs) and photographed or digitally captured with a video camera.

3. RESULTS

Figure 2 shows the fluorescence emission spectra for anti-*E. coli* O111:B4 IgM antibodies conjugated with Adirondack Green NPs, diluted to a concentration of approximately 2.8 million bacteria per milliliter. Panel A shows the spectra for the NP-conjugates before they were allowed to bind to their target *E. coli* bacteria. Note that a major emission peak appears at 520 nm, along with a minor secondary emission peak at 460 nm. This natural secondary emission peak is seen throughout the data and appears to be a common minor peak for CdSe/ZnS materials, which resides around 440 nm to 460 nm. Upon binding to the target *E. coli*, the 460 nm peak grows in intensity at the expense of the primary 520 nm peak. This indicates a transfer of energy from the 520 nm peak to the 460 nm peak. The 60 nm shift from the expected emission peak of 520 nm to the observed peak at 460 nm is impressive. However, as seen in subsequent figures, the Fort Orange NP-conjugate systems demonstrate even more remarkable wavelength shifts (Figures 3-5).

Figure 3 shows the fluorescence emission spectra for the Fort Orange NP conjugated to the *E. coli* antibodies. The primary emission peak at 605 nm dominates the spectra, but the emission peak at 465 nm is extremely low in intensity and cannot be seen on this fluorescence scan. However, as shown in Figure 4, when the NP-conjugated with *E. coli* antibodies are allowed to react and bind with *E. coli*, the 465 nm peak increases in intensity as the primary 605 nm peak is diminished. In this case, there is a 140 nm wavelength shift. This phenomenon was observed whether the *E. coli* concentrations were 0.1 mg/ml (2.8 million bacteria/ml), 0.01 mg/ml (280 thousand bacteria/ml), or 0.001 mg/ml (28 thousand bacteria/ml). As the optical path of the fluorometer passes through a sample volume of 0.1 ml, the number of bacteria being detected are 280 thousand, 28 thousand, and 2,800, respectively. Thus, the intensities of all peaks appear to increase in proportion to the concentration of target bacteria analyte.

In order to further explore this effect for a typical biowarfare agent, similar fluorescence investigations were conducted for an anthrax simulant, *Bacillus globigii* (BG) and its antibody conjugated to Fort Orange NPs. As shown in Figure 5, upon binding with their target BG spores, the BG antibody- NP conjugates show the same 140 nm shift as previously observed regardless of the BG concentration. Once again, the intensities of all peaks appear to increase in proportion to the concentration of target spore analyte. This observation is significant for the prospect of using the NP-conjugates to quantify the amount of biowarfare agent that could be detected, however, further investigation is warranted. In this case, the three concentrations of BG detected were 0.1 mg/ml, 0.01 mg/ml, and 0.001 mg/ml, representing 5 million, 500

thousand, and 50 thousand colony forming units (cfu), respectively. The detection limit of the technique for BG spores appears to be around 5 cfu per cm². In this case, wipe samples would have to be taken from a surface area of 1,000 cm² and analyzed in a laboratory fluorometer.

In all cases investigated here, the magnitude of the wavelength shift appears to be specific to the nanoparticle used as a taggant, and not to the antibody with which it is conjugated. This allows the capability of using a cocktail of nanoparticle-conjugates to identify multiplexed bacterial agents that may be present simultaneously.

Finally, to definitively confirm that antibody-NP conjugates were binding to the bacteria, fluorescence microscopy was employed and demonstrated very well-defined punctile fluorescence of the antibody-NPs on *E. coli* for both the Adirondack Green- and Fort Orange-antibody systems, as shown in Figure 6. The fluorescence images do not seem to show a noticeable color shift, which may be why this phenomenon has gone unnoticed by other investigators who did not employ spectrofluorometry.

4. DISCUSSION

Other investigators have reported mild blue shifts of 30-40 nm for CdSe/ZnS NP fluorescence due to oxidation, changes in pH, the presence of divalent cations and other environmental factors [3]. However, to the best of our knowledge this is the first report of a major blue shift due to binding of receptor-NP conjugates to bacterial surfaces. Schaertl, et al. [5] reported nanoparticle immunoassay formats which did not apparently exhibit fluorescence shifts, but did not require wash steps and therefore constituted true homogeneous assays for proteins and small molecular targets. Here those observations have been extended to the detection and possible quantitation of bacteria based on wavelength shifts.

The mechanism of the blue shift in fluorescence is unknown, but it may be due to environmental factors such as differences in hydrophobicity, hydrophilicity, pH, electric charge, etc.

5. CONCLUSIONS

In this paper, it has been shown that large NP-antibodies (150 kD IgG) exhibit dramatic blue shifts of up to 140 nm, when conjugated with nanoparticles, upon binding to the bacterial surface. Interestingly, both the Adirondack Green and Fort Orange NP-conjugates exhibited blue shifts back to a minor secondary peak in the vicinity of 440 nm to 460 nm. This 440-460 nm peak is barely present in fluorescence spectra of both kinds of

NPs alone without chemical conjugation or binding to bacteria. Both types of NPs are composed of CdSe/ZnS, but differ in average core diameter (4.3 and 6.3 nm respectively for Adirondack Green and Fort Orange according to the manufacturer; Evident Technologies), so they might be expected to share some fluorescence spectral features such as minor secondary emission peaks. The intensity (energy distribution) of this natural secondary fluorescence peak grows significantly upon binding of the NP conjugates to bacteria in several different receptor and bacterial (*Bacillus globigii* or *E. coli*) systems and that may make NP systems potentially very valuable for immunoassays and molecular biology applications, including biological warfare agent detection.

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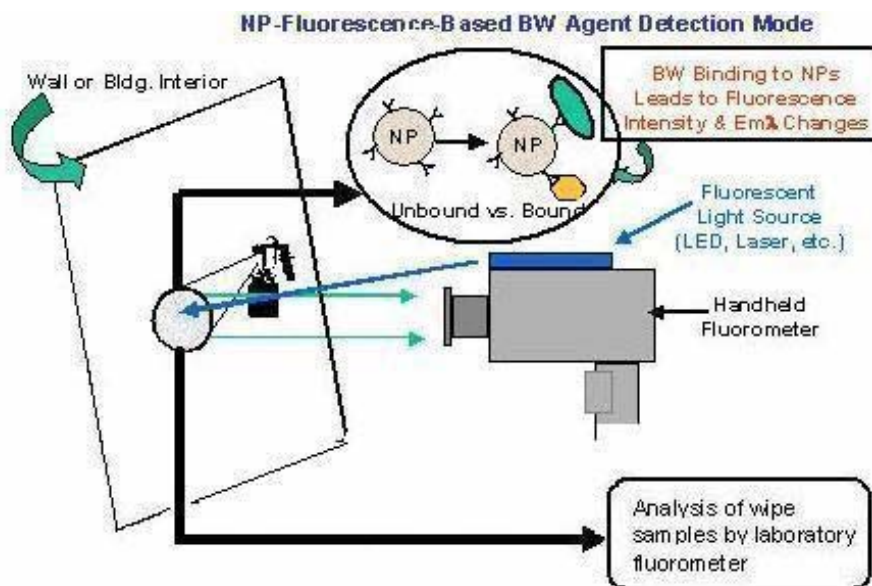


Figure 1. Schematic diagram of detection scheme for biological warfare agents using antibodies conjugated with nanoparticles.

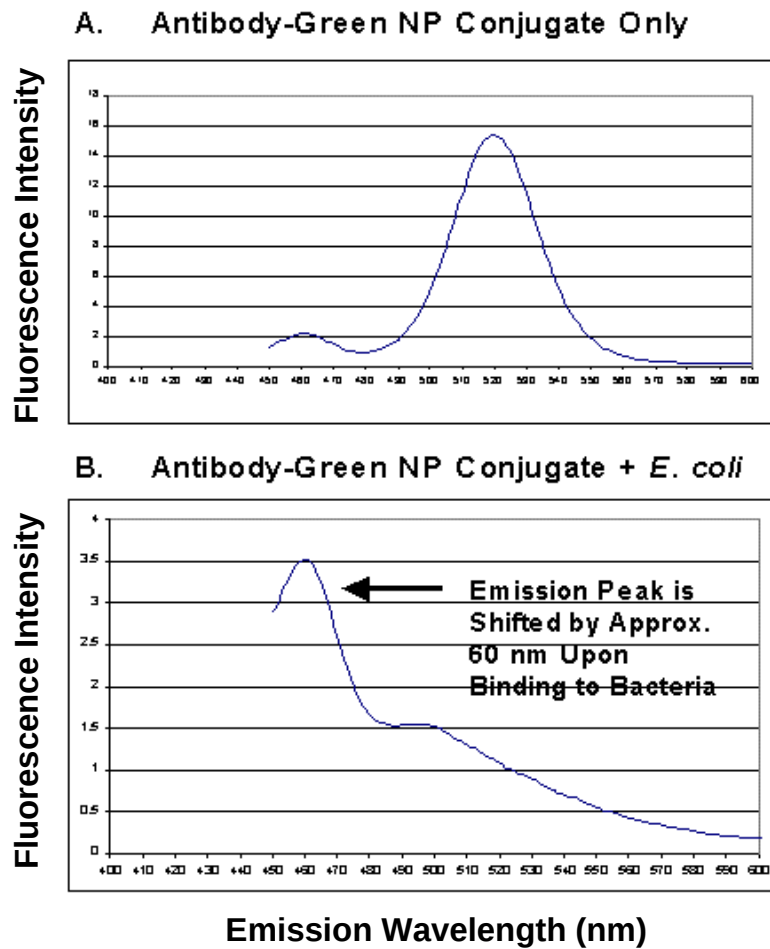


Figure 2. IgM *E. coli* antibody-Adirondack Green EviTag (NP) fluorescence spectra- Panel A: Unbound conjugates; Panel B: after binding with an estimated 2.8×10^6 *E. coli* O111:B4 bacteria.

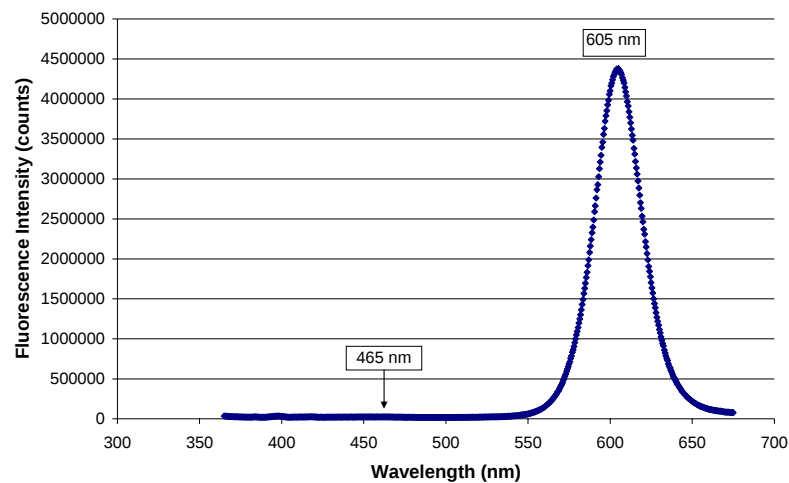


Figure 3. Fluorescence of unbound “Fort –Orange” NP conjugated to *E. coli* Antibodies. Note the primary peak at 605 nm and the absence of peak at 465 nm.

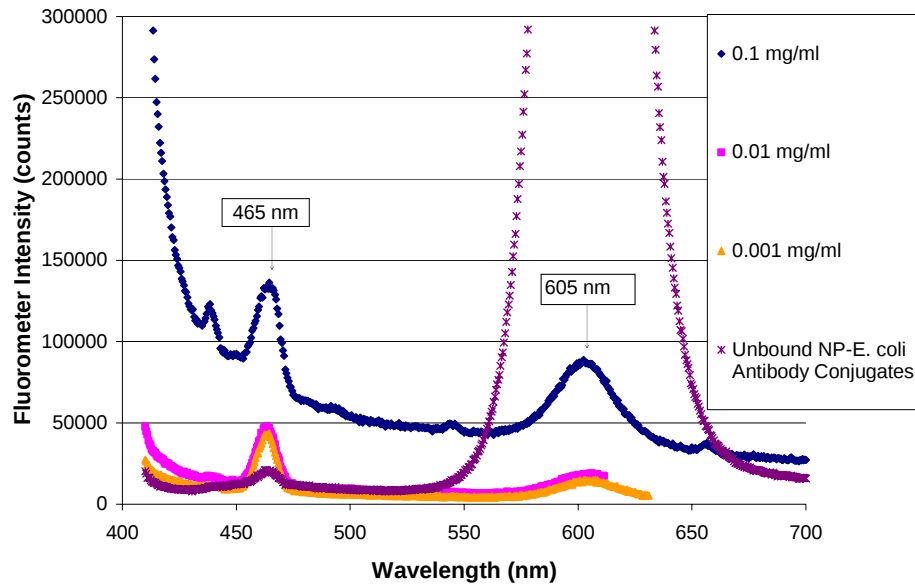


Figure 4. Fluorescence emission spectra of Fort Orange NPs conjugated to IgG *E. coli* antibody and after binding to target *E. coli* bacteria. Note the emergence of emission peaks at 465 nm for all concentrations of *E. coli*, and the decrease in intensity of the 605 nm peak as compared with the unbound NP-E.coli.

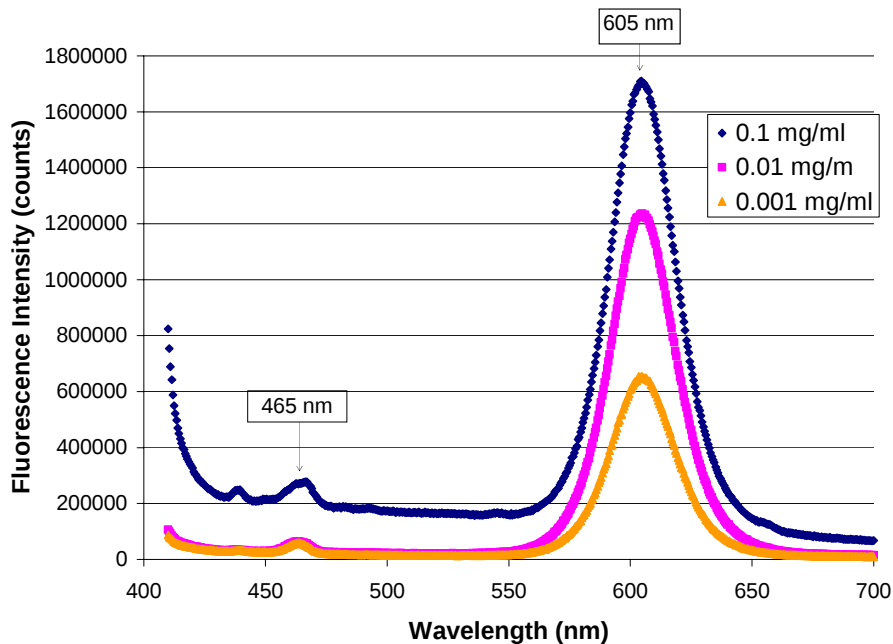


Figure 5. Fluorescence emission spectra of Fort Orange NPs conjugated to *Bacillus globigii* (BG) antibody and after binding to target BG. Note the emergence of emission peaks at 465 nm for all concentrations of BG.

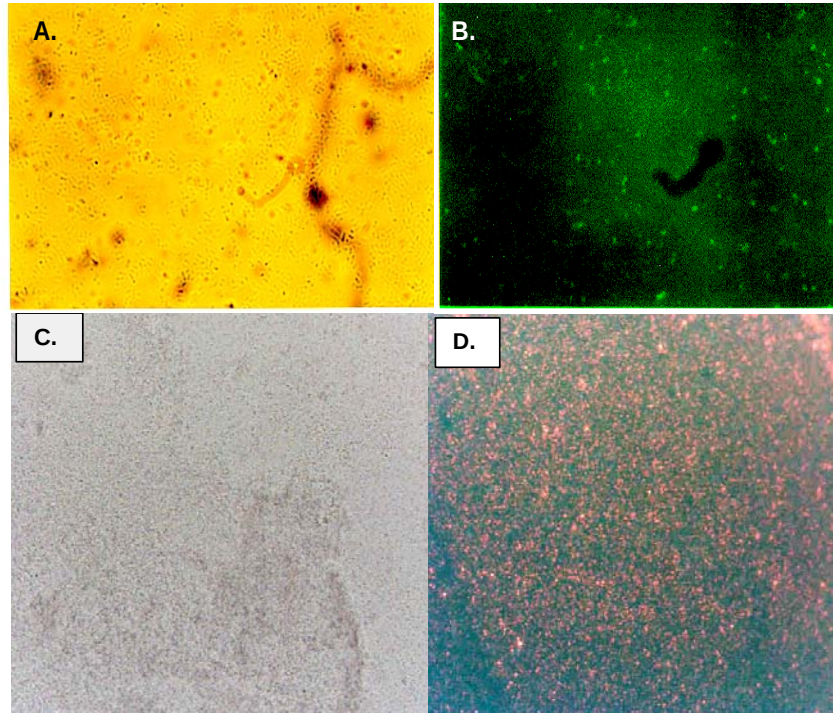


Figure 6. Panel A is a brightfield image of *E. coli* O111:B4 stained with anti-*E. coli* IgM antibody-Adirondack Green NP conjugate. Panel B shows the same sample under fluorescence microscopy using a fluorescein filter cube (blue excitation). Panel C is again a brightfield image of *E. coli* O111:B4 stained with anti-*E. coli* IgM antibody-Fort Orange NP conjugate and panel D is a blue-excited fluorescence image of the same sample. All images were taken at a total magnification of 400 X.