Mega-Nano Detection of Foodborne Pathogens and Transgenes Using Molecular Beacon and Semiconductor Quantum Dot Technologies

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***Abstract*—Signature molecules derived from *Listeria monocytogenes*, *Bacillus thuringiensis*, and *Salmonella* Typhimurium were detected directly on food substrates (mega) by coupling molecular beacon technology utilizing fluorescent resonance energy transfer (FRET), luminescent nanoscale semiconductor quantum dots, and nanoscale quenchers. We designed target DNA sequences for detecting *hlyA, Bt cry1Ac*, and *invA* genes from *L. monocytogenes*, *B. thuringiensis* and *Salmonella* Typhimurium, respectively, and prepared molecular beacons for specific targets for use in real-time monitoring. We successfully detected increased fluorescence in the presence of signature molecules at molecular beacon (MB) concentrations from 1.17 nM to 40 nM, depending upon system tested in (water, milk or plant leaves), respective target (*hlyA, Bt cry1Ac*, or *invA*) and genomic DNA target concentration (50–800 ng). We were able to detect bacterial genomic DNA derived from *L. monocytogenes* and *Salmonella* sp. in a food system, 2% milk ($>$ 20% of total volume). Furthermore, we infiltrated the *Bt cry1Ac* beacon in the presence of genomic DNA extracted from *B. thuringiensis* into *Arabidopsis thaliana* leaves and observed increased fluorescence in the presence of the target, indicating the ability to use these beacons in a plant system.**

***Index Terms*—Detection, DNA, foodborne pathogens, molecular beacon, quantum dots.**

I. Introduction

LUMINESCENT semiconductor quantum dots and nanoscale quenchers in combination with DNA recognition properties are exploited herein to fabricate molecular beacons (MBs) capable of identifying bacterial pathogens. As discussed herein, these quantum-dot-based probes provide a viable means of determining the presence of particular biological structures, including bacterial pathogens.

Bacterial pathogens are a concern for a wide range of industries (agriculture, food, cosmetic, personal care) and government agencies (DOD, FDA, USDA, DHS). Detection techniques that are able to sense and monitor low levels of pathogens rapidly, with ease, sensitivity, and selectivity in a variety of products, foods, and environments are necessary in today's global production system. Bacterial detection within these systems can be challenging because of the sample complexity both pathogen and the product or substrate where pathogens are being detected (pH, interfering substances, non-pathogenic bacteria, etc.). An ideal product protection system would provide for a rapid, real-time, high-throughput assay to monitor and detect bacterial pathogens in products, such as crops and foods, providing protection from the farm to consumer. Further, this technology could be used for the protection of our crops and foods from bioterrorism agents, such as *Bacillus anthracis*.

Foodborne illnesses are a constant threat to public health. Of the 31 major foodborne pathogens, an estimated 9.4 million people become sick with greater than 55 961 hospitalizations, and 1351 deaths occurring each year in the United States [1] and with an estimated yearly economic cost of $77.7 billion [2]. Of these illnesses, two bacterial pathogens are of importance, *Listeria monocytogenes* and *Salmonella* Typhimurium. *L. monocytogenes* is a Gram-positive intracellular pathogen that is commonly found infecting foods such as soft cheeses, deli meats, hotdogs, and raw milk [3]. The *hlyA* gene, successfully used as an amplification target using *in vitro* techniques, encodes for the production of listeriolysin, a secreted protein responsible for *Listeria monocytogenes* infection and replication [4], [5]. *Salmonella* is a Gram-negative pathogen that has greater than 60 genes coding for 200 virulence factors [6]. Foods commonly associated with *Salmonella* infection include foods of animal origin, beef, poultry, milk, and eggs [3]. There are greater than 2200 serovars of *Salmonella*, all containing the *inv* gene, which is responsible for the invasion of the bacteria into cells and has been successfully used as an amplification target for the detection of *Salmonella* using *in vitro* techniques [7], [8].

Current standard microbiological methods used for quantifying and detecting the presence of microorganisms in foods is vast; however, these methods are laborious, have limited specificity and sensitivity, and require longer periods of time for adequate identification. A new and promising detection method is the use of molecular beacons (MBs) coupled with the use of the fluorescent molecules, quantum dots (QDs). MBs are nucleic acid probes, consisting of between 15 and 25 nucleotides, with a fluorescent molecule at one end and a quencher molecule at the opposite end [9]. When the MBs are not in the presence of their target molecules, they form a hairpin structure

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where the fluorophore is in close proximity to the quencher molecule, quenching the fluorescence. However, when the target molecule is present, the MB binds to its complementary nucleic acid in the target, allowing the fluorophore to move far enough from the quencher molecule to observe and measure the emitted fluorescence. MBs utilizing QDs have been found to be more sensitive and easily detectable than traditional fluorophores and have been shown to detect multiple pathogens within a single sample [10]. While MBs have most often been used in *in vitro* techniques such as quantitative PCR, they seem to be particularly suited for *in vivo* gene expression analysis, especially for monitoring specific mRNA pools in cells [11], [12]. In addition to nucleic acids [13], [14], a variety of other different biomolecules, including proteins [15] and/or antibodies [16]–[21] have been attached to QDs for specific biological targeting of bacterial pathogens such as *Escherichia coli*, *Salmonella*, or *Shigella* and toxins such as staphylococcal enterotoxin B [21]. Furthermore, MBs have successfully been used to detect rRNA in bacterial cells [4] and mRNA in mammalian [22] and fly [23] cells. In each eukaryotic organism, the MBs were microinjected into cells and cells were visualized under fluorescence microscopy. Several combinations of fluorophore and quencher pairs are available, yielding many available colors and fluorescence resonance energy transfer pairs (FRET) [12], in which excitation light is transduced from one fluorophore to another. Bratu *et al.* [23] provide an especially sophisticated demonstration of the power of molecular beacons in visualizing mRNAs in living cells. They utilized an altered RNA backbone by substituting an oxymethyl group for the hydrogen atom at the second position of the ribose on each nucleotide, therefore conferring nuclease resistance. RNases could otherwise digest the molecular probes prior to hybridization. These researchers also utilized two different fluorophores designed to hybridize head-to-head along an mRNA strand yielding a FRET readout. In this case FRET assures that RNA probes are interacting (if fluorescence is observed) as well as avoiding endogenous autofluorescence. As a result of target hybridization, molecular beacon fluorescence could be detected in 15 min and intracellular transport of mRNA could be visualized. While advancements have been made with using QDs in plant tissues [24], the use of MBs in conjunction with QDs to target the presence or absence of specific pathogens within and on the surfaces of foods and crops has not been achieved.

The aim of this study was to develop a MB-based technology coupling QDs for detecting the bacteria, *Listeria monocytogenes*, *Bacillus thuringiensis* and *Salmonella* Typhimurium, using specific signature molecules (*hlyA*, *Bt cry1Ac*, and *invA*, respectively) for monitoring the presence of bacterial targets in real-time on several substrates (products). This technology provided a real-time monitoring technique for use in food defense and safety as well as a potentially new technique for the detection of transgenes (*Bt cry1Ac*) in plants.

II. Materials and Methods

A. Molecular Beacon Design

Molecular beacon probes were designed using BeaconDesigner™ (PREMIER Biosoft) complementary to target DNA sequences for detecting *hlyA*, *Bt cry1Ac*, and *invA* genes from *Listeria monocytogenes*, *Bacillus thuringiensis* (Gram-positive) and *Salmonella* Typhimurium (Gram-negative) respectively (Table I, Fig. 1).

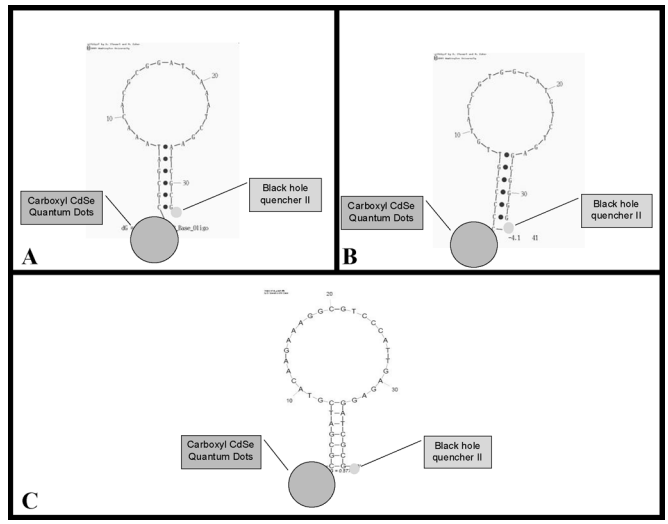

Fig. 1. Hairpin structure of MBs in quenched form. MBs designed for the detection of the *hlyA* gene of *L. monocytogenes* (A), the *invA* gene of *S.* Typhimurium (B), and the *Bt cry1Ac* gene from *Bacillus thuringiensis* (C).

TABLE I
Target DNA Sequences

Gene	Target DNA Sequence*
hlyA	GAT TT GAT CCG CGT GTT T
invA	GCT CAG ACA TGC CAC GGT ACA ACG
BtCry1AC	CTC TCA ATG GGA CGC CTT TCT TGT AC

* Target DNA sequences from the *hlyA* gene of *L. monocytogenes*, the *invA* gene of *S.* Typhimurium, and the *BtCry1AC* gene of *Bacillus thuringiensis*. The reverse complement for each target sequence was used in the construction of its individual MB and was used to target the corresponding unique sequence (signature molecule) found in the bacterial genomic DNA.

B. Molecular Beacon Synthesis

The nucleic acids used to synthesize the molecular beacons and detect *L. monocytogenes*, *B. thuringiensis*, and *S.* Typhimurium were obtained from IDT (Integrated DNA Technology Inc.). Each oligonucleotide was modified with an amide group with 12 extra carbon atoms on the $5'$ end, and a Black Hole Quencher II on the $3'$ end. EDC or EDAC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) from Thermo Scientific Pierce (Rockford, IL) served as the crosslinker to activate the carboxyl groups on the quantum dot (QD) surface to bind with the amino groups on DNA 5' terminal. Ten micromoles carboxyl-functioned QDs from eBioscience, e Fluor ™ 605 ^{NC} were used in this study. These QDs are excited by 350–500 nm light, and they emit light with a wavelength of 605 nm.

Nucleic acids were dissolved in DI water at a concentration of 100 μM and were mixed with 10 μL of 10 μM carboxyl QD in a glass tube, obtaining a final molar ratio of DNA to QD at

20 to 1. 1.7 nM of QDs were used to synthesize the *Salmonella* beacon and the *Listeria* beacon, and 0.96 mg of EDC was used as the crosslinker to activate the carboxyl groups on the QDs. Mixtures were gently stirred at room temperature for 2 h and were left stationary overnight in 4°C.

All conjugations were filtered with a 3 K MWCO membrane in 7500 × g for 15 min to remove extra salts. The filtration process was repeated five times to completely wash out extra ions. The same steps were applied to synthesize the beacon designed to detect the *Bt cry1Ac* sequence; however, the DNA to QD ratio was synthesized at 30 to 1; 0.3 nM of QDs and 0.9 μM of EDC were consumed.

C. Culture Preparation and Genomic DNA Extraction

Stock cultures of *S.* Typhimurium DT104 strain 2576 and *L. monocytogenes* strain Scott A, obtained from the Department of Food Science and Technology at the University of Tennessee, Knoxville, and *B. thuringiensis* subsp. kurstaki strain HD73 obtained from the Department of Entomology and Plant Pathology at the University of Tennessee, Knoxville, were used. Cultures were grown individually overnight in tryptic soy broth (TSB; Difco, Sparks, MD) at 30–35°C (depending upon bacteria), 150 rpm. Genomic DNA was extracted using the GenElute™ Bacterial Genomic DNA kit (SigmaAldrich, St. Louis, MO) following manufacturer's instructions and quantified using a NanoDrop (ND-1000, Thermo Scientific, Wilmington, DE).

D. Fluorescence Detection

Luminescent semiconductor quantum-dot-based molecular beacons were tested in the presence of their genomic DNA in three systems, RNase/DNase free water, 2% milk and/or co-infiltrated into 6 wk old *Arabidopsis thaliana* "Columbia" leaves. Fluorescence in presence of genomic DNA in water or 2% milk was measured using a Synergy HT microplate reader with excitation of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100. Fluorescence observed within the plant tissue or in microfuge tubes was observed visually under UV light (302 nm) (LKB 2011 Macrovue transilluminator, Bromma, Sweden).

MBs designed to detect the *S.* Typhimurium *invA* gene were measured for fluorescence alone (0 ng genomic DNA) and in the presence of two concentrations (400 and 800 ng) of genomic DNA in water and milk. MBs designed to detect *L. monocytogenes hlyA* gene were measured for fluorescence alone (0 ng genomic DNA) and in the presence of three concentrations (50 ng, 100 ng, and 200 ng) of bacterial genomic DNA in water and milk. MBs were tested at a high concentration (MB higher concentration) and at a low concentration (MB lower concentration). The final concentration of MB higher concentration for water, water diluted, milk, and milk diluted was 17.5 nM, 12.5 nM, 12.2 nM, and 8.6 nM, respectively, and the final concentration of MB lower concentration for water, water diluted, milk, and milk diluted was 8.75 nM, 6.25 nM, 6.1 nM, and 4.3 nM, respectively. Treatments termed "diluted" were diluted using water or milk to decrease the final concentration of MB, decreasing background fluorescence. Samples still contained the same quantity of genomic DNA.

For co-infiltration into plant leaves, the *Bt cry1Ac* MB probe (40 nM) was mixed with bacterial genomic DNA (100 ng or 200 ng) derived from *B. thuringiensis* or water (0 ng genomic DNA) and was infiltrated into the *Arabidopsis* leaves using a 1 ml syringe and observed for fluorescence under 302 nm UV light. Co-infiltration suspensions consisted of MB only + water (0 ng genomic DNA), MB + 200 ng bacterial genomic DNA, MB + 400 ng bacterial genomic DNA, water + 200 ng bacterial genomic DNA, water + 400 ng bacterial genomic DNA and water only.

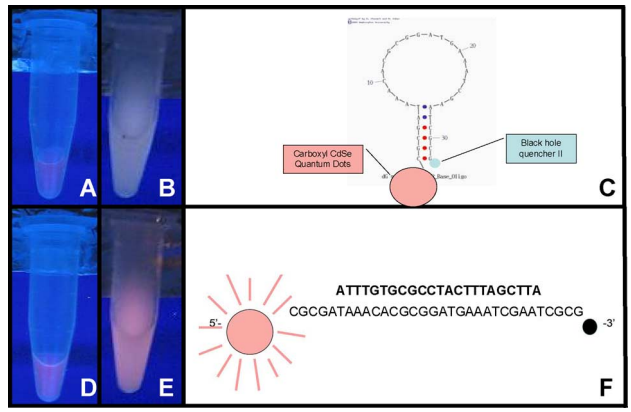

Fig. 2. Fluorescence observed in MB only in water (A) and 2% milk (B) in quenched form (C) and MB in the presence of bacterial genomic DNA in water (D) and 2% milk (E) in unquenched form (F) under 302 nm UV light. The MB was designed to detect a target sequence (*hlyA*) found in *L. monocytogenes*. Final beacon concentration was 1.17 nM (water) and 3.5 nM (2% milk), while final DNA concentration was 200 ng.

III. Results

Signature molecules (bacterial genomic DNA) derived from *L. monocytogenes*, *B. thuringiensis*, and *S.* Typhimurium were detected directly in water, milk, and/or plant tissue when in the presence of their respective quantum-dot-based MB probe. As illustrated in Fig. 2, fluorescence could be visualized with the naked eye using UV 302 nm when *L. monocytogenes* genomic DNA concentration was 200 ng when either water or milk was used as the substrate. When the lower concentration of MB probe was used, less background fluorescence was observed (Fig. 3).

In preliminary experiments, we observed that the fluorescence signal from MB alone (17.5 nM MB; 0 ng genomic DNA) was equal or lower than when in the presence of 100 or 200 ng of bacterial genomic DNA derived from *S.* Typhimurium (data not shown); therefore, a higher quantity of genomic DNA was used for these experiments. Increased fluorescence was observed for MBs designed to detect *S.* Typhimurium *invA* gene when in the presence of 800 ng of bacterial genomic DNA than in MB alone for all concentrations of MB and in all substrates. When water was used as the substrate, equal increases in fluorescence above MB alone (0 ng) were observed for both 400 and 800 ng of bacterial genomic DNA at MB concentrations of 6.25, 8.75, 12.5 and 17.5 nM [Fig. 3(A)]. When milk was used as the substrate, the increase in fluorescence above MB alone was greater for 800 ng of bacterial genomic DNA than 400 ng genomic DNA at MB concentrations of 6.1, 8.6, and 12.2 nM, However, an equal increase in fluorescence above MB alone (0 ng) was observed for both 400 and 800 ng of bacterial genomic DNA at MB concentration of 4.3 nM, indicating a higher amount of signature molecule and/or increased MB

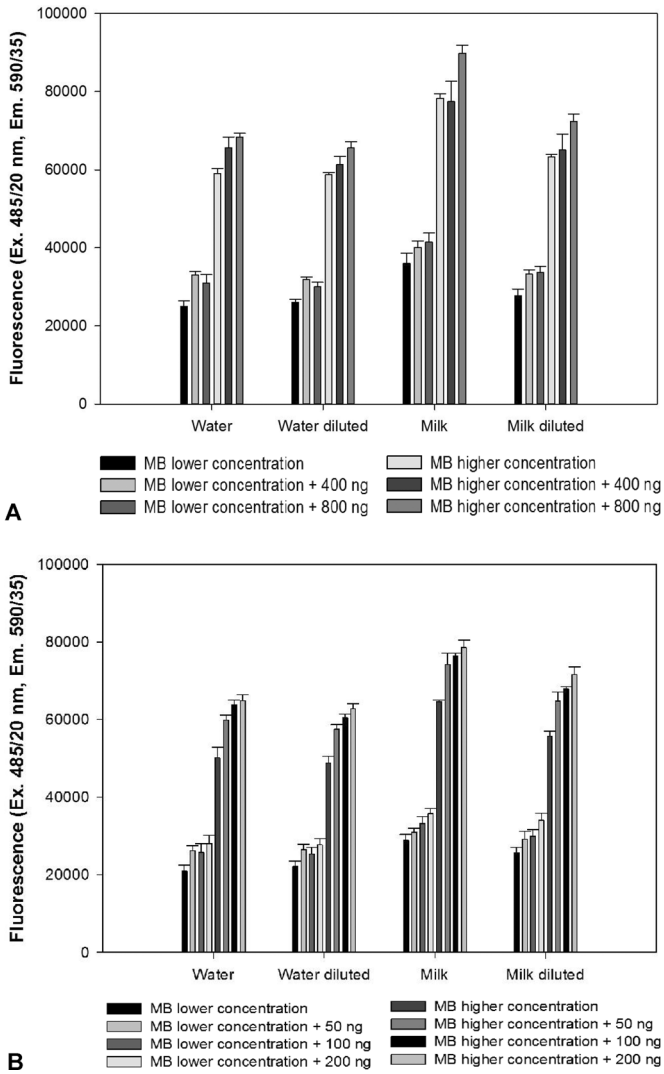

Fig. 3. Fluorescence of molecular beacon (MB) designed to detect *S.* Typhimurium *invA* gene alone (0 ng bacterial genomic DNA) and in two concentrations (400 and 800 ng) of bacterial genomic DNA (A) and fluorescence of MB designed to detect *L. monocytogenes hlyA* gene alone (0 ng bacterial genomic DNA) and in three concentrations (50 ng, 100 ng, and 200 ng) of bacterial genomic DNA (B) within water and milk using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100. Error bars report standard error. The final concentration of MB higher concentration for water, water diluted, milk, and milk diluted was 17.5 nM, 12.5 nM, 12.2 nM, and 8.6 nM, respectively, and the final concentration of MB lower concentration for water, water diluted, milk, and milk diluted was 8.75 nM, 6.25 nM, 6.1 nM, and 4.3 nM, respectively. Treatments termed "diluted" were diluted using milk or water to decrease the final concentration of MB, decreasing background fluorescence. Samples still contained the same quantity of genomic DNA (signature molecule).

concentration is necessary for detection in food substrates such as milk [Fig. 3(A)].

Similar results were observed for MBs designed to detect *L. monocytogenes hlyA* gene when in the presence of three concentrations (50 ng, 100 ng and 200 ng) of bacterial genomic DNA [Fig. 3(B)] and MB alone (0 ng). When water was used as the substrate, equal increases in fluorescence above MB alone (0 ng) were observed for 50, 100 and 200 ng of bacterial genomic DNA [Fig. 3(B)]. When milk was used as the substrate, equal increases in fluorescence above MB alone (higher concentration) were observed for 50, 100, and 200 ng of bacterial genomic DNA; however, when a lower concentration of MB was

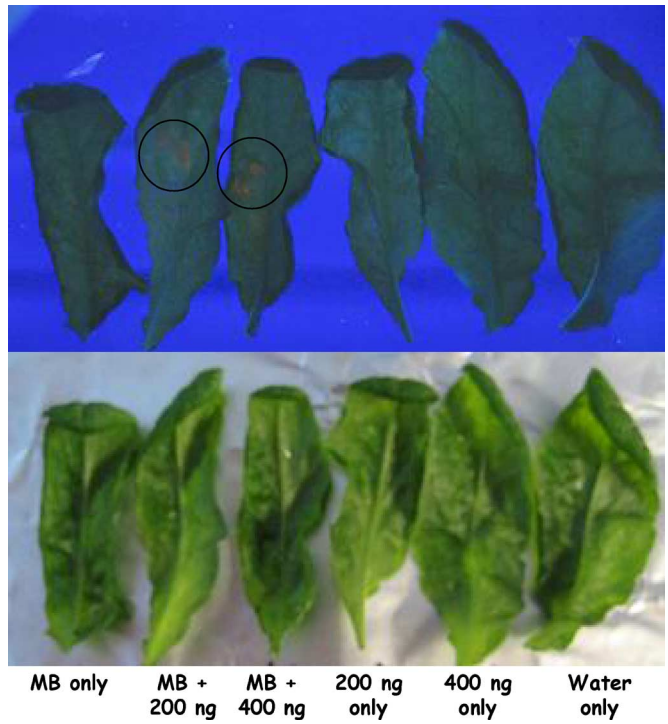

Fig. 4. *Arabidopsis* as a model system for detecting *Bacillus thuringiensis*. Fluorescence of 40 nM of molecular beacon (*Bt cry1Ac*) designed for detecting *Bt cry1Ac* gene in the presence of bacterial (*B. thuringiensis*) genomic DNA co-infiltrated into *Arabidopsis* plants observed in MB only, MB in the presence of 200 ng or 400 ng bacterial genomic DNA, 200 ng or 400 ng bacterial genomic DNA alone and water only under 302 nm UV light (above) and white light (below).

used, equal increases in fluorescence above MB alone were observed for 100 and 200 ng of bacterial genomic DNA, indicating a higher concentration of bacterial genomic DNA and MB probe are necessary for detection in a food substrate [Fig. 3(B)].

Since it was possible to observe increases in fluorescence above MB probe alone in liquid substrates (water and milk), we wanted to determine the ability to detect the presence of signature molecules within a more complex substrate, plant tissues (Fig. 4). As illustrated in Fig. 4, fluorescence could be visualized, in real-time, with the naked eye using UV 302 nm when the *Bt cry1Ac* beacon probe was co-infiltrated into *Arabidopsis* in the presence of bacterial genomic DNA (100 ng or 200 ng). An increase in fluorescence was observed above MB probe alone and no fluorescence was observed within the plant tissues for the MB probe alone, bacterial genomic DNA alone (100 ng or 200 ng) or water treatments (Fig. 4), indicating the potential use for real-time *in planta* detection (Fig. 4).

IV. Discussion

This study describes the use of MB probes coupled with QDs for the detection of signature molecules (bacterial genomic DNA) from *L. monocytogenes*, *B. thuringiensis*, and *S.* Typhimurium using several different substrates, water, milk, and/or plant tissues. MB probes were first developed in the mid-1990s by Tyagi and Kramer [9] as a means to identify target nucleic acid sequences in homogenous matrices. Modifications to this technology have allowed for the detection of bacterial pathogens *in vitro* [25]–[28], using qRT-PCR techniques. *In vivo* monitoring using MB probes has been achieved

in detecting mRNA in living cells [23], [29], [30]. Current advancements in engineering MBs for intracellular imaging have been recently reviewed by Wu *et al.* [31]. Various modifications to the MB structures have been shown to improve signal intensity, decrease background fluorescence, and provide enhanced stability [31].

The first use of an MB-PCR-based method to detect bacterial pathogens within a food system was performed by Liming & Bhagwat [27], detecting a single *Salmonella* species in fresh fruits and vegetables rapidly (with 18 h) and with moderate sensitivity (4 CFU per 25 g produce).

While *in vitro* techniques coupling QDs to MBs have been well-established, the possibility to monitor and quantify specific bacterial signatures in real-time using these mega-nanodetectors within and on the surface of plants and in food systems, such as milk, has not been fully achieved. Vasudev *et al.* [13] demonstrated the ability to visually observe an increase in fluorescence when 100 pM target DNA for the *invA* gene of *S.* Typhimurium DT104 was in the presence of 10 nM of MB. We were able to detect increases in fluorescence at similar concentrations of MB (4.3 to 17.5 nM) when genomic DNA extracted from either *L. monocytogenes* or *S.* Typhimurium was added to water or milk (Fig. 3). Similarly to Vasudev *et al.* [13], we were able to monitor for the presence of comparable signature molecules (nucleic acids) within water and milk using MBs coupled with QDs and demonstrated *in vivo* monitoring of bacterial signatures in living plant tissue.

Coupling QDs to MB probes are highly suitable for the practical applications of detecting foodborne pathogens by being able to be excited and emit signal through complex food components [32]. QDs have a ten times longer fluorescence lifetime and are more thermally stable than organic fluorophores, allowing for more stable visualization within plant tissues [10]. While advancements to this technology have been made, limitations still exist in using MB probes for the detection of bacterial signatures in complex substrates and food systems, due to the presence of interacting or inhibitory compounds, such as proteins, metabolites or other natural microflora found in plants and foods [33]–[35], and further work will need to be performed to practically implement this detection technique in the field or in industry. When monitoring for the presence of bacterial signatures using PCR-based technologies, false-negative signals may occur due to interference from these compounds [27], [33]–[35]. Furthermore, nucleic acid based detection cannot distinguish between live and dead bacterial cells. Another potential problem with detecting bacterial signatures in plants and foods is the ability to observe detectable signal from within the plant tissues and food matrices. Research has shown that the pathogens, *Escherichia coli* O157:H7 and *Salmonella*, can penetrate 20 to 200 μm below the surface of cut lettuce leaves [36], [37], escaping chlorine washes and other sanitation processes, and making detection within plant tissues essential. Hu *et al.* [24] demonstrated high efficiency transport of QDs into maize roots using a surfactant, silwet L-77, but have not coupled this nanotechnology with MB probes for use in bacterial signature detection. The surfactant assisted the movement of the QDs to tissues beyond initial contact, lending to the possibilities of detecting bacterial molecules within the tissues of plants.

Modifications to the MB structures can be made to improve sensitivity, stability, and selectivity, improving real-time detection of target molecules in living cells (reviewed in [31]) and allow for the practical implementation of this technology for use in food safety. Yang *et al.* [38] were able to improve the sensitivity and specificity of the MBs by increasing the number of DABCYL quenchers added to MBs, thereby increasing the signal-to-background ratio and enhancing fluorescence detection. An increase in the quenching ability of the DABCYL quencher was observed as the number of quenchers added to MBs increased from single to triple [38]. Such modifications may help to further decrease such background fluorescence as observed in Fig. 3.

Further, it has been shown that the half life of MBs within living cells could be as short as 30 min, after which time, nucleases begin to degrade the MBs, thereby opening up the hairpin structure and giving false-positive signals [39]. Several researchers have incorporated synthetic nucleotides to improve resistance of MBs to nuclease degradation [40]–[42]. Similar modifications would be beneficial to increase the signal above background fluorescence in such complex matrices as foods and crops.

V. Conclusion

In these proof-of-concept studies, we were able to detect increases in fluorescence when semiconductor quantum-dot—based MB probes were in contact with signature molecules (bacterial genomic DNA) derived from bacteria in water, milk, and/or plant tissue, with the aim to create an on-the-food monitoring of potential bacterial infections in real-time. We successfully demonstrated an on-the-plant method for detecting signature molecules from bacteria under UV light using a MB designed for the detection of the gene, *Bt cry1Ac*, and co-infiltrating *Arabidopsis* with genomic DNA derived from *B. thurginiensis* and its respective MB, potentiating its use in the field using a simple handheld UV device. Detection techniques that can sense and monitor low levels of bacterial pathogens with high sensitivity in a wide range of samples are a valuable technology. However, further work will need to be performed to practically implement this detection technique in the field. Additional experiments will be needed to determine sensitivity and specificity for multiple target organisms in real-world environmental samples and will be required for downstream use of this mega- nano- detection technology as well as a need to improve an accurate signal for low levels of bacterial signature molecules in complex systems, such as foods and crops. However, we envision this technology could be used in the future as a rapid, real-time, high-throughput assay to monitor and detect signature molecules from bacterial pathogens in crops, foods, and other environmental samples, providing a wide-range of protection to consumers.

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Authors' photographs and biographies not available at the time of publication.