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**ABSTRACT**

Enzymatic bio-nanotransduction is a biological detection scheme based on the production of nucleic acid nano-signals (RNA) in response to specific biological recognition events. In this study, we applied an enzymatic bio-nanotransduction system to the detection of important food-related pathogens and a toxin, *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and staphylococcal enterotoxin B (SEB) were chosen because of the implications of these targets to food safety. Primary antibodies to each of the targets were used to functionalize magnetic beads and produce biological recognition elements (antibodies) conjugated to nano-signal–producing DNA templates. Immunomagnetic capture that was followed by in vitro transcription of DNA templates bound to target molecules produced RNA nano-signals specific for every target in the sample. Discrimination of RNA nano-signals with a standard enzyme-linked oligonucleotide fluorescence assay provided a correlation between nano-signal profiles and target concentrations. The estimated limit of detection was $2.4 \times 10^3$ CFU/ml for *E. coli* O157:H7, $1.9 \times 10^4$ CFU/ml for *S. enterica* serovar Typhimurium, and 0.11 ng/ml for SEB with multianalyte detection in buffer. Low levels of one target were also detected in the presence of interference from high levels of the other targets. Finally, targets were detected in milk, and detection was improved for *E. coli* O157 by heat treatment of the milk.

Rapid and accurate detection of food pathogens is an important component of any risk management plan for food safety (19). Detection strategies that can be implemented with high-throughput designs, such as arrays or single-sample multianalyte detection, are critical for large-scale screening (17). Assessment of food products for multiple microorganisms and toxins has attained a high priority as the threat of bioterrorism continues to loom (3). There is a need to screen for both known contaminants and potential bioterror agents. To attain robust multianalyte biological detection in food and environmental samples, a flexible systems approach is desirable (31).

Several methods have been explored for the streamlining of detection for timely and multianalyte food sample analysis (1, 25, 29, 36). Four approaches have emerged that can be used in the design of a flexible multianalyte biological detection system for food products. The first is the selective concentration and purification of targets from the sample matrix. Immunomagnetic beads have been shown to be extremely useful in this process (15, 25). The second approach is the biological recognition of target organisms or molecules with biological affinity molecules. Affinity biological macromolecules used for food pathogen and toxin detection include nucleic acid hybridization probes (21), antibodies (29), lectins (13), and nucleic acid aptamers (6). The third approach is signal production and enhancement through molecular manipulation of nucleic acids. Techniques that explore the use of PCR (21), real-time PCR (18), reverse transcription PCR (30), and nucleic acid sequence–based amplification (4) have been applied to the detection of food pathogens. Additionally, the amplification of DNA sequences through PCR has been linked to antibody recognition of food pathogens and toxins by immuno-PCR techniques (20). Immunodetection amplified by T7 RNA polymerase links RNA production with antibody recognition (7, 38), but, to our knowledge, this method has not been directly applied to the detection of food pathogens.

A final approach for the rapid detection of food pathogens is the use of biosensors. Biosensors provide platforms that have the potential for miniaturization, low-cost production, and ease of use and automation. In addition, the ultimate signal for most biosensors is electronic. As a result, biosensors show great promise as deployable detection platforms and have the ability to perform built-in data processing and real-time data dissemination through existing technologies (e.g., cellular telephone, Internet). Many biosensors exist in the literature for food pathogen detection that compare with or surpass the sensitivity of other rapid techniques (1, 16, 29).

To produce a rapid and flexible multianalyte detection system for food pathogens and toxins, we have designed a system that integrates the four approaches outlined above. We use the term enzymatic bio-nanotransduction to describe this system. Enzymatic bio-nanotransduction is based on the production and measurement of biological nano-signals (nucleic acid sequences) in response to the biological recognition of the targeted organism or toxin. Specifically, biological recognition molecules (such as antibodies) are
linked to DNA templates that code for a T7 RNA polymerase promoter and a given nucleotide sequence. The specific capture and concentration of a target organism or toxin that is bound to a recognition molecule is followed by an in vitro transcription reaction of the bound DNA template. Detection of the RNA nano-signals on a detection platform is correlated with the presence or absence of the target in the original sample. By this approach, it is possible to detect multiple targets and target types (e.g., DNA, RNA, protein, whole cells) in a single sample by changing the recognition element (e.g., antibody, nucleic acid probe, aptamer, peptidomimetic) linked to the DNA template. In addition, it is possible to link this flexible detection system to nucleic acid detection platforms such as biosensors. Finally, this system is amenable to the preconcentration and purification of targets from the original sample matrix.

In this article, we describe the optimization and detection of *Escherichia coli* O157, *Salmonella enterica* serovar Typhimurium, and staphylococcal enterotoxin B (SEB) in a single sample by enzymatic bio-nanotransduction. In addition, we examine the impact of milk on the detection system. The aim of this article is to discuss and demonstrate the production of RNA (nano-signals) specific for antibody-capture and concentration of a target organism or toxin that is bound to a recognition molecule is followed by an in vitro transcription reaction of the bound DNA template. Detection of the RNA nano-signals on a detection platform is correlated with the presence or absence of the target in the original sample. By this approach, it is possible to detect multiple targets and target types (e.g., DNA, RNA, protein, whole cells) in a single sample by changing the recognition element (e.g., antibody, nucleic acid probe, aptamer, peptidomimetic) linked to the DNA template. In addition, it is possible to link this flexible detection system to nucleic acid detection platforms such as biosensors. Finally, this system is amenable to the preconcentration and purification of targets from the original sample matrix.

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### MATERIALS AND METHODS

**Materials.** Anti-SEB affinity-purified sheep immunoglobulin G was purchased from Toxin Technologies (Sarasota, Fla.). Heat-killed *E. coli* O157:H7 cells, anti-*E. coli* O157 affinity-purified goat antibodies, heat-killed *S. enterica* serovar Typhimurium cells, and anti-*Salmonella* common structural antigen (CSA) affinity-purified goat antibodies were purchased from KPL (Gaithersburg, Md.). Purified *Staphylococcus aureus* enterotoxin B was provided by Dr. Gregory Bohach (University of Idaho, Moscow). All DNA molecules used in this study were purchased from IDT Technologies (Coralville, Iowa), and the sequences are shown in Table 1. M-280 tosyl-activated magnetic beads were obtained from Dynal Biotech (Oslo, Norway). Ampliscribe T7-Flash Transcription kits were purchased from Epicentre Biotechnologies (Madison, Wis.). Sulfo-SSMC (sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate), Quanta-Blu peroxidase fluorogenic substrate, TCEP Bond Breaker solution (500 mM Tris(2-carboxyethyl)phosphine hydrochloride), and streptavidin–horseradish peroxidase were obtained from Pierce Biotechnology (Rockford, Ill.). DNA-Bind 96-well plates were purchased from Corning Inc. (Corning, N.Y.). NAP-5 size exclusion columns were purchased from Amersham Biosciences (Uppsala, Sweden). Bovine serum albumin (BSA) fraction V was purchased from FisherBiotech (Fair Lawn, N.J.). Nanosep 10K and 100K centrifugal devices were purchased from Pall Life Sciences (Ann Arbor, Mich.). All magnetic bead reaction steps were mixed with Dynal sample mixer for coating magnetic beads and captured from solution with a Dynal MPC-9600 magnet purchased from Dynal Biotech. All other solutions and chemicals were of molecular biology grade and made with 18.2 MΩ water.

**Preparation of immunomagnetic beads.** M-280 tosyl-activated magnetic beads were functionalized with goat anti-*Salmonella* CSA-1, sheep anti-SEB, or goat anti-*E. coli* O157 antibodies by mixing 50 μg of antibody with 50 μl of beads in 0.1 M Borate buffer (pH 9.5) at 37°C for 24 h, according to the manufacturer’s instructions. Beads were washed three times with phosphate-buffered saline (pH 7.2) containing 0.05% Tween-20 (PBST), and then 3% BSA (wt/vol) in PBST (PBSTB) was used to block any remaining tosyl groups and other sites of nonspecific protein adsorption on the beads. Beads were washed for the final time with PBST and stored at 4°C until used in a PBSTB solution.

**Antibody–DNA template conjugation.** A flow chart for this process is described in Figure 1. Three nano-signal–producing DNA templates were used in this study. Each double-stranded template was purchased as two complementary single-stranded molecules. The sense strands that contained a 5′ thiol modification attached to the strand through a six-carbon linker are shown in Table 1 (complements not shown). Templates were hybridized by mixing 2 nmol of each complementary DNA strand (thio DNA template 1 with complement, thio DNA template 2 with complement, and thio DNA template 3 with complement) in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) adjusted
to pH 7.0 with HCl). After initially being heated to 85°C for 10 min, the templates were allowed to hybridize for 5 h at 50°C.

Antibody DNA conjugates were made by cross-linking specific antibodies to the thiol-modified double-stranded DNA templates. A total of 77.5 µg (500 pmol) of the appropriate antibody was mixed with 1.5 mg of Sulfo-SSMC, and the volume was adjusted to 500 µl with PBS (pH 7.7). This mixture was allowed to react at 37°C for 1 h. Simultaneously, 500 to 600 pmol of double-stranded thiol DNA template was mixed with 5 µl of TCEP Bond Breaker solution in 500 µl of conjugation buffer (PBS [pH 7.2] with 1 mM EDTA) and allowed to react for 1 h at room temperature. The antibody and DNA solutions were individually placed on separate NAP-5 size exclusion columns and eluted with conjugation buffer. The modified antibody was eluted in 800 µl of conjugation buffer, while the DNA was eluted in 1 ml of conjugation buffer. The eluants were mixed, placed on nanopore 10K spin filtration devices, and spun for 5 min at 5,000 × g. The DNA and antibody were then washed from the filters in a total of 750 µl of conjugation buffer and allowed to react for 1 h. The conjugation process was monitored by comparing high-pressure liquid chromatography (HPLC) traces at 260 and 280 nm on a Shimadzu (Kyoto, Japan) SCL-10A HPLC system controller and an SPD-10 UV-Vis detector with a Bio-Silect SEC 250 column from Bio-Rad Laboratories Inc. (Hercules, Calif.). For consistency, anti- _E. coli_ O157 was conjugated to the DNA template producing nano-signal 1, anti- _Salmonella_ was conjugated to the DNA template producing nano-signal 2, and anti-SEB was conjugated to the DNA template producing nano-signal 3.

**Preparation of 96-well plates.** DNA-Bind black, 96-well plates were used to covalently attach amine-modified DNA probes (amine signal 1’, 2’, 3’, or 4’) complementary to the variable sequence of one of the four nano-signals to the surface of the plate. Wells were modified by placing 100 µl of a 0.05 M phosphate buffer with 1 mM EDTA, pH 8.5, containing 1 µM of an amine DNA probe onto the plates for 16 h at 4°C. For each sample, four wells were functionalized with the amine complement to each of the four nano-signal sequences. Following probe immobilization, wells were washed and blocked with 3% BSA in 0.5 M phosphate buffer with 1 mM EDTA, pH 8.5, for 1 h at 37°C.

**Single-target detection with enzymatic bio-nanotransduction.** Single-target detection and optimization were carried out as follows. A 100-µl sample containing the specified concentration of the target diluted in PBSTB was mixed with a single type of immunomagnetic beads (a specified amount taken from concentrate and diluted to 5 µl in PBSTB) and the matching antibody DNA conjugate (a specified amount taken from concentrate and diluted to 5 µl in PBSTB) to make a final volume of 110 µl. The sample was mixed by rotation for 1 h at room temperature. A magnet (MPC-9600) was used to collect the magnetic beads, and the beads were washed three times with PBSTB (double-autoclaved to remove any RNase activity). Following the removal of the last wash, the beads were placed in 20 µl of in vitro transcription mix (8.3 µl of RNase-free water, 1.8 µl (each) NTP (100 mM stock), 2 µl of DTT (100 mM stock), 2 µl of 10× Ampliscribe T7-Flash reaction buffer, and 0.5 µl of Ampliscribe T7-Flash enzyme solution) and rotated at 42°C. Transcription was stopped at 10 min by adding 6× SSPE (1× SSPE is 150 mM NaCl, 10 mM sodium phosphate, and 1 mM EDTA [pH 7.4]) with an additional 9 mM EDTA. The transcription mix was diluted to a total of 400 µl in 6× SSPE with a final concentration of 1 µM of the biotin detector strand. The samples were heated to 50°C for 5 min, and 100-µl aliquots of the solution were placed in three wells of a 96-well plate modified with oligonucleotide probes complementary to each of the three nano-signals (one probe type per well). Hybridization proceeded for 45 min at room temperature on a bench top rotator. The wells were washed three times with 0.25× SSC and 0.1% sodium dodecyl sulfate. One hundred microliters of streptavidin–horseradish peroxidase (1.5 µg/ml) in 3% BSA (wt/vol) in 6× SSPE was pipetted into each well and mixed for 30 min. The wells were washed three times with PBSTB, and 100-µl aliquots of Quant-a-Blu was added. The reaction was stopped at 10 min with 100 µl of Quanta-Blu stop solution.

**One-step multianalyte target detection with enzymatic bio-nanotransduction.** Multianalyte detection and optimization were accomplished by the addition of all three immunomagnetic bead types and antibody DNA conjugates to the sample solution. Sample volumes were maintained at 100 µl. For each sample, a 5-µl bead mix (a specified volume of each bead type added to PBSTB for a total of 5 µl) and 5 µl of antibody DNA conjugate mix (specified volumes of the three antibody DNA conjugates added to PBSTB for a total of 5 µl) were added, and the assay protocol outlined above was followed.

**Two-step multianalyte detection with enzymatic bio-nanotransduction.** To allow detection in more complicated sample matrices, a two-step capture-recognition protocol was followed. The first step was target capture on immunomagnetic beads. This was accomplished by adding 5 µl of an optimized bead mix (three bead types in PBSTB) to 100 µl of the sample. The sample was rotated for 1 h at room temperature, and then the magnetic beads were collected and washed two times with PBST. The last wash solution was removed, and 100 µl of PBSTB and 5 µl of antibody DNA conjugate mix (three types of antibody DNA conjugates in PBSTB) were added to the beads and rotated.

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**FIGURE 1.** Flow chart outlining the steps in the production and purification of antibody DNA conjugates. Details regarding the production can be found in the text.
Detection of targets in milk- and heat-treated samples. Targets were spiked into retail 2% milk and detected by the two-step assay described above. For heat-treated samples, heat-killed E. coli O157 cells were spiked into the sample solution (milk or PBSTB buffer) in a microcentrifuge tube and heated in a water bath at 94°C for 1 or 5 min. The samples were allowed to cool to room temperature, and the two-step assay protocol was performed. Prior to use, aliquots of the milk were spread plated on nutrient agar and found to have less than 100 CFU/ml of background microflora.

Data collection and analysis. Fluorescent readings for the 96-well plates were made on a Packard fluorescence reader with excitation and emission filters specified for use with the QuantaBlu substrate (330- and 425-nm filters) and a manual gain of 1.0. Each sample included a composite of four separate fluorescent readings (one for each nano-signal and one blank). In all cases, the nano-signal 4 measurement was used as the blank negative control to measure the background fluorescence of the assay. To limit plate-to-plate variation, each raw data measurement was normalized within the same sample set. Normalization was performed within the sample set by dividing the raw fluorescent measurement by the average fluorescent signal measured for two buffer blanks (no targets) of the same sample set. Nano-signals of the same sample were considered independently of each other; hence, normalization was performed separately for each nano-signal. For example, the fluorescent measurement for nano-signal 1 (NS1) from sample 1 (NS11) was divided by the average fluorescence of nano-signal 1 from the two buffer blanks of the sample set (average NS1BB1−2). The normalized measurement was then NS1/average NS1BB1−2. The normalized signal was used to calculate the mean and standard error of the mean with other normalized signals for the same target level from other samples and sample sets. Raw data from buffer blanks were also normalized and used to calculate mean and average deviations. By design, the mean for buffer blanks is 1, but the sample set buffer blank variability for all sample sets could be calculated. Normalized measurements for a single nano-signal under the specified conditions with no corresponding target were used to calculate the background response of the nano-signal. The limit of detection (LOD) was calculated as the lowest concentration of the target that gave a normalized signal greater than the mean of the background measurements plus three standard errors of the mean. Linear portions of the dose-response curves were fitted with trend lines by Microsoft Excel software (Microsoft Corp., Redmond, Wash.). Linear equations were used to estimate the LOD for each target by estimating the target concentration that would give a fluorescent response of three standard errors of the mean above the background from the trend line.

RESULTS

Single-target optimization. The initial step for detection optimization was to establish baseline-optimized levels for the antibody DNA conjugates and immunomagnetic beads for each target. In all experiments, the anti-E. coli O157 conjugate produced nano-signal 1, the anti-Salmonella CSA conjugate produced nano-signal 2, and the anti-SEB conjugate produced nano-signal 3. A fourth DNA template that produces nano-signal 4 has been designed, but it was not used in this study to produce signals. However, the DNA probe complement for nano-signal 4 was used to determine background fluorescence in the absence of produced nano-signals for each sample.

Various amounts of a single type of immunomagnetic beads were tested with a fixed level of a single antibody DNA conjugate for each target. At each level of beads, samples with and without a target in PBSTB were evaluated. Results were recorded as the ratio of fluorescent intensity produced from the detection of the specific nano-signal with (signal) and without (background) the presence of the target organism or target toxin. From these results, a level of beads was selected that gave the best signal-to-background ratio (0.11 μl per sample for E. coli, 0.33 μl for Salmonella, and 0.44 μl for SEB).

The optimization process was continued by determining the optimal level of antibody DNA conjugate at the same fixed level of beads as selected in the first assay. The signal-to-background ratio was used to select the level of antibody DNA conjugate resulting in the best signal-to-background ratio for that target. The optimal level of antibody DNA conjugate was found to be 0.2 μl per sample for E. coli, 1 μl per sample for Salmonella, and 1 μl per sample for SEB.

Multianalyte optimization. The inclusion of all three bead types and all three antibody DNA conjugates could influence the optimal level for a particular bead or antibody DNA conjugate. First, the specific nano-signal production, with and without a target, was measured at various levels of target-specific beads with previously optimized levels of off-target beads and all three antibody DNA conjugates. Following bead optimization for all three targets, antibody DNA conjugates for each target were optimized by measuring the specific nano-signal production with and without a target with various levels of target-specific antibody DNA conjugates and the optimized levels of all three beads and the off-target antibody DNA conjugates. Only the anti-SEB antibody DNA conjugate was found to have an optimum level different from that found in the single-target protocol (0.3 versus 1 μl). This level was only slightly better (improved signal-to-background ratio) than the previously optimized level, but it allowed lower amounts anti-SEB antibody DNA conjugate to be used in the subsequent research. All of the sensitivity assays to follow were completed with the levels of antibody DNA conjugate and immunomagnetic beads determined in the optimization.

E. coli O157 detection in buffer. The dose-response curves for the detection of E. coli O157 were determined by serial dilutions of the heat-killed organisms in PBSTB buffer. For each sample set, fluorescent measurements were normalized by dividing the raw measurement for each nano-signal by the averaged buffer blank signal of the same nano-signal from the same sample set. Each sample set contained triplicates of the same target level, and each sample set was repeated at least twice. Normalized measurements were averaged for a given target level and reported as an average normalized value plus or minus the standard error of the normalized mean.

Figure 2A shows the production of nano-signal 1 that corresponded to varying concentrations of E. coli O157.
FIGURE 2. Dose-response curves for a given target recorded in buffer. Dose-response curves in PBSTB buffer were produced by diluting E. coli O157:H7 heat-killed cells (A), S. enterica serovar Typhimurium heat-killed cells (B), or staphylococcal enterotoxin B (C) and recording the normalized fluorescence for nano-signal 1 (A), nano-signal 2 (B), or nano-signal 3 (C) with multianalyte-optimized levels of immunomagnetic beads and antibody DNA conjugates for all three targets with a single-step assay design. Inset is a magnified view of the corresponding dose-response curve. Horizontal line represents the LOD as calculated from the averaged background plus three standard errors of the mean. For E. coli O157, this cut-off value was a normalized value of 1.39. This value was based on the measurements for the buffer blanks (no targets) of this experiment, along with all of the measurements of nano-signal 1 made in other experiments with no E. coli (with off-targets) present in the sample (n = 40). The inclusion of the measurements for all the samples with no E. coli gives a more realistic assessment of the background in multianalyte assays. The equations for linear regression, the $R^2$, and the LOD based on the cut-off value of 1.39 are shown in Table 2.

Figure 3 shows an assessment of our ability to detect E. coli O157 in the presence of high levels of Salmonella and SEB. Figure 3 also shows the normalized signal profile for the detection of $3.5 \times 10^4$ CFU/ml of E. coli in the presence of $10^5$ CFU/ml of Salmonella and 10 ng/ml of SEB. It is evident that E. coli O157 can be detected at this level in the presence of the other targets. The off-targets slightly increased the variation of the 0 target level for nano-signal 1. Taken alone, this variation changed the cutoff value for E. coli to 2.23 and resulted in a change of the LOD from 2,500 to 7,000 CFU/ml.

S. enterica serovar Typhimurium detection in buffer. The changes in nano-signal 2 production with variations in heat-killed S. enterica serovar Typhimurium cell concentrations were determined in a fashion identical to E. coli O157 detection. Figure 2B shows the dose response of nano-signal 2 as represented by normalized fluorescence units. The horizontal line represents the cut-off value for Salmonella detection, which is 2.07 normalized fluorescence units. This value was calculated as described above with n = 40. The relatively high cut-off value was due to a large variation in the nano-signal 2 measurements. This was also reflected in the relatively large error bars seen in Figure 2B. The theoretical LOD for Salmonella with a cut-off value of 2.07, as shown in Table 2, is approximately $1.9 \times 10^4$ CFU/ml. However, from Figure 2B, it is evident that the measurement variation at $5 \times 10^4$ CFU/ml approaches the cut-off line.

FIGURE 3 shows an assessment of our ability to detect Salmonella at a concentration of $10^5$ CFU/ml, with $10^6$ CFU/ml of E. coli O157 and 10 ng/ml of SEB. As with E. coli, this level of target is detectable even in the presence of off-targets, and the level of nano-signal 2 shows a response to Salmonella that is similar to the results found with the detection of Salmonella alone. One difference is the increased normalized fluorescence signal with no target above the buffer blanks. Although not statistically significant ($P = 0.15$), the increased background, if taken alone, would cause an increase in the cut-off value to 2.56, which, in turn, would change the LOD to $2.9 \times 10^4$ CFU/ml from $1.9 \times 10^4$ CFU/ml.

SEB detection in buffer. The detection of SEB was determined by the standard enzymatic bio-nanotransduction protocol with serial dilutions of the toxin in PBSTB. Figure
TABLE 2. Target detection in phosphate-buffered saline with Tween-20 and BSA (PBSTB)

<table>
<thead>
<tr>
<th>Target</th>
<th>Limit of detection*</th>
<th>Equation of linear regressionb</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> O157</td>
<td>( 2.4 \times 10^3 ) CFU/ml</td>
<td>( y = 0.0002x + 0.9063 )</td>
<td>0.9986</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> serovar Typhimurium</td>
<td>( 1.9 \times 10^4 ) CFU/ml</td>
<td>( y = 0.00005x + 1.0969 )</td>
<td>0.9827</td>
</tr>
<tr>
<td>SEB</td>
<td>0.11 ng/ml</td>
<td>( y = 6.4167x + 1.0744 )</td>
<td>0.9788</td>
</tr>
</tbody>
</table>

- The limit of detection was estimated by finding the concentration of target that corresponded to the mean background signal plus 3 standard errors of the mean of the background.
- Lines were fitted to the linear portions of dose-response curves for the detection of each target in buffer (data shown in Fig. 2).

2C shows the dose-response curve for nano-signal 3 with variations in the SEB concentration. The horizontal line is a cut-off value of 1.82 normalized fluorescence units, as determined from the mean plus three standard errors of the mean (\( n = 40 \)) of nano-signal 3 measurements with no SEB present. Table 2 shows the LOD based on this cut-off value, the equation of linear regression, and the \( R^2 \) value. In this case, the fit of the line is not as good as those found with *E. coli* O157 and *Salmonella* (\( R^2 = 0.9986 \) and 0.9827, respectively), but the \( R^2 \) value for SEB of 0.9788 is acceptable. The LOD for SEB estimated from these results is 0.11 ng/ml.

Detection of SEB with a high level of off-targets was done with a concentration of 0.4 ng/ml for SEB and \( 10^6 \) CFU/ml for both *E. coli* O157 and *Salmonella*. Figure 5 shows the nano-signal profile for this experiment. As with *Salmonella*, there is a slight increase in the background signal in the presence of off-targets. If this value used to determine the cut-off value, the cut-off level is raised to 2.00 and subsequently increases the LOD for SEB from 0.11 to 0.14 ng/ml.

**Target detection in milk.** To test our system in a complex sample matrix, we chose to investigate the detection of the three targets in retail 2% milk. The detection of the pathogens and toxins in buffer was completed in a one-step capture and detection method in which immunomagnetic beads and antibody DNA conjugates were mixed together with the sample at the same time. It was determined that a two-step protocol was more appropriate for milk samples. In this case, 100 µl of the sample was mixed with 5 µl of the bead mix in PBSTB. After immunomagnetic capture, the beads were washed and placed in PBSTB buffer with antibody DNA conjugates. The standard protocol was followed from there.

Figure 6 shows the dose-response comparison for target detection by the one-step detection method in buffer or the two-step detection in milk. Panel A shows *E. coli* O157 detection, panel B shows *S. enterica* serovar Typhimurium detection, and panel C shows SEB detection. There is a clear impact of milk on the normalized signal for all three targets. Milk dramatically attenuates nano-signal production and changes the dose-response curve. In addition, milk blanks (no target) show an increase in background signal.
FIGURE 5. Detection of SEB in the presence of high levels of Salmonella and E. coli O157. All measurements were made with multianalyte-optimized levels of immunomagnetic beads and antibody DNA conjugates for all three targets with a single-step assay design. Buffer-only samples contained 100 μl of PBSTB. Dilation of targets was made in 100 μl of PBSTB. Samples of E. coli O157 and Salmonella were made at a concentration of $10^6$ CFU/ml. Samples with SEB were at a concentration of 0.4 ng/ml. Anti–E. coli O157 = nano-signal 1 (□), anti-Salmonella CSA-1 = nano-signal 2 (■), and anti-SEB = nano-signal 3 (□). Each nano-signal is represented by the average and standard error of the mean of six separate measurements.

FIGURE 6. Dose-response curves for a given target recorded in buffer and milk. PBSTB buffer curves (□) are an overlay of previous dose-response curves for each target. Dose-response curves in milk (■) were produced by diluting E. coli O157:H7 heat-killed cells (A), S. enterica serovar Typhimurium heat-killed cells (B), or staphylococcal enterotoxin B (C) in milk and recording the normalized fluorescence for nano-signal 1 (A), nano-signal 2 (B), or nano-signal 3 (C) in a two-step multianalyte detection. Inset is a magnified view of the corresponding dose-response curve. Horizontal line represents the LOD as calculated from average normalized background for the particular nano-signal (1 = A, 2 = B, and 3 = C) in milk plus 3 standard errors of the mean (n = 6).

and variability compared with buffer blanks for both SEB and E. coli. With this as a basis, the LOD cut-off value is increased from 1.39 to 1.95 for E. coli. A more dramatic impact is seen with SEB, in which the cut-off value increases from 1.82 to 3.75. Salmonella does not show a change in the cut-off value, but a significant decrease in sensitivity is seen with milk due to low signal response.

Previous research in our laboratory has shown an improvement of immunomagnetic capture with heat treatment of milk (unpublished data). As a result, we chose to examine the impact of the heat treatment of milk on the signal for the detection of E. coli O157 with enzymatic bio-nanotransduction. Milk and buffer samples with and without 6.8 × 10^4 CFU/ml of E. coli O157 were heat treated for 0, 1, or 5 min at 94°C. These samples were then used in a two-step enzymatic bio-nanotransduction assay. Table 3 shows the results for the normalized fluorescence of nano-signal 1. Two percent milk clearly caused a dramatic decrease in the normalized signal compared to the buffer. However, a significantly increased signal was noted in a 1-min heat treatment, with additional improvement at 5 min of heat treatment. Background signals and detection of E. coli in buffer are relatively unaffected by heat treatment.

DISCUSSION

To apply enzymatic bio-nanotransduction to the detection of important food-related pathogens and toxins, it was necessary to optimize the system for detection. Our first approach was to optimize the level of immunomagnetic beads and antibody conjugates for each target. We then proceeded to investigate the optimal level for detection in the presence of off-target beads and antibodies. Optimal levels were not significantly different in the presence or absence
TABLE 3. Improvement of detection signal with heat treatment of milk

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normalized fluorescence (nano-signal) 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBSTB buffer only</td>
<td>1 ± 0.32</td>
</tr>
<tr>
<td>6.8 × 10^4 E. coli O157 in PBSTB buffer</td>
<td>7.62 ± 0.11</td>
</tr>
<tr>
<td>2% milk only</td>
<td>0.60 ± 0.08</td>
</tr>
<tr>
<td>6.8 × 10^4 E. coli O157 in 2% milk</td>
<td>2.5 ± 0.602</td>
</tr>
<tr>
<td>PBSTB buffer only heat treated for 1 min</td>
<td>1.1 ± 0.12</td>
</tr>
<tr>
<td>6.8 × 10^4 E. coli O157 in PBSTB buffer heat treated for 1 min</td>
<td>7.1 ± 0.15</td>
</tr>
<tr>
<td>2% milk only heat treated for 1 min</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td>6.8 × 10^4 E. coli O157 in 2% milk heat treated for 1 min</td>
<td>5.00 ± 0.72</td>
</tr>
<tr>
<td>PBSTB buffer only heat treated for 5 min</td>
<td>0.94 ± 0.24</td>
</tr>
<tr>
<td>6.8 × 10^4 E. coli O157 in PBSTB buffer heat treated for 5 min</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>2% milk only heat treated for 5 min</td>
<td>0.7 ± 0.06</td>
</tr>
<tr>
<td>6.8 × 10^4 E. coli O157 in 2% milk heat treated for 5 min</td>
<td>6.84 ± 0.86</td>
</tr>
</tbody>
</table>

*Four different sample types (phosphate-buffered saline with Tween-20 and BSA [PBSTB], E. coli O157 in buffer, milk only, and E. coli O157 in milk) were heat treated at 94°C for 0, 1, or 5 min. After being allowed to cool to room temperature, the samples were used in a two-step enzymatic bio-nanotransduction assay. Each measurement was made in triplicate, and values are reported as means ± standard errors of the mean. Results for the detection of E. coli O157 in milk are shown in bold.

Salmonella is the variability and the generally higher background level than that found with E. coli detection. This results in an LOD that is almost 10-fold higher than the LOD for E. coli. Another group with the same heat-killed organisms and antibodies showed a similar difference in the LOD between E. coli and Salmonella (12). It seems likely that the difference between the two targets is the affinity of the antibody. However, in our study, it is possible that the conjugation process has a differential impact on the two antibodies.

Unlike the pathogen targets where PCR provides sensitive detection, PCR cannot be used as a rapid method for the detection of SEB. PCR has been used to detect the presence of organisms containing the gene for SEB production (27). However, antibody detection schemes form the basis of the detection of the protein itself. Detection of SEB is accomplished through the modified Ouchterlony precipitation test (2), passive latex agglutination (2), ELISA (14), immunomagnetic flow cytometry (26), immunomagnetic electrochemiluminescence (22, 37), and biosensors (10). Our results show an LOD for SEB of 0.11 ng/ml, which is comparable to that found in a majority of the methods listed above. Only electrochemiluminescence has shown a significantly lower LOD of 0.5 pg/ml (37).

Our results provide evidence of the ability to simultaneously detect all three targets without a significant impact on the sensitivity. In fact, the slight change in sensitivity with high levels of off-targets found in our study could be because of the limited number of samples used, resulting in a higher average variation than would be found with repeated tests. Continued testing of the impact of off-target and nontarget pathogens and molecules will be required for a definitive assessment of specific sensitivity in a multianalyte scheme. Our system is designed and optimized for multianalyte detection in a single sample rather than the single-detection concept used in many of the approaches listed above. As a result, we have had to trade some sensitivity for multianalyte capabilities. The current proof of concept model does not take full advantage of the multiplexing ability of this approach, as samples ultimately have to be split across the wells of a microplate. However, applications of this approach could easily be transitioned to nano-signal detection on a hybridization array that would include nucleic acid biosensors (11, 33). Additionally, enzymatic bio-nanotransduction may provide a link between antibody target recognition and charge-based detection of nucleic acids on field-effect transistor biosensors. Field-effect transistor biosensors have the potential for the low-cost mass production of detection equipment, but they require surface-bound charge densities that are most easily realized with nucleic acids (5).

Application of this system to more complicated sample matrices was examined with milk. In general, milk seems to decrease the normalized signal for all three targets while dramatically increasing the background for SEB. The overall result is a decrease in sensitivity for all targets in milk. This does not seem to be a result of the impact of the milk on the transcription process (nano-signal production), as results in our laboratory have shown that milk does not affect...
the transcription of DNA templates attached to magnetic beads following the washing steps as they were performed in our detection assays (data not shown). Other food products may not have a similar impact on detection; however, it will be important to investigate alterations to the system that will provide enhanced detection in milk, an important commercial product for contamination monitoring (35).

One possible modification is the use of the heat treatment for samples prior to detection. Our sensitivity results show that there is a dramatic improvement in the detection of E. coli O157 in milk with 1 to 5 min of heat treatment at 94°C. Additional studies will need to be completed to optimize this process and investigate the impact on the other targets.

Even with the current limitations, our sensitivity results in buffer compare well with the sensitivity of the multianalyte array biosensor (10, 32), which is a well-studied sensor capable of both pathogen and toxin detection in food products. In this proof of concept model for the application of enzymatic bio-nanotransduction to targets important for food protection, we chose to examine our ability to detect E. coli O157, S. enterica serovar Typhimurium, and SEB. All three targets are listed as category B priority agents by the National Institute of Allergy and Infectious Diseases, with the potential for exposure to the agents from accidental and intentional contamination of food products. The ability to detect all three targets from a single sample has important implications for the potential of a rapid high-throughput system for food pathogens with bio-nanotransduction. Also, we were able to provide evidence for the simultaneous detection of whole pathogens and toxins in a single sample.

ACKNOWLEDGMENT

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REFERENCES


