Immunosensor for rapid extraction/detection of enteric pathogens

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An antibody-based sensor (immunosensor) has been developed that features an all-in-one extraction and detection of the enteric pathogen E. coli O57:H7 using the same redox-active polyaniline-coated iron oxide nanoparticles (PIONs). Capture efficiency for E. coli O57:H7 is shown to be 81–99% in various food matrices with varying properties. The immunosensor’s detection range is 105 to 107 CFU/mL with a detection limit of 5 CFU. Furthermore, magnetic separation is showing great promise as an alternative to existing conventional sample processing systems. Given the bacteria’s low contamination level in food and water, this biosensor technology has great potential in monitoring enteric microbial contaminants in the food supply chain where simplicity, sensitivity, and ease of use are important.

Keywords: Biosensors; Diarrheal Pathogens; Food Safety; Magnetic Nanoparticles.

INTRODUCTION

There are approximately 500 species of commensal bacteria that colonize the human gastrointestinal tract, producing disease when natural defenses are compromised1. Microbial pathogens in general are a major cause of foodborne illness outbreaks in the US and around the world, leading to deaths, chronic health issues, loss in productivity and jobs, impacted food supply, and overall economic chaos. Global food trade, extensive production, and complex supply chain contribute toward an increasing number of microbial food safety outbreaks2. Additionally, food safety is increasingly relevant as more foods are globally-sourced, processed, and shipped faster than ever before3. While the US food supply is among the safest in the world, the federal government estimates that there are 48 million cases of foodborne illness annually (1 in 6 Americans), 128,000 hospitalizations and 3,000 deaths45. An updated estimate by the Centers for Disease Control and Prevention (CDC) shows that the aggregated annual cost of foodborne illness in the US associated with 31 known pathogens and a broad category of unspecified agents is $77.7 billion67. Of these 31, 14 pathogens account for 95% of illnesses and hospitalizations and 98% of deaths in the US with an average estimated cost of $14.0 billion and an average loss of 61,000 quality-adjusted life years (QALYs) per year8. The principal invasive intestinal bacterial pathogens of food-animal origin are Campylobacter, Salmonella, Listeria, Escherichia coli O157 (and other Shiga toxin- and enterotoxin-producing strains of E. coli), Yersinia, and Vibrio9. Other reports indicate that the top 5 pathogens contributing to domestically acquired foodborne illness resulting in hospitalization are Salmonella spp., Campylobacter spp., Toxoplasma gondii, and Escherichia coli O15710. E. coli O157:H7 has been associated with the development of renal hemolytic uremic syndrome (HUS), a disease involving the digestive system that could lead to kidney failure10. First identified in 1982, E. coli O157:H7 is the dominant enterohemorrhagic serotype underlying foodborne human infections in North America and increasingly the cause of outbreaks in a number of meat products, dairy products, and fresh produce1112. E. coli O157:H7, also a biodefense agent, has a very low median infective dose of 23 cells13. The average cost per case for E. coli O157:H7 infection in the US is $10,0486.

Food itself is a major impediment to food safety controls as it goes through production, processing, packaging, distribution, and retail. Some food products do not undergo any bacterial kill-step between production and consumption. In a comprehensive report by CDC covering 1998–2008, 1 in 5 illnesses were linked to leafy green vegetables, more than any other type of food5. Juice-associated outbreaks implicated apple juice, orange juice, and other fruit juices14. The 10 worst US food illness outbreaks in 2014 included milk, a basic and necessary food for many children and adults15.

The gold standard method for identifying and enumerating microbial pathogens, such as E. coli O157:H7, is overnight culture enrichment following the procedure by FDA’s Bacteriological Analytical Manual16. The bacteria is plated on selective media and followed by a biochemical test, which are laborious and time consuming (24–48 hours). With increasing deadly food poisoning outbreaks, the gold standard is no longer adequate. Continued reliance on culture enrichment is a major roadblock to rapid pathogen detection in food17. The 2011 Food Safety Modernization Act (FSMA) aims to ensure that the US food supply is safe by shifting the focus from responding to contamination to preventing it18. Preventive food safety demands rapid, sensitive, specific, and economical means of extracting and detecting pathogens from food sources, environment, and clinical samples.

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there are major efforts in developing faster, more sensitive and more convenient methods for the rapid and reliable detection of foodborne pathogens, such as polymerase chain reaction, DNA hybridization, latex agglutination, enzyme immunoassays, phenotype microarrays, and various forms of sensors. However, while these approaches have shortened the time to detection, many of these methods detect at best $10^3$–$10^4$ colony forming units (CFU) of the target pathogen and require clean samples, thus lengthy culture enrichment steps are still necessary. For example, most commercially available immunoassays require 18–42 hours of turnaround time and provide only presence/absence results. Table 1 presents 10 commonly used immunoassays in the food and environmental industries. The turn-around time includes sample preparation and detection. Test result is either qualitative (presence or absence) or quantitative measure of the bacteria. The separation and subsequent concentration of bacterial cells from a complex matrix during sample preparation continues to be a stumbling block in the advancement of rapid methods for the detection of pathogens. Rapid detection technologies could be enhanced if the bacteria can be separated, concentrated and purified from the sample matrix before detection. This approach would facilitate the detection of multiple bacterial strains, remove matrix-associated reaction inhibitors, provide adequate sample size reduction, and detect low levels of pathogens. Thus, this paper presents a novel technique where bacterial extraction and detection are all accomplished by the same redox-active magnetic nanoparticles. Iron oxide nanoparticles are coated with polyaniline, a highly redox-active polymer, and functionalized with antibody to extract the pathogen from the complex food matrix. The same redox-active nanoparticle is used to report the pathogen (detection) through its redox signature by cyclic voltammetry on an antibody-functionalized electrode sensor. The extraction/detection assay and immunosensor technology is demonstrated in drinking water and in complex matrices, such as milk, apple juice and spinach, making this a simple technology that has the potential for point-of-use food poisoning evaluation.

Table 1 Commercially available immunoassays for detection of E. coli O157: H7 in food.

<table>
<thead>
<tr>
<th>Name</th>
<th>Turn-around time (h)</th>
<th>Test results</th>
</tr>
</thead>
<tbody>
<tr>
<td>BioControl – Assurance EIA</td>
<td>18–28</td>
<td>Presence/absence (P/A)</td>
</tr>
<tr>
<td>BioControl – Transia</td>
<td>17–24</td>
<td>P/A</td>
</tr>
<tr>
<td>bioMerieux – VIDAS</td>
<td>18–24</td>
<td>P/A</td>
</tr>
<tr>
<td>TECRA E. coli O157 : H7 Via</td>
<td>20</td>
<td>NA</td>
</tr>
<tr>
<td>BioControl – VIP</td>
<td>8–18</td>
<td>P/A</td>
</tr>
<tr>
<td>EMD – Singlepath</td>
<td>18–24</td>
<td>P/A</td>
</tr>
<tr>
<td>EMD – Duopath Verotoxin</td>
<td>24+</td>
<td>P/A</td>
</tr>
<tr>
<td>Meridian – ImmunoCardSTAT</td>
<td>24+</td>
<td>P/A</td>
</tr>
<tr>
<td>Neogen – Reveal for E. coli</td>
<td>8–20</td>
<td>P/A</td>
</tr>
<tr>
<td>Dupont – Qualicon Lateral</td>
<td>18</td>
<td>P/A</td>
</tr>
</tbody>
</table>

Figure 1 shows a schematic of the extraction and detection assay and immunosensor using the redox property of polyaniline coating.

### METHODS

#### Chemicals and reagents

Aniline, ammonium persulfate, iron (III) oxide nanopowder, hydrochloric acid (HCl), methanol, diethyl ether, glutaraldehyde, peptone water, phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO). Protein A/G was purchased from Thermo Fisher Scientific (Rockford, IL). Affinity purified monoclonal and polyclonal antibodies for E. coli O157: H7 were obtained from Meridian Life Science (Sacramento, CA). BBL™ Trypticase™ Soy broth, BBL™ TSA II Trypticase™ Soy agar, BBL™ MacConkey II agar with Sorbitol, and Chrom agar were purchased from Becton, Dickinson and Company (Sparks, MD). Milk, spinach, and apple juice were purchased from a local store. Drinking water was collected from a private well. All chemical reagents were of analytical grade. All solutions and buffers were prepared in deionized water (Millipore Direct-Q system).

#### Bacterial isolates and culturing

Characterized strains of E. coli O157: H7 (Sakai strain), Bacillus cereus and Shigella boydii were obtained from the Nano-Biosensors Laboratory collection at Michigan State University. Bacterial cultures were grown following standard procedures. Briefly, colonies were isolated on TSA II Trypticase™ Soy agar by streak-plate method using frozen bacterial stocks stored at $-70^\circ$C. This was followed by inoculation of a single colony in 9 mL of BBL™ Trypticase™ Soy broth and...
incubation at 37°C overnight. One mL volume of this liquid culture was then transferred to 9 mL of BBL® Trypticase® Soy broth to produce a stock culture. The stock culture was stored at 37°C and used within 2 days. Prior to each experiment, a fresh bacterial culture was grown by transferring 1 mL of the stock culture to 9 mL of BBL® Trypticase® Soy broth and incubating for 6 hours at 37°C. A series of 10-fold serial dilution was prepared from the fresh culture using 0.1% (w/v) peptone water. Viable cells were enumerated by conventional microbial plating on BBL® TSA II Trypticase® Soy agar for Bacillus cereus and Shigella boydii and on Chrom agar with Sorbitol for E. coli O157:H7. All experiments were conducted in a certified Biosafety Level II cabinet.

Preparation of polyaniline-coated magnetic nanoparticles and antibody conjugation

Polyaniline-coated iron oxide nanoparticles (PIONs) were synthesized according to our published procedure with minor modification. Briefly, 0.650 g of gamma iron (III) oxide (γ-Fe₂O₃) nanoparticles were dispersed in a solution containing 0.4 mL of aniline, 50 mL of 1M HCl, and 10 mL deionized water by sonication in an ice-bath for 1 hour. This was followed by a slow addition of 20 mL of the oxidant, ammonium persulfate (0.2M), under sonication. Formation of polyaniline coating over γ-Fe₂O₃ nanoparticles was confirmed by a color change from rust brown to dark green. After 2 hours, the solution was filtered through a 2.5 μm pore-sized qualitative grade filter (Ahlstrom, grade 601). The supernatant was successively filtered through a 1.2 μm pore-sized nitrocellulose filter (Millipore). The solid thus obtained was washed with 10 mL of 1M HCl followed by 10 mL of 10% methanol and finally with 10 mL of diethyl ether and dried overnight under vacuum at room temperature. The solid was ground to a fine powder and stored in a vacuum desiccator at room temperature. The PIONs have been previously characterized in terms of size, conductivity and magnetization.

The PIONs were then functionalized with E. coli O157:H7-specific monoclonal antibodies (mAb) following our published procedure with slight modification. Ten mg/mL of PIONs were dispersed in 0.01M PBS (pH 7.4) under sonication for 15 minutes. Protein A/G (0.25 mg/mL) were added to the PIONs and the solution was incubated on a rotisserie-style tube rotor (Labquake, Thermo Scientific, MA) for 1 hour at room temperature. Then, 2.5 mg/mL of mAb were added to the PION solution and allowed to hybridize on a tube rotor at room temperature. After 1 hour, the mAb-PIONs were magnetically separated using a commercial commercial Glutaraldehyde + gold nanoparticles. After adding 25 μL of protein A/G at 25 μg/mL, E. coli O157:H7-specific polyclonal antibodies (5 μg/mL, pAb) were washed with 1 mL of 0.01M PBS containing 0.05% Tween-20 for 5 minutes and stored at 4°C for future use.

Immunosensor fabrication

The immunosensor was fabricated using a screen-printed carbon electrode (SPCE, Gwent Inc., UK) following a similar procedure as reported previously. Each SPCE was washed with 1 mL of sterile water and dried for 15 minutes at room temperature. Glutaraldehyde solution (25 μL of 2.5 mM) was pipetted onto the working electrode and incubated at 4°C for 1 hour. This was followed by three washings with 1 mL of sterile water each and drying for 15 minutes at room temperature. Gold nanoparticles (synthesized in-house, 30 nm in diameter) were added (25 μL) on the working carbon electrode. Then, 25 μL of protein A/G at 25 μg/mL was applied to the working area of the electrode, incubated for 1 hour at room temperature, washed 3 times with 1 mL of sterile water and dried. Finally, 25 μL of E. coli O157:H7-specific polyclonal antibodies (5 μg/mL, pAb) were added and dried for 15 minutes at room temperature followed by washing and drying. Figure 2 shows a schematic of the immunosensor.

Bacterial capture with polyaniline-coated magnetic nanoparticles

Serial dilutions of E. coli O157:H7 were prepared using 900 μL of sterile 0.01M PBS and 100 μL of stock culture and sprayed on 25 g of spinach to simulate contamination. The contaminated spinach was washed with 150 mL of sterile water before being aseptically transferred to a Whirl-pak bag. Then, 100 mL of 0.01M PBS were added into the Whirl-pak bag. The bag was then placed in the stomacher for 2 minutes to homogenize the spinach sample. Then, 100 μL of the resulting liquid from the homogenized spinach sample was added to 900 μL of 0.01M PBS and serially diluted into samples. Then, 200 μL of mAb-PION were added to the samples. The sample was allowed to react for 30 minutes and then magnetically separated. The supernatant was discarded. The cell-PION complex was washed with 1 mL 0.01M PBS containing 0.05% Tween-20 for 5 minutes and magnetically separated again. The PION-cell was re-suspended in 1 mL of PBS and 100 μL was plated on Chrom agar plates. The plates were incubated for 24 hours at 37°C and the colonies were counted. Extraction efficiency was calculated by dividing the log plate count with PION by the log plate count without PION. Milk and apple juice were prepared in the same manner. After adding 200 μL of mAb-PION to 99 mL of each liquid food sample, the procedure follows the method as in spinach above.

Detection was accomplished only for water and milk samples. One hundred microliters of the PION-cell complex were pipetted on the modified SPCE and incubated for 30 minutes at room temperature. The electrodes were rinsed with 1 mL of sterile water and dried for 15 minutes at room temperature. Then, 100 μL of 0.1M HCl was pipetted onto the SPCE and cyclic voltammetry (CV) was conducted to visualize the polyaniline redox signature in the captured E. coli O157:H7 complex as shown in Fig. 2. Cyclic voltammograms were generated on a 263A potentiostat/galvanostat (Princeton Applied Research, MA).

RESULTS AND DISCUSSION

PIONs were synthesized by polymerizing aniline around a cluster of iron oxide nanoparticles. Figure 3 shows transmission electron microscope (TEM) images of the iron oxide nanoparticles (a) and PIONs (b). From the TEM image, about 10–20 iron oxide nanoparticles comprise a PION.

The PIONs were then functionalized with anti-E. coli O157:H7 monoclonal antibodies and used to capture or extract the bacteria.
Figure 4 shows scanning electron microscope (SEM, JOEL 7500F, acceleration voltage of 5 kV) images of mAb-PIONs before (a) and after bacterial capture (b). The image in Fig. 4b shows that mAb-PION is attached to the bacterium *E. coli* O157:H7. The monoclonal antibody was prepared using purified *E. coli* serotype O157:H7 lipopolysaccharide (LPS) and its O polysaccharide moiety (Meridian Life Sciences). The cell envelope of Gram-negative bacteria, such as *E. coli*, is surrounded by a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane containing LPS. It is therefore likely that the mAb-PION has attached to the LPS in the cell wall of *E. coli* O157:H7.

Food samples representing past illness outbreaks (spinach, milk, and apple juice) were artificially contaminated with various concentrations of *E. coli* O157:H7 culture. Capture efficiency (CE) was calculated by counting the plated bacteria with PION and without PION after magnetic separation. A total of 109 experiments (*n* = 109) were conducted to generate the data in Table 2. More experiments were conducted on milk hence the higher *n* (64 experiments) compared to spinach (19) and apple juice (26). At all contamination levels (10⁰–10⁴ CFU/mL), the overall CE for spinach, milk, and apple juice are 92%, 99%, and 81%, respectively. Milk (*n* = 64), with a pH of 6, has the highest CE despite the fat and protein contents. PBS (pH 7.4) was used to rinse the spinach and the rinsate was used as sample (*n* = 19). Apple juice has a pH of 3.5–4 (acidic) and has the lowest CE (*n* = 26). The reduction in CE for apple juice could be attributed to the antibody being denatured with the acidic sample. Antibodies operate well at 6.6–8.3. At both sides of the maximum, the antigen-antibody reaction is strongly inhibited. For example, at pH 5.0 or 9.5, the equilibrium constant is reported to be 100-fold lower than at 6.5–7.0. Extreme pH values induce marked

### Table 2. Capture Efficiency of PION in Various Food Matrices.

<table>
<thead>
<tr>
<th>Concentration (CFU/mL)</th>
<th>Spinach</th>
<th>Milk</th>
<th>Apple juice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CE</td>
<td>Std Dev</td>
<td>n</td>
</tr>
<tr>
<td>10⁰</td>
<td>93%</td>
<td>4.2</td>
<td>3</td>
</tr>
<tr>
<td>10¹</td>
<td>94%</td>
<td>4.6</td>
<td>6</td>
</tr>
<tr>
<td>10²</td>
<td>92%</td>
<td>3.0</td>
<td>4</td>
</tr>
<tr>
<td>10³</td>
<td>90%</td>
<td>3.1</td>
<td>6</td>
</tr>
<tr>
<td>Average</td>
<td>92%</td>
<td>3.8</td>
<td>99%</td>
</tr>
<tr>
<td>Total number of experiments</td>
<td>19</td>
<td>64</td>
<td>26</td>
</tr>
</tbody>
</table>

**Figure 3** Transmission electron microscope images of the iron oxide nanopowder (a) and polyaniline-coated iron oxide nanoparticles (b).

**Figure 4** SEM images of polyaniline coated magnetic particles immunofunctionalized with *E. coli* O157:H7-specific monoclonal antibodies (a) and *E. coli* O157:H7 cell captured by the magnetic nanoparticles (b).
conformational changes in the antibody molecule that probably destroy the complementarity with the antigen\textsuperscript{43}.

Bacterial detection was carried out on an antibody-functionalized SPCE. Figure 5 shows the antibody-modified SPCE surface before immunosensing (a) and after immunosensing with bacterial attachment (b). The image shows that the bacterium was captured by the polyclonal antibody on the SPCE surface. The immunogen used in the preparation of the polyclonal antibody was the whole *E. coli* O157: H7 organism that retained the native structure of the O and H antigens (Meridian Life Sciences) thus, the attachment of the antibody is either on the O or H antigen surface of the bacterium.

Detection was achieved by the redox activity of PION. Figure 6 shows a typical cyclic voltammogram of a sample without bacteria (black, blue line) and with bacteria (red line). PION has a CV peak at −0.28 V indicative of polyaniline. The blank samples have polyaniline signal even though no bacteria were present. This might be due to non-specific attachment of PIONS on the SPCE surface via antibody-antibody interactions and/or physical adsorption. Nonetheless, the immunosensor with bacteria shows a higher peak current than the blank samples.

Figure 7 shows the results for detection of *E. coli* O157: H7 in peptone water and skim milk expressed as signal-to-noise (SNR) ratio. In peptone water (blue diamonds), detection was successfully achieved at bacterial concentration ranging from $5 \times 10^1$ CFU/mL to $5 \times 10^5$ CFU/mL. It should be noted that since only 100 µL of the solution was loaded onto the immunosensor, the sensor could then detect close to 5 CFUs. In milk (pink squares), the detection range is $5 \times 10^2$–$5 \times 10^5$ CFU/mL. Milk was not tested at one log lower (10\textsuperscript{1}) concentration because it is difficult to confirm the bacterial count by plating at a lower concentration. Besides, data show that milk has higher average signal than water at all concentrations which would indicate that the biosensor should be able to detect the bacteria at similar lower concentration. Figure 7 also shows that the standard deviation increases as the concentration decreases. This is expected as the bacteria are more dispersed in the sample matrix at lower concentration and pipetting them out for detection is influenced by their dispersion.

Immunosensor specificity was tested using two non-target bacteria, *Shigella boydii* and *Bacillus cereus*. The immunosensor detected *E. coli* O157: H7 only and not *Bacillus cereus* and *Shigella boydii* even with the non-target bacteria at a higher concentration of $10^5$ CFU/mL as shown in Fig. 8. This result indicates that the immunosensor has high specificity. Both the non-target bacteria were not captured by the mAb-PION as confirmed by plating. Figure 9 shows the results for the detection of *E. coli* O157: H7 in 100 mL of ground water sample. The immunosensor could detect bacterial concentration from $3 \times 10^2$ CFU/mL to $3 \times 10^5$ CFU/mL. Again, with sample size being 100 µL, there were about 30 CFU on the immunosensor surface. This low-level detection can be extremely
is demonstrated for 5 CFUs. The wide variance is something that will affect the performance of the biosensor.

Additional work on sensitivity and specificity will strengthen the robustness. The biosensor will need to be validated in more varied food and clinical samples to demonstrate robustness. Additional work on sensitivity and specificity will strengthen the performance of the biosensor.

This biosensor technology has great potential in monitoring microbial contaminants in food production and supply chain settings where simplicity, sensitivity, and ease of use are important. This technology has promise to help in preventing and reducing illness due to microbial contamination.

Furthermore, magnetic separation is showing great promise as an alternative to existing conventional sample processing systems, such as centrifugation, filtration, and microfluidics. Centrifugation can be time-consuming and requires an expensive equipment; filtration or microfluidics is prone to clogging. The use of PIONs for extraction and detection will allow for rapid processing of high-volume samples relevant in the food industry and would be useful in life-saving and time-sensitive food safety decision-making.

ACKNOWLEDGMENTS
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