Rapid Detection of *E. coli* O157:H7 on Turnip Greens Using a Modified Gold Biosensor Combined with Light Microscopic Imaging System

Mi-Kyung Park and Jun-Hyun Oh

Abstract: This research aims to demonstrate the feasibility of a modified gold biosensor to detect *E. coli* O157:H7 in leafy turnip greens. The gold biosensor was modified with dithiobis-succinimidyl propionate (DSP) and/or protein A or G. The gold biosensor modified with DSP (Gold-DSP) was combined with a light microscopic imaging system (LMIS). The optimal concentration and specificity of anti-*E. coli* O15 polyclonal antibodies (pAbs) on the biosensor were determined. The reliability of Gold-DSP biosensor was investigated by determining the sensitivity, specificity, and limit of detection (LOD) of the Gold-DSP combined with LMIS. The Gold-DSP combined with LMIS was applied to turnip greens for *E. coli* O157:H7 detection. The modification of Gold biosensor with DSP significantly increased the detected number of *E. coli* O157:H7. The specificity of pAbs was sufficient to react with target *E. coli* O157:H7 among the tested bacterial culture. The optimum concentration of pAbs was determined as 200 μg/mL. The sensitivity, specificity, and LOD of Gold-DSP combined with LMIS were determined as 100%, 90%, and 10^3 CFU/25 mm^2, respectively. When applied to turnip greens, the Gold-DSP combined with LMIS could detect 2641 $\pm$ 394 and 15383 $\pm$ 3853 cell/mm^2 with the initial concentrations of 10^1 and 10^2 CFU/25 g turnip greens, respectively, after 10 h-enrichment. Overall, this research suggested that the Gold-DSP combined with LMIS could be used to detect *E. coli* O157:H7 on turnip greens qualitatively and quantitatively.

Keywords: biosensor, dithiobis-succinimidyl propionate, *E. coli* O157:H7, polyclonal antibodies, turnip greens

Introduction

The increased consumption of fresh fruits and vegetables may be associated with an increase in fresh produce-related foodborne diseases (Meng and Doyle 2002; Sivapalasingam and others 2004; Heaton and Jones 2008; Lynch and others 2009). Recently, *E. coli* O157:H7 outbreaks have become more problematic and have caused serious concerns in the fresh produce industry (Erkan and Vural 2008; Pangloli and others 2009). Turnip greens (*Brassica rapa* subgroup rapifera) are a common leafy vegetable that are widely consumed in daily diets, providing reliable and affordable vitamins and minerals in most developing countries (Mosha and others 1995). Turnip greens are often sold in local grocery stores or at farmers' markets, and the safety of turnip greens with respect to pathogens is of concern in the local markets. Therefore, it would be ideal for fresh produce such as leafy turnip greens to be inspected systematically with a practical real-time and on-site method in the field as well as critical control points along the entire supply chain.

Considerable effort has been directed towards the development of rapid and practical detection methods such as polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), and several types of biosensor methods to substitute for the laborious and time-consuming conventional detection methods (Skottrup and others 2008; Velusamy and others 2010). Among those alternative methods, biosensor methods have been demonstrated for the potential feasibility of practical on-site detection of foodborne pathogens (Lazcka and others 2007; Ricci and others 2007; Velusamy and others 2010; Su and others 2011). Although extensive studies have been performed over the past decades, the most popular and promising biosensor methods include surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) biosensors for practical applications (Ricci and others 2007; Skottrup and others 2008). The primary principle of SPR and QCM biosensor methods is based on the detection of subtle changes caused by the interactions between the target microorganisms and the specific antibody-immobilized biosensor platforms (Ricci and others 2007; Skottrup and others 2008). The advantages of the SPR or QCM biosensors as direct and label-free detection methods are the simplicity and rapidity of these methods. In addition, these methods do not require the level of operator expertise and complicated sample preparation steps compared with PCR (Lazcka and others 2007; Ricci and others 2007). Biacore SPR sensor platforms are the most widely used and are commercially available with various functionalities (Skottrup and others 2008). In further improvement of the SPR method, Spreeta^{TM} sensors have been developed to overcome the lack of portability and field applications. Spreeta SPR systems have been demonstrated for on-site and in laboratory detection for toxins and pathogens such as *Camplyobacter*, *E. coli*, and *Listeria monocytogenes* (Chinowsky and others 2007; Nanduri and others 2007; Feltis and

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others 2008; Skottrup and others 2008). However, since these SPR and QCM methods are based on the subtle changes at the sensor surfaces, the detection sensitivity and reliability could be affected from the binding of other co-existing items in the food as well as the target bacteria (Ricci and others 2007; Velusamy and others 2010).

The modifications and improvement of biosensors have been attempted recently (Zhang and others 2006; Guntupalli and others 2007; Velusamy and others 2010). The gold biosensor combined with light microscopic imaging system (LMIS) was developed to detect *Salmonella* in fresh milk and chicken products (Zhang and others 2006; Park 2009). In the gold biosensor combined with LMIS, a nanometer scale gold layer was coated on a glass platform to facilitate the antibody immobilization on the sensor platform. The specific antibodies against *Salmonella* were then immobilized with a direct adsorption method as a biosensing element to bind with target bacteria during incubation. Bound *Salmonella* on a gold sensor can be visualized and enumerated using a light microscope connected with a cooled digital charged-coupled device (CCD).

The overall goal of this research is to demonstrate the feasibility of a modified gold biosensor combined with LMIS to detect *E. coli* O157:H7 in turnip greens as a rapid analytical detection method in a food testing laboratory. The specific objectives of this research were to (1) improve the binding efficiency of the gold biosensor with *E. coli* O157:H7, (2) optimize and characterize the gold biosensor by determining the optimal concentration of antibodies, specificity, and detections limits of the biosensor, and (3) apply the modified gold biosensor combined with LMIS to detect *E. coli* O157:H7 in turnip greens.

Materials and Methods

Bacterial species and culture conditions

The bacterial species tested in this research were obtained from the Food Microbiology Lab. at Auburn Univ. (Auburn, Ala., U.S.A.). Novobiocin and nalidixic resistant *E. coli* O157:H7 204P and *E. coli* O157:H7 301C were cultured in 20 mL modified EC broth (Difco Lab., Sparks, Md., U.S.A.) with 100 ppm novobiocin and 10 ppm nalidixic acid (mEC + nn broth) for 16 h at 37 °C. The bacterial species except *Listeria* species listed in Table 1 were cultured in Trypticase® Soy Broth (TSB, Difco Lab.) for 16 h at 37 °C. *L. monocytogenes* were cultured in TSB containing 0.6% yeast extract (TSBYE) for 16 h at 37 °C.

After cultivation, each bacterial culture was washed with sterilized phosphate buffered saline (PBS) (pH 7.2) (Sigma-Aldrich Co., St. Louis, Mo., U.S.A.) 3 times by centrifugation at 5000 × *g* for 5 min. The collected bacterial cells were re-suspended in sterilized PBS, and the bacterial concentration was adjusted to approximately 10^9 CFU/mL using a pre-constructed standard curve determined by the optical density at 640 nm for further experiments.

Preparation of gold biosensors immobilized with anti-*E. coli* O15 polyclonal antibodies

A microscopic cover glass with a thickness of 0.17 mm was cut into 5 × 5 mm^2 using a micro-dicing saw (MPE Inc., Grass Valley, Calif., U.S.A.). The cut glass squares were first ultrasonically cleaned in acetone followed by ethanol and finally rinsed with deionized (D.I.) water. The cleaned glass squares were coated with a 140 nm thick gold layer using a Pelco SC-6 sputter coater (Ted Pella Inc., Redding, Calif., U.S.A.) for the preparation of the gold sensor platform (Zhang and others 2006; Park 2009).

An anti-*E. coli* O15 polyclonal rabbit IgG antibodies (pAbs, 4 mg/mL) purchased from LifeSpan BioSciences Inc. (Seattle, Wash., U.S.A.) was immobilized onto the gold biosensor. To form the measurement biosensor, the gold sensor platform was incubated with a 100 μL pAbs at 37 °C for 2 h in a 96-well plate (Whatman Inc., Clifton, N.Y., U.S.A.). A control biosensor was similarly prepared, substituting D.I. water for pAbs. After washing with PBS 3 times, both types of sensors were blocked with 100 μL 1% bovine serum albumin (BSA, Sigma-Aldrich Co.) at 22 °C for 1 h. Finally, the sensors were washed 3 times with PBS and air dried for further use (Park 2009).

Detection of bacteria on the pAbs-immobilized gold biosensor combined with LMIS.

The pAbs-immobilized gold biosensors were immersed into a sample suspension containing selected bacterial concentrations and incubated at 37 °C for 1 h in a rotary shaker. After incubation, the pAbs-immobilized gold biosensors were washed with D.I. water, dried, and then treated with OsO_4 (Sigma-Aldrich Co.) for at least 1 h to fix the bacteria on the sensor and to protect the bacteria from any structural damages (Zhang and others 2006). The detection of captured bacteria on the biosensor was performed in combination with LMIS. The images of bacterial cells on the biosensor were viewed and enumerated using a CCD camera equipped with the light microscope (Nikon Eclipse L150, Nikon Instruments Inc., Melville, N.Y., U.S.A.) with 1000 times magnification. The captured bacteria cells were enumerated from 10 randomly selected areas on the surface of gold biosensor. The detected bacterial number on a biosensor was averaged from the counted bacterial numbers in each area and expressed as cell per square millimeter (cell/mm^2).

Modification of gold biosensor

The gold biosensor was modified with dithiobis-succinimidyl propionate (DSP), protein A, or recombinant protein G to enhance the immobilization of antibodies. For the modification of the gold biosensor with DSP (hereafter Gold-DSP biosensor), 200 μL 2 mM DSP (Thermo Sci., Rockford, Ill., U.S.A.) in DMSO solution was added onto the surface of gold biosensors. For modifications of biosensors with protein A (hereafter Gold-Protein A) or protein G (hereafter Gold-Protein G), 50 μL protein A (Sigma-Aldrich Co.) or protein G (Sigma-Aldrich Co.) were dissolved in 20 mM phosphate buffer (pH 7.4) and added onto the surface of the gold biosensors. Then, 50 μL 0.1 M sodium-acetate buffer (pH 4.69) were added onto the surface of gold biosensors immediately to optimize the adsorption of protein A or protein G onto the surface of the gold biosensor (Babacan and others 2000).

The Gold-DSP biosensors were further modified with protein A (hereafter Gold-DSP-Protein A) or protein G (hereafter Gold-DSP-Protein G), following the similar procedures for protein modifications. The modifications of Gold-DSP, Gold-Protein A, Gold-Protein G, Gold-DSP-Protein A, and Gold-DSP-Protein G were continued by incubating at 22 °C for 2 h, washing gently with PBS buffer 4 times, and air-drying. The 5 modified biosensors were immobilized with pAbs, following the immobilization procedures described in the previous section as a measurement sensor. The control sensor was prepared without pAbs immobilization procedure.

Determination of optimum concentration of pAbs

The Gold-DSP biosensor was incubated with a 100 μL pAbs diluted in PBS to concentrations of 12.5, 25, 50, 100, 200,

and 400 μg/mL for 2 h at 22 °C to determine the optimum concentration of pAbs for immobilization. After incubating the pAbs-immobilized Gold-DSP biosensor in the presence of *E. coli* O157:H7, the number of captured *E. coli* O157:H7 on the biosensor was counted using a LMIS, following the procedures described in the previous section.

Specificity of pAbs-immobilized Gold-DSP biosensor

Antibody immobilized Gold-DSP biosensors were prepared following the procedures described in the previous section. The pAbs-immobilized gold biosensors were immersed into a bacterial suspension containing selected bacterial concentrations (10^6 and 10^8 CFU/mL) for the specificity test and followed the procedures described in the previous section (detection of bacteria on the pAbs-immobilized gold biosensor combined with LMIS). Indirect ELISA was also conducted with the pAbs diluted 600 times and 3000 times from the original concentration (4 mg/mL) to confirm the specificity of the Gold-DSP biosensor combined with LMIS. The indirect ELISA was performed following the general procedure previously developed by Zhang and others (2006).

Reliability of Gold-DSP biosensor combined with LMIS

An *E. coli* O157:H7 cocktail containing both *E. coli* O157:H7 204P and *E. coli* O157:H7 301C was used as a true positive sample, and a bacterial cocktail containing the strains of *L. monocytogenes* 7757, *L. monocytogenes* 1911, *S. enterica* Typhimurium, and *S. enterica* Enteritidis was used as a true negative sample. An aliquot of 100 μL (10^8 CFU/mL) from true positive and negative cocktail samples was applied to the pAbs-immobilized Gold-DSP biosensor and followed the procedures described in the previous section (detection of bacteria on the pAbs-immobilized gold biosensor combined with LMIS).

The sensitivity and specificity of the biosensors were calculated using the following equations reported by Eijkelkamp and others (2009) with minor modification: Sensitivity (%) = the number of true positives results/the number of true contaminated sample × 100 = the number of true positive results/(the number of true positive results + the number of false negative results) × 100; Specificity (%) = the number of true negative results/(the number of contaminated sample with true negative bacteria × 100 = the number of true negative results/(the number of false positive results + the number of true negative results) × 100.

Fully grown *E. coli* O157:H7 cocktails cultivated in mEC + nn broth were serially diluted from 10^2 to 10^9 CFU/mL and 100 μL of *E. coli* O157:H7 cocktails were added on to both the pAbs-immobilized Gold-DSP biosensor and the control biosensor to determine the limit of detection of the biosensor with *E. coli* O157:H7. The number of captured *E. coli* O157:H7 on the biosensor was counted using a LMIS.

Application of Gold-DSP biosensor combined with LMIS to detection for *E. coli* O157:H7 on turnip greens

Turnip greens were purchased from a local farmers' market (Tuskegee, Ala., U.S.A.) and sanitized with 200 ppm-chlorine to minimize the background bacteria potentially present on the surface of the turnip greens. After rinsing the sanitized turnip greens with sterilized water 10 times, the turnip greens were trimmed to 25 g under a biosafety cabinet. Prior to applying the method, *E. coli* O157:H7 was enriched to increase the bacterial number. An aliquot of 100 μL *E. coli* O157:H7 cocktail in PBS (pH 7.4) with the concentrations of 10^2 and 10^3 CFU/mL were spot-inoculated on turnip greens by depositing droplets at 20 locations and 100 μL blank PBS (pH 7.4) was also applied to turnip greens as a negative control. Then the turnip greens were kept at room temperature for 90 min to allow *E. coli* O157:H7 to attach on the surface of turnip greens. The *E. coli* O157:H7inoculated on turnip greens were put into a sterile stomach bag containing 225 mL mEC + nn broth and blended in a Seward 400 circulator stomacher (Seward Co., Seward, England) for 2 min. Then the homogenized turnip greens were transferred into an Erlenmeyer flask and enriched up to 10 h at 37 °C in an orbital shaker with the shaking rate of 250 rpm. During enrichment, 100 μL was taken from the mixture at the time periods of 2, 4, 6, 8, and 10 h, and plated on triplicate Sorbitol MacConkey agar (SMAC, Difco Lab.) containing 100 ppm novobiocin and 10 ppm nalidixic acid. The SMAC plate was selected as a selective media to differentiate *E. coli* O157:H7 from other *E. coli* (Feng and others 1998; Chen and Frankel 2005). An aliquot of 25 mL was filtered through glass wool and centrifuged at 5000 × *g* for 10 min. After washing with sterilized PBS, the collected *E. coli* O157:H7 cells were suspended in 1 mL sterilized PBS. An aliquot of 100 μL mixture was finally added onto the both a pAbs-immobilized Gold-DSP biosensor and control sensor. The number of captured *E. coli* O157:H7 on the biosensor was counted using a LMIS.

Statistical analysis

The experiments were performed triplicate and the experimental results were expressed as means with the standard deviation (SD) of means. Comparisons between various treatments and/or groups were conducted using one-way analysis of variance (ANOVA) and Tukey–Kramer multiple comparisons test. Differences were considered to be statistically significant if the "*P*-value" was less than 0.05 or 0.001. Statistical analysis was performed using GraphPad and InStat version 5 programs (GraphPad, San Diego, Calif., U.S.A.).

Results and Discussion

Modification of gold biosensor

The random orientation of antibodies during direct physical adsorption can be improved by using an alternative covalent attachment method that involves chemical binding between antibodies and chemical materials such as DSP and 11-mercaptoundecanoic acid (MUA) (Oh and others 2005; Jung and others 2008). The covalent attachment method was reported more stable than the physical adsorption method due to the bi-functional cross-linking where one functional group reacted with the sensor platform and the other group interacted with antibody. Recently, protein A, recombinant protein G, and their derivatives have been applied to interact with the Fc region of antibodies to increase the desirable orientation and anchoring on the biosensor platform (Jung and others 2008; Skottrup and others 2008).

The direct modification of Gold biosensors with protein A or recombinant protein G did not significantly increase the detected number of *E. coli* O157:H7 on the biosensor compared to the Gold biosensor without any modification (Figure 1). However, the Gold biosensor modified with DSP only (Gold-DSP) and Gold biosensor modified with DSP and protein A (Gold-DSP-Protein A) exhibited significant increases in the binding efficiencies with *E. coli* O157:H7 ($P < 0.05$), exhibiting 1955 ± 516 and 2030 ± 475 cell/mm^2. Although there was no significant difference between these 2 biosensors, DSP modified biosensor showed increased binding efficiencies with *E. coli* O157:H7.

In comparison, a gold-coated QCM biosensor modified with DSP exhibited a greater binding efficiency with *Salmonella* spp. than that of a biosensor modified with protein A only (Park and Kim 1998). Also, a gold-coated SPR biosensor previously modified with MUA and protein G improved the binding efficiency with *Salmonella* Typhimurium, as compared to a gold biosensor treated with protein G only (Oh and others 2005). DSP, as a bi-functional cross-linker, could bind covalently with anti-*E. coli* pAbs through *N*-hydroxysuccinimide ester in DSP and adsorb chemically onto the gold biosensor platform (Ye and others 1997). In turn, the properly bound antibodies on the gold biosensor could increase the binding efficiency of the biosensor with *E. coli* O157:H7. Finally, since there was no significant difference between the Gold-DSP biosensor and the Gold-DSP-protein A biosensor, the Gold-DSP biosensor was determined as the optimum biosensor for *E. coli* O157:H7 detection.

Determination of optimum concentration of pAbs

The optimum concentration of anti-*E. coli* pAbs was determined and presented in Figure 2. The concentrations of pAbs greater than 50 μg/mL exhibited significant increases in the detected numbers of *E. coli* O157:H7 ($P < 0.05$). Since the greatest detected number of *E. coli* O157:H7 was observed at the concentration of 200 μg/mL, the optimum concentration of pAbs was determined as 200 μg/mL in this research. The apparent decrease in binding efficiency at concentrations greater than 200 μg/mL may be attributed to the steric hindrance caused by crowded antibodies on the biosensor.

Specificity of pAbs-immobilized Gold-DSP biosensor

The specificity of Gold-DSP biosensor compared with indirect ELISA tested with selected bacterial strains is presented in Table 1. The anti-*E. coli* pAbs exhibited significantly higher reactivity with both *E. coli* O157:H7 204P and 301C than the reactivity with other strains of *Escherichia* tested ($P < 0.05$ or $P < 0.001$). Indirect ELISA method exhibited that the absorbance of both *E. coli* O157:H7 204P and 301C were greater than the cut-off value, 0.271. Although there was slight reactivity with *Shigella sonnei* ATCC25931, *E. coli* GM2163, and *E. vulneris* ATCC29943, and *E. vulneris* ATCC33832 at the concentration of 10^8 CFU/mL, the reactivity was considered negligible ($P > 0.001$). Therefore,

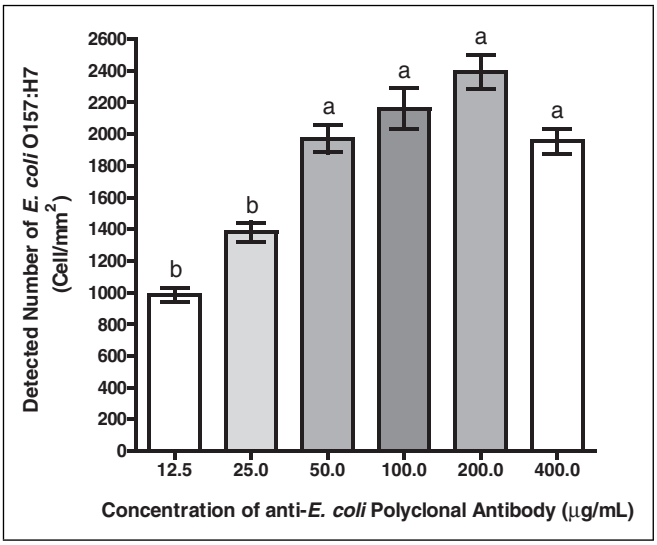

Figure 2–The effect of concentration of anti-*E. coli* O15 polyclonal antibodies (pAbs) on the binding efficiency of gold biosensor modified with DSP (Gold-DSP). Vertical bars represent standard deviation ($N = 30$), and different letters indicate significantly different means among treatments at $P < 0.05$.

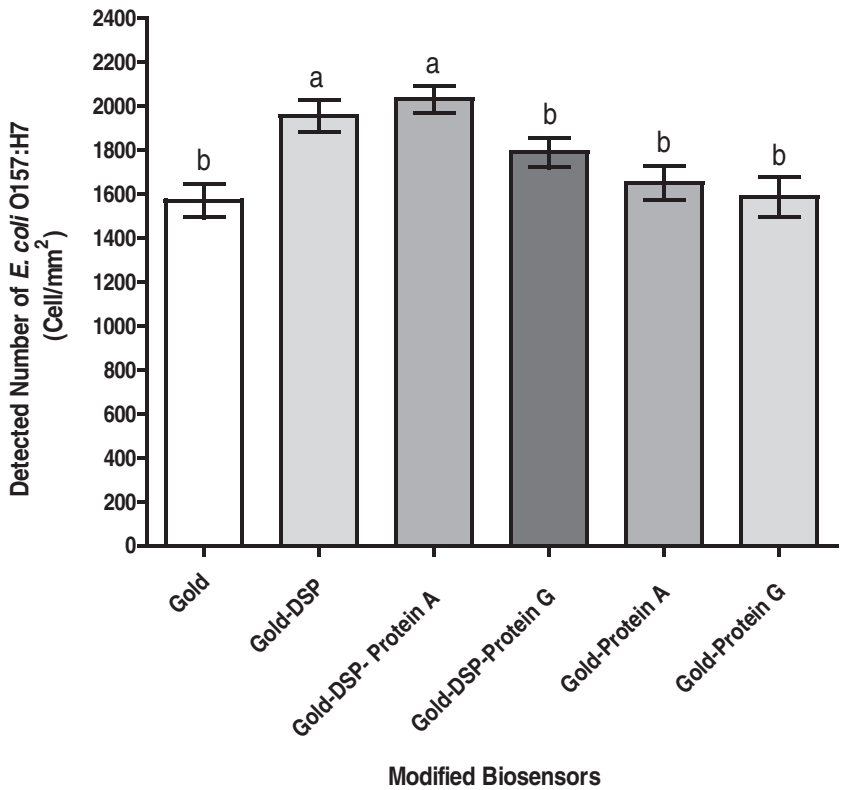

Figure 1–The binding efficiency of gold biosensors (Gold) modified with DSP (Gold-DSP), protein A (Gold-Protein A), or recombinant protein G (Gold-Protein G). Vertical bars represent standard deviation ($N = 30$), and different letters indicate significantly different means among treatments at $P < 0.05$.

it was concluded that the anti-*E. coli* pAbs was sufficiently and significantly specific with the pathogenic *E. coli* O157:H7 204P and 301C.

Reliability of the Gold-DSP biosensor combined with LMIS

The sensitivity is defined as the probability of a sample testing positive if a contamination was truly present, whereas specificity represents the probability of a test being negative if a contamination is truly absent (Eijkelkamp and others 2009). The cut-off value for consideration as positive result was determined as 74 cell/mm^2 obtained from the average of detected numbers of *L. monocytogenes* and *Salmonella enterica* in Table 1. The calculated sensitivity and specificity of Gold-DSP combined with LMIS were 100% and 90%, respectively. Although there have not been established clear acceptance criteria for sensitivity and specificity (Eijkelkamp and others 2009), these results are similar or greater than to those from the Pavic and others' study (2010) with sensitivity of 92% and specificity of 95%. In addition, these results are similar to those from the AOAC and ANFOR-approved Vidas® *Salmonella* assay with sensitivity of 93% and specificity of 96%, respectively (Pavic and others 2010). Therefore, it was assumed that this method was not perfect but competitive with other method.

Table 1– The comparison of specificity determined by an anti-*E. coli* O15 polyclonal antibodies (pAbs)-immobilized Gold-DSP biosensor combined with LMIS with indirect ELISA testing among selected bacteria.

		Detection method	
Bacteria	Bacterial conc. (CFU/mL)	Gold-DSP biosensor combined with) LMIS (Cell/mm^2)	Indirect ELISA (O.D.)
E. coli O157:H7 204P	10^6	2311 ± 395*	0.347 ± 0.015[a]
	10^8	20447 ± 3975***	1.139 ± 0.024[a]
E. coli O157:H7 301C	10^6	2090 ± 471*	0.548 ± 0.025[a]
	10^8	18623 ± 3702***	1.124 ± 0.018[a]
E. coli GM2163	10^6	106 ± 167	0.176 ± 0.009
	10^8	851 ± 730	0.364 ± 0.040
E. blattae ATCC33429	10^6	66 ± 77	0.072 ± 0.010
	10^8	85 ± 93	0.124 ± 0.040
E. blattae ATCC33430	10^6	43 ± 57	0.098 ± 0.015
	10^8	79 ± 84	0.139 ± 0.024
E. fergusonii ATCC35469	10^6	66 ± 97	0.148 ± 0.017
	10^8	105 ± 93	0.224 ± 0.018
E.hermannii ATCC33650	10^6	39 ± 47	0.076 ± 0.009
	10^8	51 ± 58	0.097 ± 0.020
E. vulneris ATCC33832	10^6	101 ± 97	0.147 ± 0.015
	10^8	451 ± 257	0.219 ± 0.024
E. vulneris ATCC29943	10^6	137 ± 205	0.149 ± 0.007
	10^8	669 ± 327	0.257 ± 0.000
Salmonella enterica	10^6	23 ± 38	0.085 ± 0.001
Typhimurium	10^8	91 ± 99	0.164 ± 0.013
Salmonella enterica	10^6	41 ± 39	0.141 ± 0.004
Enteritidis	10^8	77 ± 51	0.243 ± 0.094
Salmonella enterica Misson	10^6	38 ± 29	0.019 ± 0.009
	10^8	60 ± 75	0.106 ± 0.013
Listeria monocytogenes 7738	10^6	15 ± 68	0.022 ± 0.001
	10^8	67 ± 99	0.100 ± 0.010
L. monocytogenes 1911	10^6	38 ± 53	0.016 ± 0.003
	10^8	86 ± 56	0.076 ± 0.006
Listeria innocua	10^6	24 ± 29	0.046 ± 0.005
ATCC33090	10^8	65 ± 68	0.089 ± 0.005
Citrobacter freundii SA5606	10^6	31 ± 59	0.057 ± 0.001
	10^8	73 ± 52	0.077 ± 0.013
Staphylococcus aureus	10^6	46 ± 99	0.123 ± 0.008
ATCC6538	10^8	41 ± 49	0.133 ± 0.013
S. sonnei ATCC25931	10^6	106 ± 158	0.105 ± 0.011
	10^8	338 ± 174	0.195 ± 0.028
Yersinia enterocolitica M22	10^6	51 ± 99	0.023 ± 0.008
	10^8	84 ± 84	0.133 ± 0.013
Klebsiella oxytoca	10^6	84 ± 98	0.029 ± 0.002
ATCC13182	10^8	167 ± 149	0.170 ± 0.049
Negative control	10^6	0.0 ± 0.0	0.007 ± 0.001
(distilled water)	10^8	0.0 ± 0.0	0.011 ± 0.002

[a] The numbers indicate that the absorbance value was greater than the cut-off value (0.271 = mean of the negative control + 0.250) ($N = 3$) (Lieve and van Poucke 1990).
* and *** indicate that the detected number of foodborne pathogenic bacteria was significantly different at $P < 0.05$ and $P < 0.001$, respectively.
The entire bacterial species tested were obtained from the Food Microbiology Lab. at Auburn Univ. (Auburn, Ala., U.S.A.).

The numbers of *E. coli* O157:H7 detected by Gold-DSP combined with LMIS from the selected concentrations are presented in Figure 3 and 4. As the inoculated concentration of *E. coli* O157:H7 increased, the detected numbers of *E. coli* O157:H7 also increased. The detected numbers of *E. coli* O157:H7 on the Gold-DSP sensor were 99 ± 55, 484 ± 199, 4373 ± 703, 19196 ± 2115, and 34215 ± 3070 cell/mm^2 for the inoculated concentrations of 10^3,10^4, 10^5, 10^6, and 10^7 CFU/25 mm^2, respectively. The control sensors exhibited negligible detection of *E. coli* O157:H7 (less than 7 cell/mm^2) over the entire range of concentrations. At the inoculated concentrations of 10^8 CFU/25 mm^2, *E. coli* O157:H7 was often observed in a clustered form consisting of several cells, possibly resulting in inaccurate counts. An appropriate range of bacterial concentration for this method from 10^3 to 10^6 CFU/25 mm^2 is proposed to prevent false counting and facilitate the enumeration process in practical applications.

The limit of detection (LOD) was defined as the lowest concentration of *Salmonella* inoculated on a sensor required for at least single bacterial cell detection with minimum 50% positive rate among 15 observations per sensor (Eijkelkamp and others 2009). Based on the definition, the LOD of Gold-DSP combined with LMIS in this research was determined as 10^3 CFU/25 mm^2. The LOD of Gold-DSP combined with LMIS in this research was greater than the LODs of SPR or QCM. Oh and others (2005) reported that the LOD of protein G treated gold biosensor modified with a self-assembled monolayer (SAM) of MUA was determined as 10^5 CFU/mL for *E. coli* O157:H7 detection using the SPR method. The LOD of gold-DSP biosensor combined with a flow injection analysis (FIA) was determined to be 10^5 CFU/mL to detect *Salmonella typhimurium* (Ye and others 1997), and the LOD of a QCM method to detect *E. coli* was determined to be 10^6 CFU/mL (Ivnitski and others 1999). In addition, the LOD of the pAbs-immobilized Gold-DSP combined with LMIS is comparable to several commercial rapid detection kits; the LOD of RapidCheck® Lateral Flow Test Kit is 10^4 CFU/mL, which is designed to detect viable *E. coli* O157 in raw beef after enrichment, and the LOD of Singlepath® *E. coli* O157 Rapid Lateral Flow Assay is 10^4 to 10^6 CFU/mL after enrichment (Tokarskyy and Marshall 2008).

Application of Gold-DSP biosensor combined with LMIS to detection of *E. coli* O157:H7 on turnip greens

As presented in Figure 5, *E. coli* O157:H7 did not grow during 4 h enrichment at the initial inoculations of both 10 CFU and 10^2 CFU/25 g turnip greens. However, the number of *E. coli* O157:H7 were increased to 6.2 log CFU/25 g turnip greens from 10 CFU/25 g turnip greens, and 7.8 log CFU/25 g turnip greens from 10^2 CFU/25 g turnip greens after 10 h-incubation time. Since the LOD of the Gold-DSP biosensor combined with LMIS was determined as 10^3 CFU/25 mm^2 in this research, *E. coli* O157:H7 was not detected by the Gold-DSP biosensor from an enrichment period of less than 8 h. In fact *E. coli* O157:H7

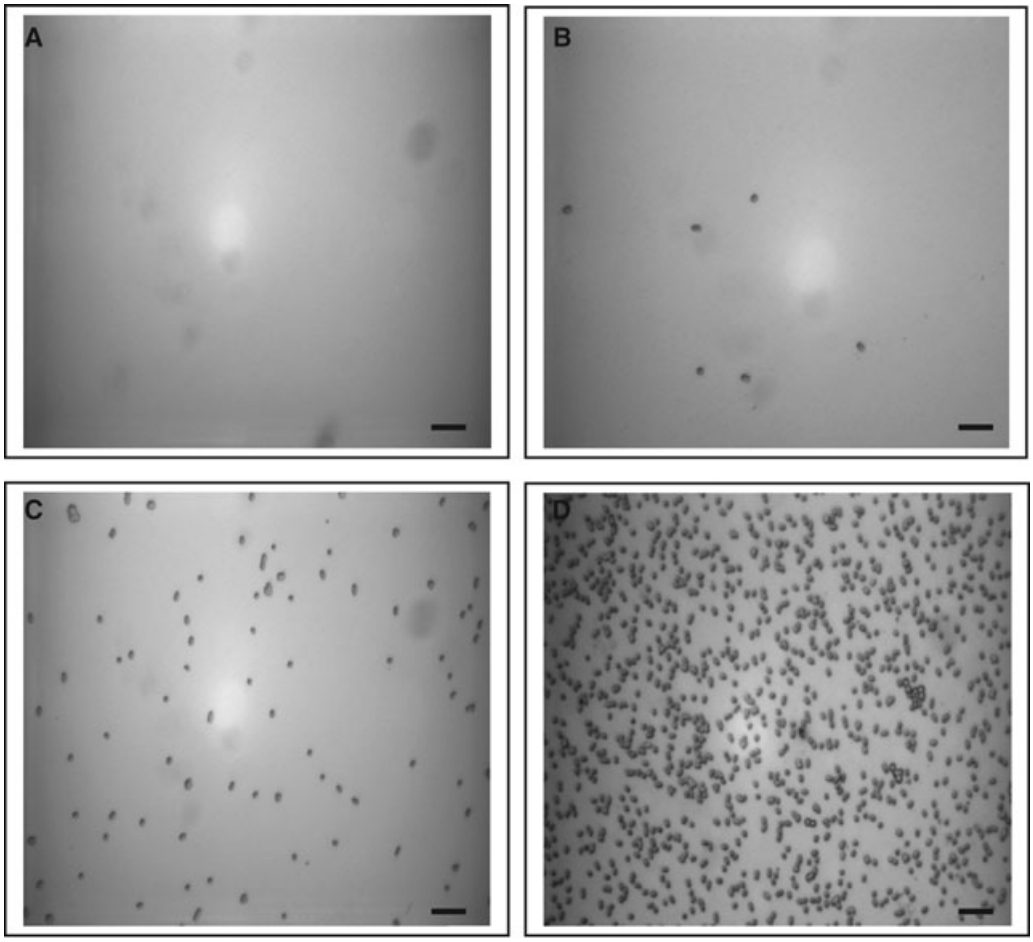

Figure 3–The light microscopic images of *E. coli* O157:H7 detected on the gold biosensors modified with DSP (Gold-DSP). The detection was performed with Gold-DSP control sensor for the concentration of (A) 10^6 CFU/25 mm^2 and with pAbs-immobilized Gold-DSP measurement sensors for the concentrations of (B) 10^4 CFU/25 mm^2, (C) 10^5 CFU/25 mm^2, and (D) 10^7 CFU/25 mm^2. The bar in the image represents 5 μm in length.

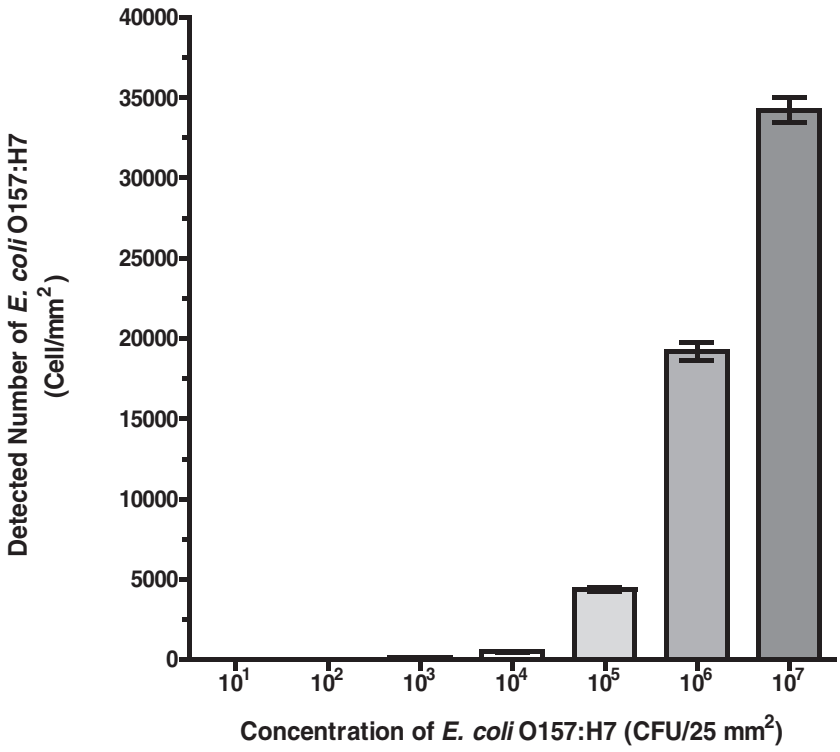

Figure 4–The detected number of *E. coli* O157:H7 using the gold biosensor modified with DSP (Gold-DSP) combined with LMIS at selected bacterial concentrations. Vertical bars represent standard deviation ($N = 30$).

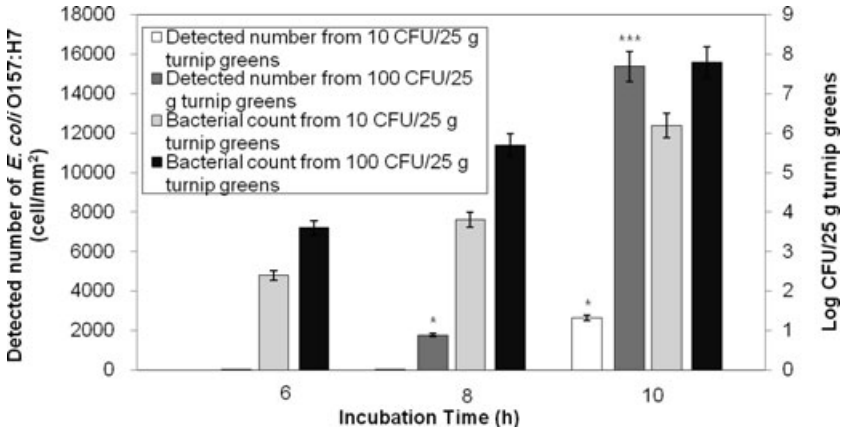

Figure 5–The detected number and bacterial count of *E. coli* O157:H7 in turnip greens after enriching for selected time periods using the Gold-DSP measurement sensor combined with LMIS. The initial concentrations of *E. coli* O157:H7 prior to enrichment were 10 and 10^2 CFU/25 g turnip greens. * and *** indicate that the detected number of *E. coli* O157:H7 was significantly different at $P < 0.05$, and $P < 0.001$, respectively. Vertical bars in detected number present standard deviation ($N = 30$) and vertical bars in bacterial count present standard deviation ($N = 3$).

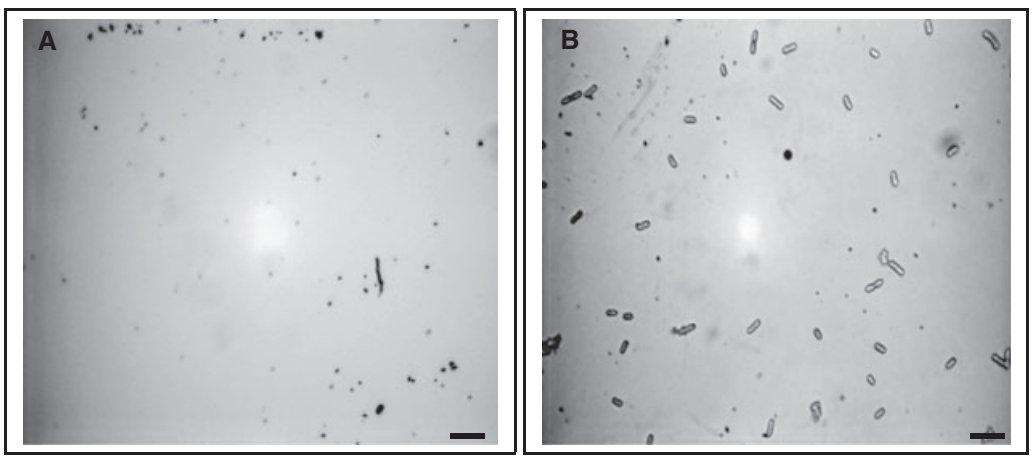

Figure 6–The light microscopic images of *E. coli* O157:H7 detected on the Gold-DSP biosensors. The detection was performed with the Gold-DSP biosensor after 10 h-enrichment with the inoculation of PBS buffer (A) and after 8 h-enrichment with the initial inoculation of 10^2 CFU/25 g turnip greens. The bar in the image represents 2.5 μm in length.

was detected from the 8 h enrichment with initial concentration of 10^2 CFU/25 g turnip greens, exhibiting 1767 ± 233 cell/mm^2 (Figure 5). The detected number of *E. coli* O157:H7 increased significantly as the bacterial number increased during 10 h enrichment, exhibiting 2641 ± 394 and 15383 ± 3853 cell/mm^2 with the initial inoculation of 10 CFU/25 g turnip greens and 10^2 CFU/25 g turnip greens, respectively ($P < 0.05$ or $P < 0.001$). The control sensor did not detect *E. coli* O157:H7 at any enrichment times (data not presented), which was also confirmed by the light microscopic images of *E. coli* O157:H7.

In general, the sensitivity of biosensor when applied to a food system may be significantly decreased, presumably due to interference with the food particles. Figure 6 presents the light microscopic images of food particles and *E. coli* O157:H7 detected by the Gold-DSP biosensor. Unlike the application of pure culture presented in Figure 3, unwanted attachment or binding by the particles of turnip greens to the biosensor were observed in Figure 6. Significant amounts of food particles were randomly attached on the Gold-DSP biosensor after 10 h enrichment without bacterial inoculation (Figure 6A). Although the pAbs on the biosensor were able to bind specifically with the target bacteria and BSA could minimize the nonspecific binding, it was impossible to completely prevent the nonspecific binding on the biosensor. The nonspecific binding or attachment is one of great concerns when rapid biosensor methods are applied for practical purposes (Lazcka and others 2007; Ricci and others 2007; Velusamy and others 2010). Even though there were nonspecific binding of biosensor with food particles, the Gold-DSP biosensor was able to differentiate between *E. coli* O157:H7 and the food particles by observing the images from the combined LMIS (Figure 6B).

The total time for detecting *E. coli* O157:H7 using the Gold-DSP biosensor requires several minutes if the bacterial concentrations are greater than the detection limit. Other rapid detection methods such as SPR and QCM require several minutes for detection; however, these methods are so sensitive to mass change that the methods can provide a false-positive results if there is nonspecific binding with food particles (Ricci and others 2007; Velusamy and others 2010). The significant advantage of using the Gold-DSP biosensor combined with LMIS was that the Gold-DSP biosensor was able to detect specifically *E. coli* O157:H7, and eliminate the false counts interfered by the nonspecifically bound food particles in food by observing the light microscopic images.

Conclusions

This research suggests that the Gold-DSP combined with LMIS could be applied to detect *E. coli* O157:H7 in real food systems as demonstrated in turnip greens. The Gold-DSP biosensor possesses a substantial advantage in that the biosensor can eliminate possible interference of the bacteria with unwanted food particles, when combined with LMIS. The detected *E. coli* O157:H7 on the Gold-DSP can be easily differentiated from the nonspecific binding of food particles and counted from the images observed by LMIS.

Therefore, the Gold-DSP biosensor combined with LMIS can be used as a reliable, reproducible, and promising rapid detection method for *E. coli* O157:H7 in fresh produce products.

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