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In-situ immuno-gold nanoparticle network ELISA biosensors for pathogen detection

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ABSTRACT

Food poisoning microorganisms that contaminate food products and compromise food safety and security have been considered a major health threat and a serious concern for food producers and processors. Developing sensor technologies that are rapid for sensitive and selective detection and quantification of pathogens is a high priority for scientists in academia, state and federal research institutes, and industries. In this work we propose an *in-situ* immuno-AuNP network-based ELISA biosensor integrated with a sample concentration step based on immuno-magnetic separation to detect pathogenic microorganisms with high sensitivity. The sensor system was optimized by the specific formation of immuno-AuNP network onto the antigenic site present at the outer membrane surface of bacteria and the analytical concept was validated by a microtiter immunoassay. The *in-situ* network biosensor was able to detect pathogens at extremely low numbers: 3 cells/mL of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in buffer and 3 CFU/mL of *E. coli* O157:H7 and 15 CFU/mL of *S. typhimurium* in real sample conditions within 2 h of inoculation. The ability to monitor target bacteria with improved analytical sensitivity compared to the current techniques presents a unique opportunity for routine monitoring to improve the safety of foods.

1. Introduction

Well known highly pathogenic bacteria such as *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Salmonella enteritidis*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Listeria monocytogenes* which usually cause severe foodborne illness are some of the key threat agents known to compromise food security (Kim et al., 2007; Zhang et al., 2009). Because of their fast replication under normal environmental conditions, even at low concentration these organisms pose a serious threat to human health. Early detection of these bacteria in very low numbers is very crucial not only to prevent the consumption of contaminated foods by consumers but also to alert a foodborne disease outbreak well in advance (Jadhav et al., 2012; Tauxe, 2002). The key focus of this work is in developing technology to detect target bacteria with high sensitivity.

In the past decades, the presence of pathogenic bacteria in contaminated foodstuffs has been detected using analytical techniques such as typical colony counting (Reissbrodt, 2004; Scotter et al., 2001; Velusamy et al., 2010), genetic-based polymerase chain reaction (PCR) (Navas et al., 2006; Ueda et al., 2006) and immunoassay-based methods (Byrne et al., 2009) such as enzyme-linked immunosorbent assay (ELISA) (Sun et al., 2005) and lateral flow-based immunochromatographic assay (ICA) (Seo et al., 2009; Wang et al., 2012). However, these approaches have significant disadvantages especially in the early identification of contaminating microorganisms because they are time-consuming (*e.g.*, one to several days), not very sensitivity, and involves laborious multiple steps. In addition, it is essential that most of the current methods stated above usually require a longer cultivation time so that the number of bacteria are sufficient to be detectable by the analytical method. Here we develop a sensitive and easy-to-use analytical method that will circumvent the current technical drawbacks and can be implemented for routine testing.

We propose a new immunoassay entitled '*in-situ* immuno-gold nanoparticle network ELISA' in combination with immuno-magnetic separation to advance the present status of heterogeneous ELISA technique for bacteria detection. To improve its analytical performance in direct detection of pathogens in food matrices, we employed magnetic and gold nanoparticle both of which were coupled with antibodies specific to the target bacterium. By using the magnetic component of the sensing scheme immuno-magnetic concentration (IMC) was first performed to separate and concentrate the target bacteria suspended either in buffer or in liquid foods. Next, gold nanoparticles 30 nm in size were functionalized with secondary antibodies to recognize and bind to complementary targets on the cell surface to form a network structure that can grow with time to enhance the signal in the network structure (Lee et al., 2011; Lee and Irudayaraj, 2009). We expect our newly designed enhanced ELISA approach based on nanoparticle assembly in conjunction with the IMC approach can detect pathogens

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rapidly and at a very high level of sensitivity without the need for enumeration in real food samples.

2. Materials and methods

2.1. Materials

Monoclonal antibodies (mAb) which are specific to *E. coli* O157:H7 and *S. typhimurium* were purchased from Abcam (Cambridge, MA). Polyclonal antibodies (pAb) raised from goat which are reactive to *E. coli* O157:H7 and *S. typhimurium* were purchased from KPL (Gaithersburg, MD). Dynabeads (M-270 amine-functionalized) was obtained from Invitrogen (Carlsbad, CA). Four different species of bacteria, *E. coli* O157:H7, *S. typhimurium*, *S. enteritidis*, and *L. monocytogenes* were supplied through the Center for Food Safety Engineering consortium at Purdue. Sodium citrate, chloroauric acid ($HAuCl_4$), casein (sodium salt type, extracted from milk), Tween 20, tetramethyl benzidine (TMB), ethanolamine were obtained from Sigma (St. Louis, MO). 2-(*N*-morpholino) ethanesulfonic acid (MES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), rabbit anti-goat IgG antibody and two HRP-labeled antibodies (mouse anti-goat antibody and mouse anti-rabbit antibody were purchased from Pierce (Rockford, IL)).

2.2. Preparation of immuno-magnetic beads

For antibody coupling to the magnetic beads (2.8 μm in diameter) pre-functionalized with amine, monoclonal antibodies specific to *E. coli* O157:H7 and *S. typhimurium* were respectively conjugated with the magnetic beads according to the manufacturer's instruction. Briefly, the beads (33 μL of 30 mg/mL) were washed three times with 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, pH 4.5, and then chemically coupled with the corresponding antibody (10 μg) followed by the addition of 50 μM EDC dissolved in MES. This reaction was carried out at room temperature for 2 h. The residual active surfaces of the immuno-beads were blocked with 1 M ethanolamine (12.2 μL), pH 8.5 for 15 min followed by adding 5% (w/v) casein dissolved in PBS buffer (casein-PBS) for final blocking and maintained at room temperature for 1 h. The immuno-bead conjugates for the detection of *E. coli* O157:H7 and *S. typhimurium* were separated and washed three times using a magnet and then finally resuspended in 1 mL of 0.5% (w/v) casein-PBS. The conjugates were stored at 4 °C until further use.

2.3. Synthesis of antibody-coupled gold nanoparticles

Gold nanoparticles with a mean diameter of 30 nm were chemically synthesized using sodium citrate as a reducing agent (Pong et al., 2007). Two anti-bacteria goat polyclonal antibodies, *i.e.*, anti-*E. coli* O157:H7, anti-*S. typhimurium* and rabbit anti-goat IgG antibody (host animal is rabbit; the antibody recognizes antibodies raised in a goat host) (15 μg) were added to the respective gold nanoparticle solution (1 mL) that was adjusted to a pH of 8.5, followed by gentle shaking at room temperature for 1 h. To block the residual gold surfaces, 5% casein dissolved in phosphate buffer (PB), pH 7.4, was added to the mixture and then reacted for 30 min. After centrifugation at 14,000 rpm for 30 min, the supernatant was discarded and the pellet was then resuspended with 0.5% casein-PB (final volume of 50 μL, 20-fold concentrate). The conjugates were then stored at 4 °C until used.

2.4. Enzyme-linked immunosorbent assay

Monoclonal antibody specific to *E. coli* O157:H7 (3 μg/mL, 100 μL) diluted in PBS was coated on the polystyrene microwell surfaces at 37 °C for 1 h under humid conditions. This incubation procedure will be adopted in all subsequent steps and followed by a washing step with DI water. The washing was performed by adding 300 μL of DI water into the microwell and was repeated five times. To block residual surfaces, the sample was treated with 0.5% casein-PBS (200 μL). Six different concentrations (0, 1×10^3, 1×10^4, 1×10^5, 1×10^6, and 1×10^7 cells/mL) of *E. coli* O157:H7 diluted in 0.5% casein-PBS containing 0.1% Tween 20 (100 μL) were added to each microwell. Two types of antibodies, an anti-*E. coli* O157:H7 goat antibody (1 μg/mL, 100 μL) and 30 nm-sized gold nanoparticle coupled with the same antibody (1 to 100 dilution from stock solution, 100 μL), were reacted as control and primary antibody-AuNP respectively. To assess the effect of secondary antibody-AuNP, rabbit anti-goat IgG antibody (1 to 200 dilutions, 100 μL) was added to the primary antibody-AuNP. For the tertiary antibody-AuNP formation, goat anti-rabbit IgG antibody (1 to 200 dilutions, 100 μL) was reacted with the secondary antibody-AuNP. Two HRP-labeled antibodies, mouse anti-goat IgG antibody and mouse anti-rabbit IgG antibody (1 to 5000 dilution, 100 μL) were further reacted and then the colorimetric substrate mixture (50 mM sodium acetate buffer, pH 5.1: 1% (w/v) TMB: 3% (v/v) H_2O_2 = 1000: 10: 1 as volume ratio) (200 μL) was finally added and the reaction maintained for 15 min at room temperature to obtain the respective signal. The optical intensity of each method was measured using the Versamax™ absorbance microplate reader (Molecular Device; Sunnyvale, CA).

2.5. Analytical Procedure of in-situ immuno-AuNP network assay

The *E. coli* O157:H7 and *S. typhimurium* samples serially diluted in PBS (0, 1×10^0, 1×10^1, 1×10^2, 1×10^3, 1×10^4, and 1×10^5 cells/mL) from stock solution (3×10^{10} and 1×10^{11} cells/mL for *E. coli* O157:H7 and *S. typhimurium* respectively) were reacted with the corresponding immune beads (100 μL) prepared with gentle shaking at room temperature for 30 min followed by separation of the supernatant using a magnet (AM10026, Invitrogen). Supernatant was discarded by suctioning followed by addition of 500 μL of DI water and gently mixed. This process was repeated three times. This separation method was identically applied at every step unless specifically noted. To form a sandwich complex, 30 nm-sized AuNPs coupled with the primary goat antibody specific for the corresponding bacteria (*i.e.*, *E. coli* O157:H7 or *S. typhimurium* respectively) were mixed (1:200 dilution, 200 μL) with the beads respectively and incubated at room temperature for 30 min. Secondary antibody-AuNP, rabbit anti-goat IgG antibody (1 to 200 dilution, 200 μL) was reacted with the primary antibody-AuNP at the same reaction conditions. Tertiary antibody-AuNP, goat anti-rabbit IgG antibody (1 to 200 dilutions, 200 μL) was further reacted with the secondary antibody-AuNP. Two HRP-labeled antibodies, mouse anti-goat IgG antibody and mouse anti-rabbit IgG antibody (1 to 5000 dilution, 200 μL) were finally incubated. After removing unbound HRP-conjugate, the colorimetric signal was obtained by the identical method described above. The standard curves obtained from the signals were linearized by logit-log transformation (Cho et al., 2006) for quantification and the limits of detection of this method was calculated at three standard deviation at zero dose (Cho et al., 2006) of bacteria.

2.6. Cross-reactivity test

Specificity experiments for the *in-situ* immuno-AuNP network-based biosensor were performed by using the following bacteria species: *E. coli* O157:H7, *S. typhimurium*, *S. enteritidis*, and *L. monocytogenes*. The analytical procedure was the same as described above except for the reaction step used for capturing the analyte. *S. typhimurium*, *S. enteritidis*, and *L. monocytogenes* were used to test the specificity of *E. coli* O157:H7 analysis and *E. coli* O157:H7, *S. enteritidis*, and *L. monocytogenes* were used to test the specificity for *S. typhimurium* detection. The bacteria concentration was maintained at a high level (1×10^8 cells/mL) in casein-PBS for the cross-reactivity experimental runs.

2.7. Real food sample test

Reduced fat milk, ground beef, and pineapple juice were employed as sample matrices for the detection of pathogens in food. *E. coli* O157:H7 and *S. typhimurium* were used in this study. According to the standard manual for sample preparation (Muldoon et al., 2012), 10 g of the ground beef was mixed with 10 mL of sterilized PBS and *E. coli* O157:H7 pre-determined by colony counts on Lysogeny broth (LB) agar plate was then inoculated in the matrix. 10 mL of the milk and juice were also used as sample matrices to assess the presence of *E. coli* O157:H7 and *S. typhimurium* respectively by inoculation of a pre-determined count. After this artificial inoculation, food samples were held in sterile plastic culture tube with a screw cap at 37 °C for 2 h with gentle shaking at 200 rpm. The pH of the juice sample was adjusted to a pH of 7 after inoculation using 1 M NaOH. Next, 100 μL of immunomagnetic bead was applied to the sample and reacted with gentle shaking at room temperature for 30 min followed by retrieval of the magnetic bead using a magnet. All experiments were repeated in triplicate.

3. Results and discussion

3.1. Analytical concept

The novel immuno-analytical strategy based on enhanced ELISA and IMC proposed for the detection of pathogenic microorganisms consists of three different key elements: gold nanoparticle (AuNP)-antibody probes and two enzyme-coupled antibodies for immuno-AuNP network structure formation on the outer membrane of bacterium (Fig. 1) for enhanced detection. First, anti-bacteria primary antibodies, raised from goat, coupled to AuNP, 30 nm in diameter will be reacted with the pathogen to bind to one of the antigenic sites of the bacteria. Next, rabbit anti-goat IgG secondary antibodies, conjugated with AuNP are positioned onto the primary antibody-AuNP and tertiary antibodies, produced by goat, coupled to the same size of AuNP are then reacted against the secondary antibody-AuNP to build the nano structure assembly. Since this is a colorimetric assay, two enzyme (*e.g.*, HRP)-labeled antibodies that are reactive to goat IgG antibody and rabbit IgG antibody respectively, raised from mouse are employed for signal enhancement of the network structure (Fig. 1). Unlike common ELISA, the assay can be performed on magnetic beads functionalized with monoclonal antibodies in which the target bacterium could be captured and separated as a first step (Voitoux et al., 2002). This *in-situ* immunoassay is able to provide several advantages towards the detection of bacteria, for instance, it incorporates a bacteria capture and concentration step by a simple magnetic concentration step and enhances the sensitivity of the assay by the network reaction and structure formation.

To maximize analytical efficiency of the *in-situ* immuno-AuNP assay, it is crucial to optimize binding conditions between antibodies and AuNP (Yang et al., 2009). It has been reported that three major forces are highly related to this antibody-AuNP complex formation, *e.g.*, hydrophobic interactions, ionic interactions, and dative binding (Cvak et al., 2012; Rudolf et al., 2009). Hydrophobic linkages are due to attraction between hydrophobic regions of antibody and surface of gold nanoparticle, while ionic interactions are due to the positive charges of amino acids in the antibody molecule and negative charges are presented by the functionality of the AuNP surface. Compared to these non-covalent linkages, dative binding due to covalent linkage between the sulfhydryl groups of the antibody and gold surface results in strong binding between the components. Among these three major forces, the non-covalent linkages, *i.e.*, hydrophobic and charge interactions can be controlled, by optimizing the charge distribution of the antibody. When the net charge of the antibody is zero, the degree of hydrophobicity of antibody is maximized (Nakanishi et al., 2001), allowing for the antibody molecules in this state to be attached to the AuNP with maximum efficiency. The optimal conjugation conditions can be determined by estimating the amount of antibody molecules on AuNPs by quantitative immunoassay on a microtiter plate. Since the signal intensity produced from this assay format is directly proportional to the amount of enzyme-coupled antibody, higher the concentration of antibodies (*i.e.*, primary, secondary, and tertiary) on AuNP, the higher is the signal.

Although many antibody-AuNP conjugates are involved in the network formation, non-specific interactions between the different elements could result in increased noise in the assay system developed. Excessive use of antibody-AuNPs beyond optimal amount tends to increase the background signal due to non-specific binding of antibody molecules and gold nanoparticles in the reaction. Hence a titration step is necessary to optimize the antibody-AuNP conjugates used in

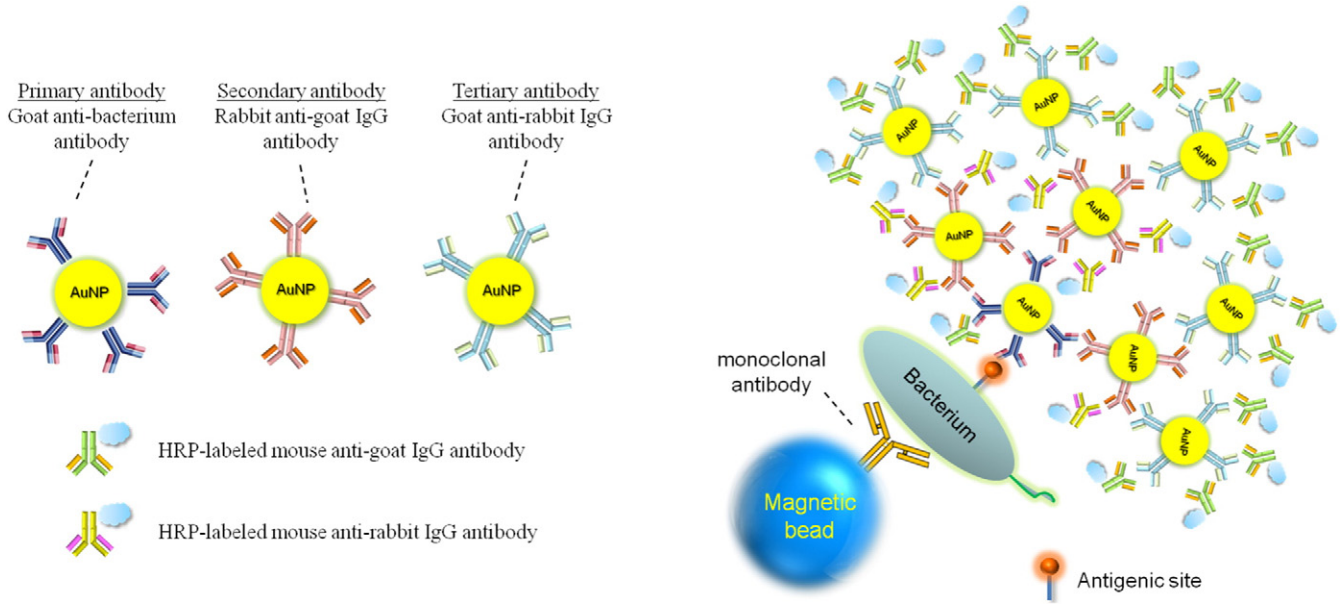

Fig. 1. Schematic of the *in-situ* immuno-AuNP network biosensor for the detection of food pathogens. Various antibody components (primary, secondary, and tertiary) coupled to AuNPs and HRP-labeled antibodies were used to form the network for signal enhancement. Target bacterium captured by immuno-magnetic beads exposes many antigenic sites on the outer membrane that are able to form the network allowing for several HRP-labeled antibodies to bind to the network complex to enhance the signal to detect low numbers of bacteria.

assay development. In addition, improper surface blocking of AuNP may also lead to a high possibility of the unwanted phenomena (for example, non-specific interaction among the components caused by improper surface blocking). Our experiments show that treatment of the AuNP surface with casein exhibits a better blocking efficiency compared to bovine serum albumin which is usually used as a common blocking agent (Ramos-Vara, 2005; Vogt et al., 1987).

3.2. Proof of analytical concept

Experimental validation of the *in-situ* immuno-AuNP network concept was performed with ELISA using immobilized monoclonal antibody specific to *E. coli* O157:H7 with a microtiter well. After the binding of the analyte at various concentrations (0 to 1×10^7 cells/mL of *E. coli* O157:H7), four types of sandwich complexes can be individually formed by building the following patterns: (a) goat anti-*E. coli* O157:H7 antibody followed by mouse anti-goat IgG antibody labeled with HRP, which were used as a conventional control, (b) anti-*E. coli* O157:H7 antibodies raised from goat which were conjugated with AuNP, (c) rabbit anti-goat IgG antibody coupled to AuNP were formed by a binding complex with (b), (d) the immuno-AuNP network was finally constructed by binding goat anti-rabbit IgG antibodies conjugated with AuNP to the (c) complex. After network formation, two HRP-labeled antibodies, specific to goat IgG antibody and rabbit IgG antibody respectively, were reacted and bound for signal generation.

Results in Fig. 2 show that the dose response of the immuno-AuNP network-based methods, *i.e.*, (b), (c), and (d), against *E. coli* O157:H7 show better performances compared to the conventional method (a) that uses goat anti-*E. coli* O157:H7 antibody followed by mouse anti-goat IgG antibody coupled HRP without AuNP. Particularly, in the case of (c) and (d), we were able to detect 1×10^4 and 1×10^3 cells/mL of *E. coli* O157:H7 respectively with the microtiter assay, increasing the analytical sensitivity by approximately 1000-fold compared to the conventional, *i.e.*, 1×10^6 cells/mL (a). Difference in analytical evaluations was due to differences in the number of HRP coupled antibodies for signal generation. As the network was grown on the antigenic site, shown in Fig. 1, the antibody components forming the network complex that are bound to the AuNP conjugates were conducive to the binding of the HRP-labeled antibodies. Accordingly, as the immuno-AuNP layers increase, the higher is the signal from the network complexes. Our observation shows that for a network layer greater than three, *i.e.*, primary, secondary, and tertiary immuno-AuNP complex, a remarkable increase in the background signal could be noted, with a significant amount of noise affecting the performance of the overall assay. To circumvent this drawback, the amount of antibody-AuNPs used in the assay needs to be optimized and the appropriate assay conditions need to be determined (data not shown).

3.3. Analytical performance of the in-situ network assay

After optimization of analytical conditions as described above, the immuno-AuNP network assay was applied for *in-situ* detection of bacteria in conjunction with the immuno-magnetic separation technique. To separate and concentrate the target bacteria with high efficiency, magnetic beads of size 2.8 μm were used compared to smaller size of beads because these beads yielded faster separation under a low magnetic field using a simple magnet (Catalog# AM10026, Invitrogen). In addition, compared to the conventional two-dimensional ELISA approach, the *in-situ* immuno-magnetic bead-based assay proposed has the potential for higher signal enhancement due to the network structure presented by the different antibody-AuNP and antibody-HRP conjugates. Under these conditions, the analytical performance of the *in-situ* immuno-AuNP network assay was demonstrated to detect *E. coli* O157:H7 and *S. typhimurium* captured by magnetic beads (Fig. 3).

Results show an increase in the limit of detection (LOD) of the two bacteria by an approximate 10^5-fold compared to that possible by the conventional microtiter-based ELISA shown in Fig. 2. To determine the detection capability of this assay format, we calculated the limit of detection (LOD) from each data point in the curve. The LOD was considered as the standard deviation at zero dose (*i.e.*, 0 cells/mL, of bacteria as described below) by multiplying the signal at zero dose by three standard deviation (Binder and Archimbaud, 2000; Cho et al., 2006). The LOD of the two pathogens tested was found to be ~3 CFU/mL, indicating a very high level of sensitivity provided by the enhanced *in-situ* network technique developed in this work. Such high level of sensitivity is primarily possible because of the active network assembly of nanoparticles captured by the magnetic beads, while in the conventional ELISA under 2-D conditions, capturing bacteria only depends on passive diffusion of bacteria to the immobilized antibody, which requires a long reaction time.

A high level of sensitivity is possible by employing the immunomagnetic beads, where the reaction volume containing bacteria is reduced from 10 mL to 100 μL, whereby theoretically increasing the initial concentration by 100-fold. Second, the *in-situ* assay is governed by combining both active and passive diffusion, providing a more appropriate condition for forming the complexes between bacteria and the immuno-magnetic beads than the two-dimensional assay. Third, the surface area of the immuno-magnetic beads is much larger than that of microtiter plate, and hence facilitates the formation of the

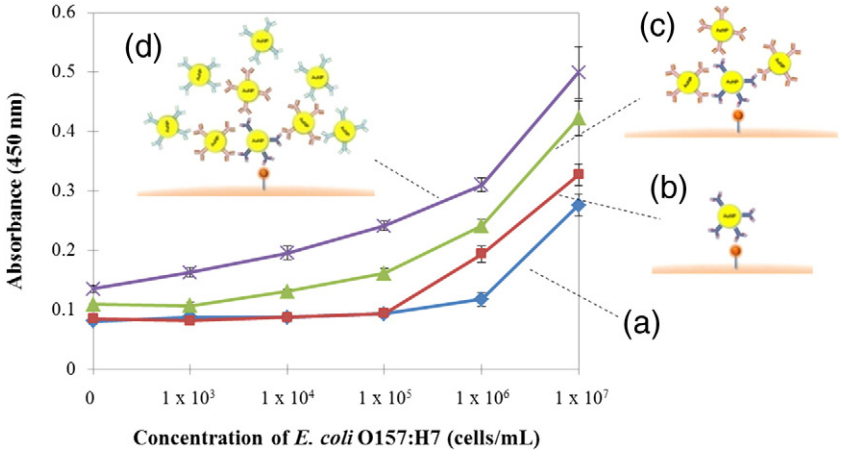

Fig. 2. Proof of the analytical concept of *in-situ* immuno-AuNPs network-based method by ELISA on microtiter plate. *E. coli* O157:H7 diluted to different concentration (0 to 1×10^7 cells/mL) was used as a model analyte. Comparison was made with the conventional method that only uses anti-*E. coli* O157:H7 polyclonal antibody (a), primary antibody-AuNPs (b), secondary antibody-AuNPs (c), and tertiary antibody-AuNPs (d) by sequential formation of the network on the surface of the bacterium at its antigenic site. Details are provided in the text.

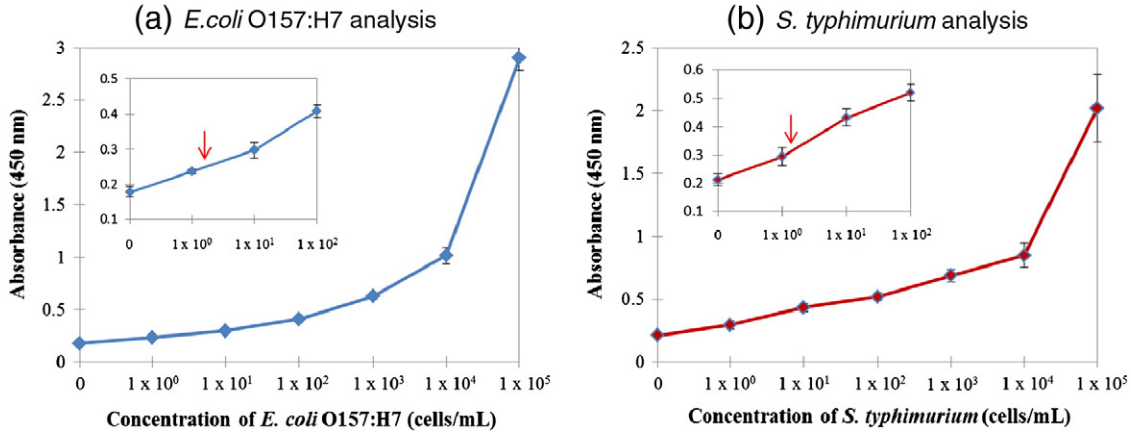

Fig. 3. Performance analyses of *in-situ* immuno-AuNP network assay on the magnetic bead against *E. coli* O157:H7 and *S. typhimurium* respectively. Dose response curves for *E. coli* O157:H7 (a) and *S. typhimurium* (b) represents target bacteria concentration. Limit of detection indicated in the inset for each analysis can be calculated by standard deviation at zero dose of bacteria used.

complex more effectively as described. For these reasons, we expect the sensitivity of the *in-situ* network assay to be significantly higher than the conventional ELISA approaches.

3.4. Cross-reactivity analysis

To examine the specificity of the *in-situ* immuno-AuNP network assay, testing for cross-reactivity is an important step in assay development because many antigenic sites of the bacteria cell surface, *e.g.*, lipopolysaccharides (LPS) and the different types of surface antigens (K-, O-, and H-antigens), are analogous to those on other genera and species (O'Neil and Marquis, 2006). Four microorganisms, *E. coli* O157:H7, *S. typhimurium*, *S. enteritidis* as gram negative and *L. monocytogenes* as gram positive were used to evaluate the specificity of *E. coli* O157:H7 and *S. typhimurium* respectively. The analysis was performed under a high concentration (*e.g.*, 10^8 cells/mL) of each bacteria to yield a reliable performance of the biosensor developed in this research.

From the results shown in Fig. 4, it was obvious that the *in-situ* immuno-AuNP network-based ELISA biosensor has a high degree of specificity to target bacteria without significant cross-reactivity towards other bacteria even when these are present in higher concentrations. (Here, 1×10^8 cells/mL is regarded as a higher concentration) For the specific detection of *E. coli* O157:H7, the biosensor did not cross-react with other bacteria, *i.e.*, *S. typhimurium*, *S. enteritidis*, and *L. monocytogenes*. For *S. typhimurium* analysis, the biosensor was highly specific to *S. typhimurium*. A possible reason for such high specificity is the use of monoclonal antibody immobilized onto the magnetic beads for target capture. Proper use of monoclonal antibody can also help prevent cross-reactivity with non-target bacteria.

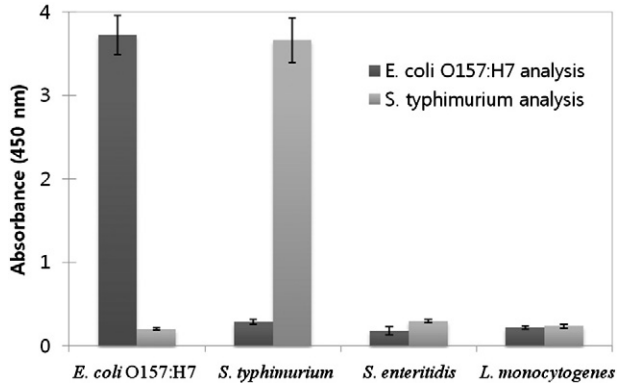

Fig. 4. Cross-reactivity results of the proposed approach compared to the different species of pathogenic bacteria (*E. coli* O157:H7, *S. typhimurium*, *S. enteritidis*, and *L. monocytogenes*). This assay was performed at high concentration (*i.e.*, 1×10^8 cells/mL) of each bacteria species.

3.5. Performance of the in-situ immuno-AuNP network assay in food samples

As a final step of validation, the sensor performance was demonstrated in three food matrices: 2% reduced-fat milk and ground beef for *E. coli* O157:H7 and pineapple juice for *S. typhimurium* by artificially inoculating the bacteria into the corresponding sample (Son et al., 2007; Wang et al., 2011). For inoculation, the concentration of bacteria was determined by the conventional method, *e.g.*, colony counting, to validate this assay, which resulted in $0, 3 \pm 2$, and 15.7 ± 6.1 CFU/mL for *E. coli* O157:H7 and $0, 3 \pm 2$, and 15.3 ± 7.5 CFU/mL for *S. typhimurium* (see Fig. 5). Two hours after inoculation each sample was analyzed by the *in-situ* network assay in triplicate.

Based on the results, it is clear that the LOD for *E. coli* O157:H7 by the *in-situ* assay in both sample matrices, *i.e.*, 2% reduced fat milk (a) and ground beef (b), was 3 CFU/mL within 2 h of incubation. In case of *S. typhimurium* analysis, even though the sensor system did not show an identical performance as the *E. coli* analysis a LOD of 15 ~ CFU/mL was achieved (c). Such difference in sensitivity may be due to the low pH of pineapple juice (Hennion and Pichon, 2003) possibly interfering with antibody-antigen interaction. From the results, it should be noted that the sensitivity of the *in-situ* network biosensor in real samples was nearly identical to those in the buffer system, indicating that the assay is robust. Accordingly, compared to other methods used for bacteria detection, a pre-enrichment process was not required because of its high level of sensitivity.

In conclusion, the *in-situ* immuno-AuNP network ELISA biosensor proposed in this study, demonstrates a high level of sensitivity and specificity for bacteria detection in selected liquid food samples. The biosensor was able to detect key pathogens at extremely low concentrations, 3 cells/mL for *E. coli* O157:H7 and *S. typhimurium* in buffer, and 3 CFU/mL of *E. coli* O157:H7 and 15 CFU/mL of *S. typhimurium* in liquid food samples tested within 2 h. The analytical performance of the proposed assay was superior to those of other commercialized test kits as well as various types of biosensors introduced in the literature (Gupta et al., 2006; Ravindranath et al., 2011; Yu et al., 2006) with regard to simplicity, sensitivity, and reduction in assay time. We expect the network structure-based biosensor concept developed in this study could be utilized for rapid screening of food samples to enhance the safety and to report on low levels of microbial contamination. We expect the methods be applicable to detect other target cells and protein biomarkers at extremely low concentration.

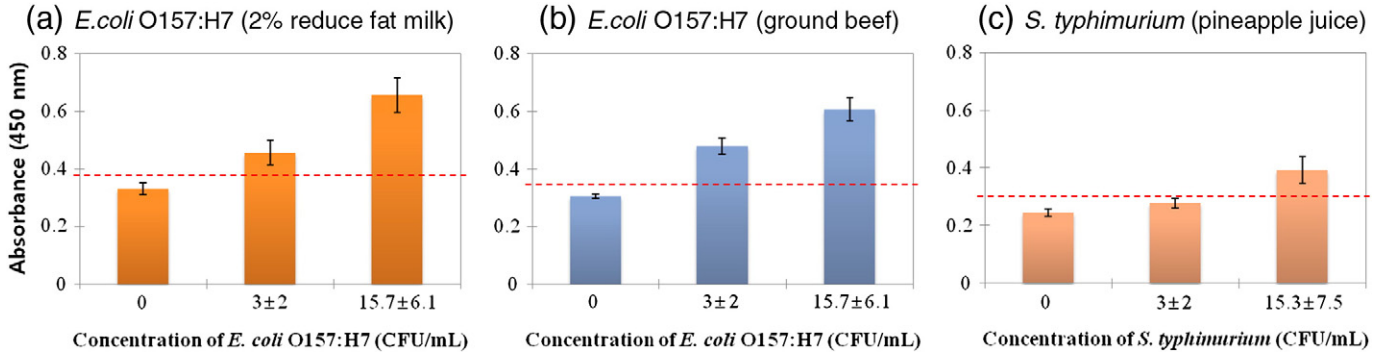

Fig. 5. Performance analyses in food sample matrices. 2% reduced fat milk (a) and ground beef (b) were used for *E. coli* O157:H7 detection and pineapple juice (c) was used for *S. typhimurium*. To validate the initial number of bacteria inoculated, colony counting was performed at the same time when inoculated. The red dash line indicates the limit of detection for each assay.

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