Biosensors and Bioelectronics 57 (2014) 143–148

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

In-situ fluorescent immunomagnetic multiplex detection of foodborne pathogens in very low numbers

Il-Hoon Cho [a], Lisa Mauer [b], Joseph Irudayaraj [a,b,*]

[a] Bindley Bioscience and Birck Nanotechnology Center, Department of Agricultural & Biological Engineering, Purdue University, West Lafayette, IN 47907, United States

[b] Department of Food Science, Purdue University, West Lafayette, IN 47907, United States

ARTICLE INFO

Article history:
Received 24 December 2013
Received in revised form
1 February 2014
Accepted 5 February 2014
Available online 19 February 2014

Keywords:
Foodborne pathogens
Rapid detection
Signal enhancement
Magnetic separation
Fluorescent detection
Highly sensitive

ABSTRACT

Consumption of foods contaminated with pathogenic bacteria is a major public health concern. Foods contain microorganisms, the overwhelming majority of which are nonpathogenic, some are responsible for food spoilage, and some cause serious illness leading to death or a variety of diseases in humans. The key challenge in food safety is to rapidly screen foods to determine the presence of pathogens so that appropriate intervention protocols can be pursued. A simple fluorometric immunological method in combination with a magnetic concentration step was developed for rapid detection of target bacteria with high sensitivity and specificity in less than 2 h without enumeration. The method constitutes performing an *in-situ* immunoassay on a magnetic bead through the formation of a sandwich complex of the target bacteria and the probe (detection antibody—denatured BSA labelled with fluorophores) followed by the release of fluorophores by means of enzymatic digestion with proteinase *K*. The limit of detection (LOD) was <5 CFU/mL of the tested pathogens (*Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes*) in buffer. When the pathogens were inoculated in foods (spinach, chicken, and milk), the LOD was under 5 CFU/mL for *E. coli* O157:H7, *S. typhimurium* and *L. monocytogenes*. Furthermore, the method was highly specific in detecting the target pathogens in a multiplex format. The developed *in-situ* fluorescent immunomagnetic sensor approach offers distinct advantages because it is rapid, highly sensitive, and easy to use and could therefore be potentially used as a pathogen screening tool.

1. Introduction

The annual burden of foodborne illness, particularly United States, as estimated by the Centers for Disease Control and Prevention, is 48 million cases of foodborne illness, 128,000 hospitalizations, and 3000 deaths along with a cost of $152 billion in medical expenses, lost productivity and business, lawsuits, and compromised branding (Scallan et al., 2011; Zhang et al., 2009). Efforts to improve the safety of the food supply are highlighted by the FDA Food Safety Modernization Act (Drew and Clydesdale, 2013). This regulation requires enhanced detection of pathogens in both imported and domestically produced foods coupled with effective intervention methods to achieve food safety. However, mandated testing has not yet been implemented, in part due to the lack of technology capable of meeting the requirements. A working premise is that rapid and comprehensive pathogen detection methods will enable implementation of effective strategies to avoid, or when needed intervene in, the distribution of foods that have been contaminated with foodborne pathogens.

The complexity of food matrices, the wide variety of microorganisms therein, and the varying growth and replication traits of target pathogenic microorganisms that, when present, are often in much smaller numbers than the nonpathogenic microorganisms, pose significant challenges for pathogen detection. Conventional methods used to identify pathogens in contaminated foodstuffs include: colony culture (Reissbrodt, 2004; Velusamy et al., 2010), polymerase chain reaction (PCR) (Malorny et al., 2008; Navas et al., 2006), and immunoassays (*e.g.*, enzyme-linked immunosorbent assays (ELISA) and immunochromatographic assays) (Delehanty and Ligler, 2002; Seo et al., 2010), often in combination with selective enrichment. However, these conventional methods are time-consuming, laborious, and/or lack in sensitivity in part due to the requirement of cultivation steps. Thus, alternate approaches are needed for rapid detection. Biosensors have been developed and documented to improve the limit of detection (LOD) and/or

* Corresponding author at: Purdue University, Department of Agricultural and Biological Engineering, 225 South University Street, West Lafayette, IN 47907, United States. Tel.: +1 765 494 0388; fax: +1 765 496 1115.
E-mail address: josephi@purdue.edu (J. Irudayaraj).

http://dx.doi.org/10.1016/j.bios.2014.02.012
0956-5663

time to result, based on electrochemical (Patel et al., 2011; Setterington and Alocilja, 2012; Smietana et al., 2011) and optical (Li et al., 2011; Smietana et al., 2011; Subramanian et al., 2006a, 2006b) techniques, including surface enhanced Raman spectroscopy (SERS) (Craig et al., 2013; Wang et al., 2010, 2011b) methods. However, a need still exists to develop highly sensitive easily implementable biosensors for onsite detection of pathogens in complex food matrices.

To address this need, an *in-situ* immunomagnetic bead with enhanced fluorescence approach was developed and applied for the detection of three prevalent foodborne pathogens, *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes*. Factors critical to the suitability of detection methods and sensors involved in food analysis were investigated, including sensitivity, limit of detection (LOD), detection time, and ease of use.

2. Materials and methods

2.1. Materials

Monoclonal antibodies specific to *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* and a polyclonal antibody raised from rabbit specific to *L. monocytogenes* were purchased from Abcam (Cambridge, MA). Polyclonal antibodies raised from goat which are reactive to *E. coli* O157:H7 and *S. typhimurium* were purchased from KPL (Gaithersburg, MD). Dynabeads (M-270, NH_2-functionalized), protein ladders (pre-stained) for SDS-PAGE, and two maleimide-functionalized Alexa Fluor™ dyes (532 and 647) were obtained from Life Technologies (Carlsbad, CA). *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* were obtained from the culture collection at the Center for Food Safety Engineering consortium at Purdue University. Casein (sodium salt), Tween 20, tetramethyl benzidine (TMB), ethanolamine, urea, bovine serum albumin (BSA), Sephadex G-15, dialysis tubing, and proteinase K were obtained from Sigma (St. Louis, MO). 2-(*N*-morpholino) ethanesulfonic acid (MES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxy-(6-amidocaproate) (LC-SMCC), 2-iminothiolane, dithiothreitol (DTT), fluorescein-5-maleimide (maleimide-FITC), and mouse anti-goat IgG labeled with horseradish peroxidase (HRP) were purchased from Pierce (Rockford, IL). Source of the antibodies and bacteria used in this study are provided in Supporting information (S-5).

2.2. Preparation of bacteria

E. coli O157:H7, *S. typhimurium*, and *L. monocytogenes* were respectively cultured in 500 mL of Luria-Bertani (LB) media at 37 °C with shaking, for 18 h and then harvested by centrifugation (5000 rpm, 20 min) and stored in 50 mL of sterilized PBS (phosphate buffered saline, 10 mM phosphate and 150 mM NaCl). The concentration of bacteria in these stock solutions was determined in triplicate by enumeration on plate count agar following incubation at 37 °C for 24 h.

2.3. Preparation of immuno-magnetic beads

For coupling antibodies to the beads, monoclonal antibodies specific to *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* were respectively immobilized onto amine-functionalized beads as described elsewhere (Cho and Irudayaraj, 2013). Briefly, the beads (1 mg) were washed with 0.1 M MES buffer, pH 4.5, and then reacted with the corresponding antibody (10 μg) by adding 50 μM EDC at room temperature for 2 h. For blocking the EDC-activated sites of antibody and the bead surfaces, 1 M ethanolamine (12.2 μL), pH 8.5 and 0.5% (w/v) casein dissolved in PBS buffer was sequentially reacted for 15 min and 1 h, respectively. After washing with 0.5% (w/v) casein-PBS three times, the conjugates for detection of the three pathogens were stored at 4 °C until use.

2.4. Conjugation of antibody with fluorescent dBSA

BSA (0.25 mg) was treated with 1 mL of 8 M urea in de-ionized water (DIW) for 30 min followed by the addition of 20 mM of DTT in DIW for 30 min at 37 °C with gentle shaking to ensure complete denaturation. The urea and DTT were removed by gel filtration with a Sephadex G-15 column (volume 10 mL) and the protein fractions identified by Bradford assay were immediately mixed with the three maleimide-functionalized dyes (*i.e.*, FITC, Alexa Fluor 532 and 647) respectively and maintained at room temperature for 1 h. The excess dyes were consecutively removed by dialysis and gel filtration on Sephadex G-15 chromatography. The dBSA bearing fluorescent dyes were coupled to 50-fold molar excess of LC-SMCC for 1 h followed by purified on Sephadex G-15 column. The fluorescent dBSA was coupled to antibody treated with 10-fold molar excess of iminothiolane (reagent used for thiolation of antibody) at room temperature for 2 h.

2.5. SDS-PAGE analysis

Protein samples, *i.e.*, native BSA, anti-*L. monocytogenes* antibody and the fluorescent dBSA conjugate, and protein marker were treated with sample buffer without mercaptoethanol (non-reducing condition) and loaded on an 8% polyacrylamide gel to verify the conjugation efficiency of the synthesized IFdBSA conjugate. For electrophoretic separation, two voltages (80 and 120 V) were sequentially applied for 20 and 60 min respectively using a Mini-Protein Tetra Cell apparatus (Bio-Rad, Hercules, CA). The separation gel was sequentially immersed in staining solution including 0.1% Coomassie blue R-250 for 15 min and destaining solution for 1 h for developing the image.

2.6. In-situ fluorescent bead assays

E. coli O157:H7, *S. typhimurium*, and *L. monocytogenes* samples serially diluted in PBS (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}, 1×10^{11}, and 5×10^9 cells/mL for *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* respectively) were first reacted with the corresponding beads (50 μL) with gentle mixing at room temperature for 20 min followed by separation of the supernatant using a magnet (AM10026, Life Technologies). The supernatant was discarded by pipetting and the bead was carefully washed with sterilized PBS. For probe attachment, 0.01 μg/mL of the fluorescent dBSA conjugate (200 μL) for each bacteria in 0.5% casein-PBS containing 0.1% (v/v) Tween 20 (casein-PBS-Tw) was mixed with the bead and incubated at room temperature for 20 min. The beads were washed with casein-PBS-Tw three times and then proteinase K solution (200 μL) diluted 100 times in reaction buffer (20 mM Tris–HCl, pH 8.0, 20 mM NaCl and 10 mM $CaCl_2$) was mixed with the beads. This enzymatic digestion was maintained at room temperature for 30 min. The fluorescent signals obtained from the supernatant after the digestion step were measured by a fluorescence spectrophotometer (Cary Eclipse, Agilent Technologies) at different excitation and emission wavelengths (*i.e.*, 495/519 nm, 531/554 nm, and 650/668 nm for FITC, Alexa532, and Alexa647, respectively).

2.7. Multiplex assays

Bacteria were prepared in two concentrations (0 and 100 cells/mL) by using the blank, uninoculated samples, for the zero dose and serial