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Anal. Chem., **2009**, 81 (8), 3094-3101• DOI: 10.1021/ac9000833 • Publication Date (Web): 24 March 2009

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Targeted Capture of Pathogenic Bacteria Using a Mammalian Cell Receptor Coupled with Dielectrophoresis on a Biochip

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Efficient capture of target analyte on biosensor platforms is a prerequisite for reliable and specific detection of pathogenic microorganisms in a microfluidic chip. Antibodies have been widely used as ligands; however, because of their occasional unsatisfactory performance, a search for alternative receptors is underway. Heat shock protein 60 (Hsp60), a eukaryotic mitochondrial chaperon protein is a receptor for *Listeria* adhesion protein (LAP) during *Listeria monocytogenes* infection. This paper reports application of biotinylated Hsp60 as a capture molecule for living (viable) *L. monocytogenes* in a microfluidic environment. Hsp60, immobilized on the surface of streptavidin-coated silicon dioxide exhibited specific capture of pathogenic *Listeria* against a background of other *Listeria* species, *Salmonella, Escherichia, Bacillus, Pseudomonas, Serratia, Hafnia, Enterobacter, Citrobacter,* and *Lactobacillus*. The capture efficiency of *L. monocytogenes* was 83 times greater than another *Listeria* receptor, the monoclonal antibody, mAb-C11E9. Additionally, the capture rate was further increased on a Hsp60-coated biochip by 60% when a dielectrophoresis force was applied for 5 min at the beginning of the final 1 h incubation step. Our data show that Hsp60 could be used for specific detection of *L. monocytogenes* on a biochip sensor platform.

The foodborne pathogen *Listeria monocytogenes* is a Gram-positive intracellular bacterium that causes human listeriosis affecting pregnant women, newborns, the elderly, and immunocompromised individuals. The symptom starts as flu-like and can progress to septicemia and meningitis. Infection often results in abortion in pregnant women.[1] Major sources of outbreaks are fully processed ready-to-eat (RTE) foods, which can be contaminated by poor hygienic practices during post-processing handling and packaging. Because of the high risk of *L. monocytogenes* association with RTE products and consequent fatality rate, the U.S. government has established the zero-tolerance policy for RTE foods.[2] *L. monocytogenes* has the highest average fatality rate of 20−30% among foodborne pathogens. As of 1999 in the U.S.A., 2,493 cases, 2,298 hospitalizations, and 499 deaths occurred because of listeriosis.[3] The estimated annual foodborne illness cost due to *Listeria* is about $2.3 billion, which is equivalent to that for *Salmonella*, though the number of outbreaks are much lower.[4] Therefore, it is crucial to develop a cost-effective and specific detection system for *L. monocytogenes*.

There are six species in the genus *Listeria* and only *L. monocytogenes* is considered to be of public health concern. This can be problematic because *Listeria*-positive food samples presumably contain low numbers (about 10%) of *L. monocytogenes* and high numbers (about 90%) of *L. innocua* or other *Listeria* spp.[5] Moreover, *L. innocua* has a higher exponential growth rate of 1.69 h^{-1} as compared to *L. monocytogenes* which has a growth rate of 1.32 h^{-1}, and the difference in these growth rates can hinder the detection of pathogenic bacteria.[6] Numerous conventional methods for detection of *Listeria* spp. are available and are highly effective; however, rapid, sensitive, and specific assays are still lacking for *L. monocytogenes*.[7]

Biosensors consisting of microfluidic devices that capture targets and then use changes in impedance to detect capture have

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10.1021/ac9000833 CCC: $40.75
Published on Web 03/24/2009

potential for foodborne microbial analysis.[8,9] Antibodies have been widely used for biosensors because of their specific, strong, and stable binding to antigens.[5,10,11] However, antibodies may not be the perfect bioreceptor because of possible variations in antigen binding, instability when bound to solid surfaces, and limited binding sites.[12] Additionally, environmental factors or stress may affect antibody-specific antigen expression of bacterial cells thereby limiting antibody-based capture and detection on biosensor platforms.[13−15]

Pathogen or toxin-specific receptor molecules have been used as substitutes for antibodies for specific capture or binding and detection of pathogens or toxins. Synthetic or natural glycoconjugate, lectin, were used to bind bacterial lipopolysaccharides for detection of Gram-negative bacteria on electrochemical biosensors.[16,17] Carbohydrate derivatives were used for detection of cholera toxin[18,19] and Shiga toxin.[20] Protein molecules such as agglutinin was used for detection of *Staphylococcus aureus*,[21] bacteriophage for *Escherichia coli* O157:H7,[22] and lysozyme for *E. coli* and *L. monocytogenes*.[23] Often these receptors lack specificity which limits their use for some types of biosensors.

Listeria adhesion protein (LAP) in *L. monocytogenes* is a housekeeping enzyme, designated alcohol acetaldehyde dehydrogenase, with a molecular mass of 104 kDa[24] that interacts with the host cell receptor, heat shock protein 60 (Hsp60)[25] during the intestinal phase of infection.[25] In this study, we demonstrate the use of Hsp60 as a potential bioreceptor for specific capture of *L. monocytogenes* cells on the biochip sensor platform and compare the performance of Hsp60 with a monoclonal antibody as biorecognition molecule.

EXPERIMENTAL SECTION

Bacterial Cultures and Growth Media. *L. monocytogenes* F4244 (serotype 4b), *L. ivanovii* SE98, *L. innocua* F4248, *L. welshimeri* ATCC 35897, *L. seeligeri* SE31, *L. grayi* ATCC 19120, *Bacillus cereus* 4AC, *Salmonella enterica* serovar Enteritidis, *E. coli* O157:H7, *Proteus vulgaris*, *Enterobacter aerogenes*, *Staphylococcus epidermidis* 8VO5B, *S. aureus* ATCC 51740, *Pseudomonas aeruginosa* ATCC 10145, *Citrobacter freundii* ATCC 3624, *Hafnia alvei, Serretia marcesens* ATCC 14756, *Lactobacillus rhamnosus* GG, *Lac. casei* KCTC 3103, *Lac. acidophilus* NRRL B1910 and *Leuconostoc mesenteroides* from our culture collection were used in the experiments. *Listeria* species were cultured in brain heart infusion (BHI, Difco Laboratories, Sparks, MD) or Luria−Bertani (LB, 0.5% NaCl, 1% tryptone peptone, and 0.5% yeast extract) media at 37 °C for 16 to 18 h. LAP-deficient mutant *L. monocytogenes* strain KB208[24] and GFP-expressing *L. monocytogenes* V7 were grown in BHI or LB containing erythromycin (5 μg/mL and 10 μg/mL, respectively) at 42 °C for 20 to 24 h and 37 °C for 16 to 18 h, respectively. All other bacteria were grown in BHI medium at 37 °C for 16 to 18 h except for lactic acid bacteria, which were cultured in deMann Rogosa Sharpe (MRS, Difco Laboratory) broth. The growth conditions for lactic acid bacteria were; *Leu. mesenteroides* at 30 °C, and *Lac. rhamnosus* at 37 °C under 7% CO_2.

Biotinylation of Hsp60 and mAb-C11E9. Purified recombinant Hsp60 from human promyelocytic leukemia cell (HL-60) was purchased from Assay Design (Ann Arber, MI). Monoclonal antibody-C11E9[26] specifically reacts to *N*-acetyl muramidase A (NAM)[13] of *L. monocytogenes* and was purified by immunoaffinity column. Sulfo-NHS-LC conjugated biotin (10 mM; Pierce, Rockford, IL) was prepared in deionized (DI) water and conjugated to the primary amine group of Hsp60 and mAb-C11E9 for biotinylation according to manufacturer's instruction. In short, Hsp60 (1.2 mg/mL) was allowed to react with 10 mM biotin reagent for 2 h on ice and then the unbound biotin was removed by Zeba desalt spin column (Pierce, Rockford, IL). The mole-to-mole ratio of biotin to Hsp60 was measured using HABA (4′-hydroxyazobenzene-2-carboxylic acid; Pierce) and biotinylated Hsp60 (B-Hsp60) had 2.44 moles of biotin/mol and 2.82 moles of biotin/mol for mAb-C11E9.

Immobilization of Hsp60 onto Silicon Dioxide (SiO_2) Wafer for Capture of Bacteria. Wells to hold the solutions on SiO_2 surface of the biochip were prepared by polydimethylsiloxane (PDMS). Elastomer base and curing agent were mixed with a ratio of 10:1 (w:w), poured into a Petri dish, and cured in a hard-bake oven at 120 °C for 10 min (Dow Corning Corp, Midland, MI). The baked PDMS layer with a well (23.04 mm^2 ± 1.17 mm^2) punched on the center was placed on top of the SiO_2 substrate (Panels A and B of Figure 1). Then, biotinylated bovine serum albumin (B-BSA, Sigma) at 2 mg/mL in PBS (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, and 0.5 M NaCl, pH 7.4) was added on the chip, which was placed inside a covered Petri dish and incubated at ambient temperature for 30 min. After washing the surface twice with 0.5% Tween 20 in PBS (PBS-T) and once with PBS, streptavidin (1 mg/mL, Sigma) was incubated on the wafer for 15 min. B-Hsp60 or B-mAb C11E9

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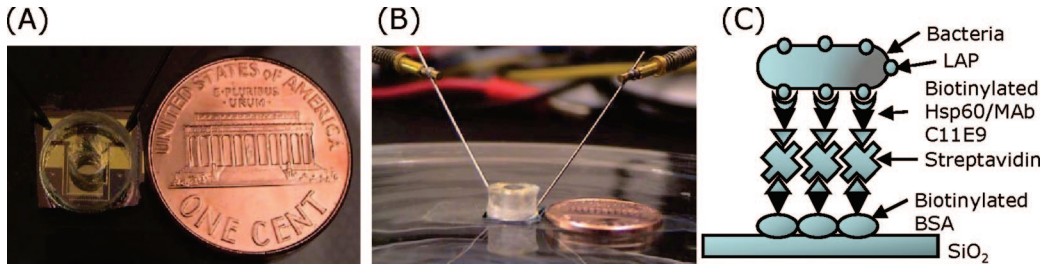

Figure 1. SiO_2 wafer with dielectrophoresis set up. (A) Top view of DEP biochip with PDMS wells. (B) Side view of DEP biochip connected with function generator by micromanipulators and probe tips. (C) Schematic diagram showing bioreceptor (Hsp60 or mAb-C11E9) immobilization onto the SiO_2 surface. LAP; *Listeria* adhesion protein. The illustration is not drawn to scale.

(1 μg/mL) was added and the wafer was incubated with living (viable) bacteria (5×10^7/well) at ambient temperature for 2 h[27] (Figure 1C).

After incubation was completed, the bacterial cells were fixed by 10% formalin, stained with propidium iodide, and enumerated under fluorescence microscopy (Leica, Nuhsbum Inc. Bannockburn, IL). Binding efficiency of *L. monocytogenes* was compared by the net difference between bindings of bacterial cells to Hsp60 coated or non-Hsp60 coated wafer surface. Digital images from the microscope were captured with the SPOT software v. 4.6 (Diagnostic Instruments Inc., Sterling Heights, MI). At least nine images were taken for each die, and the average cell number was calculated. To estimate the number of captured bacteria on the entire chip surface, the bacterial count was calculated by normalizing cell counts for each microscopic image, 130 μm × 98 μm, to a total area of the silicon die, 23.04 mm^2 ± 1.17 mm^2 (eq 1).

$$\text{Total estimated captured bacteria} = \text{average cell number/image} \times \frac{\text{Total wafer surface area } (23.04\ \text{mm}^2)}{\text{Microscopic image area } (0.013\ \text{mm}^2)} \quad (1)$$

Analysis of Hsp60 and *Listeria* Interaction by ELISA. Freshly grown bacterial cultures were centrifuged and resuspended in 0.1 M carbonate coating buffer (68 mM $NaHCO_3$, 32 mM Na_2CO_3, pH 9.6) to obtain a cell population of ~10^9 cfu/mL. Each culture was distributed (0.1 mL/well) to a 96-well microtiter plate (Dynex, Chantilly, VA) and incubated at 4 °C overnight. The plates were washed four times with PBS-T and blocked with 2% BSA. One hundred microliters of Hsp60 (1 μg/mL) were added and incubated at 37 °C for 1 h. Hsp60 specific rabbit polyclonal antibody (1.4 μg/mL) was added to determine the bound Hsp60 to *Listeria*. The polyclonal antibody (PAb) was generated by immunizing two female rabbits held in a pathogen-free environment with the recombinant Hsp60 (Assay Designs) at Small Animal facility in the Department of Biological Science laboratory at Purdue University. Serum was collected after the booster injection. Anti-Hsp60 PAb was purified using protein G column (GE Life Sciences, Pittsburgh, PA), concentrated to 1.4 mg/mL, and stored in PBS with addition of 0.01% thimersol. After the addition of anti-Hsp60 PAb, goat anti-rabbit-peroxidase conjugated antibody (0.4 μg/mL, Jackson Immuno Research Laboratories, West Grove, PA) was added and incubated at 37 °C for 1 h. Then *o*-phenylenediamine (OPD, Sigma) with 10% (v/v) hydrogen peroxide was added and the color intensity was measured at 492 nm.[26] In a parallel experiment, Internalin A specific receptor, E-cadherin (1 μg/mL) was added to the wells, and binding of *L. monocytogenes* to E-cadherin was determined by using monoclonal anti-E-cadherin antibody (1 μg/mL; R&D Systems, Inc. Minneapolis, MN). Sheep anti-mouse-peroxidase conjugated antibody (0.4 μg/mL, Jackson Immuno Research Laboratory) and OPD with H_2O_2 substrate was used to complete the assay as above.

Analysis of Capture Limit for Hsp60 on SiO_2 under Static Condition. A fresh culture of *L. monocytogenes* cells was washed and serially diluted from 5×10^7 cfu/well to 5×10^2 cfu/well in 0.1 M carbonate coating buffer, pH 9.6. Diluted *L. monocytogenes* cells were added to wells in each SiO_2 wafer, incubated and washed with PBS-T as described in the Immobilization of Hsp60 onto SiO_2 Wafer section (see above). Cells were fixed by 10% formalin, stained with propidium iodide, and examined using the fluorescence microscope. The capture limit was determined by quantifying the captured bacteria for each dilution of *L. monocytogenes* cells used onto the wafer. To determine the capture limit values on 96 microtiter plate, captured cells were sequentially reacted with Hsp60, anti-Hsp60 antibody, anti-rabbit-alkaline phosphatase conjugated antibody (0.4 μg/mL, Jackson Immuno Research Laboratory), and the substrate, 4-methylumbelliferyl phosphate (MUP, Sigma).[28] The fluorescence intensity was quantified in a spectrophotometer (SpectraMax, Gemini) using excitation and emission wavelengths of 360/440 nm.[28]

Enhanced Capture Efficiency by DEP. The capture efficiency of Hsp60 was further enhanced by combining with dielectrophoresis (DEP; panels A and B of Figure 1). Devices with interdigitated electrodes for DEP were fabricated by metal evaporation of 50 nm Ti and 250 nm of Au, 25 μm wide and 25 μm spacing, onto a 3500 ± 200 Å thermal SiO_2 layer grown on a silicon substrate. The geometries of the devices and PDMS wells were kept similar to the rest of experiments (Immobilization of Hsp60 onto SiO_2 Wafer) of this study for consistency, except the Au/Ti electrode structures for DEP. The DEP forces were generated by applying an 10 V (peak to peak) Ac voltage at 1 kHz across the interdigitated metal electrodes[29,30] (Figure 1A). Twenty microliter samples were used for each experiment, and experimental procedures for immobilization were the same

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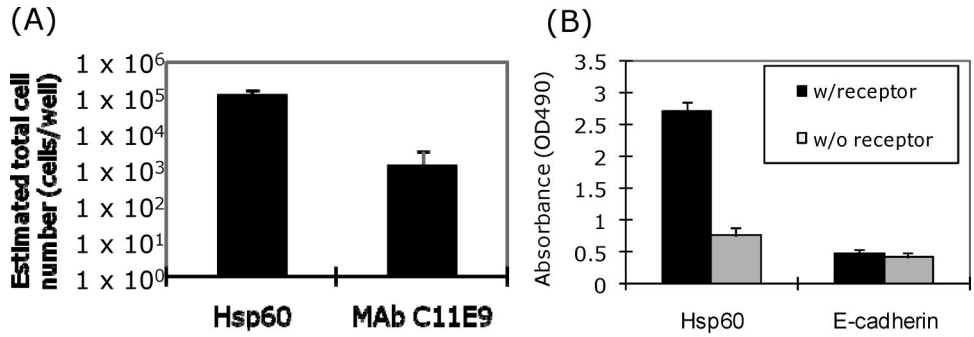

Figure 2. Analysis of *L. monocytogenes* cells binding to different bioreceptors. (A) Binding to Hsp60 and mAb-C11E9 immobilized on SiO_2 surface and cells were counted under microscope after staining with propidium iodide. (B) Comparative binding analysis of *L. monocytogenes* to Hsp60 and E-cadherin as determined by indirect ELISA. Hsp60-mediated capture was significantly ($P < 0.05$) greater than mAb-C11E9 (A) or E-cadherin (B).

as the direct biochip method, except that the DEP force was applied at the final incubation step for enhancing the capture of the bacteria on the surface of the biochips.

Microfluidic Biochip Fabrication. The microfluidic device was made by plasma-enhanced bonding of PDMS channels on top of 3500 ± 200 Å thermal SiO_2 on a silicon substrate.[31,32] The channel size was 25 μm high and 250 μm wide and was made by pouring PDMS on a SU-8 photoresist master mold, followed by a curing step at temperature of 85 °C for 45 min on a hot-plate. The SU-8 mold was made by spin-coating SU-8 25 at 500 rpm, 5 s and 2000 rpm, 40 s for a thickness of 25 μm. Photolithography steps of SU-8 mold includes 3 min soft-bake at 65 °C, exposure to a dose of 420 mJ/cm^2, postbake of 1 min at 65 °C on a hot-plate, followed by the development of the photoresist using a SU-8 developer solution (Microchem, Newton, MA) and isopropyl alcohol rinse. PTEE ultramicrobore tubing (Cole-Parmer Instrument, Vernon Hill, IL) with an inner diameter of 152.4 μm and outer diameter of 406.4 μm were inserted at the ends of the channel for the delivery of chemicals and cells for immobilization and capture. The microfluidic chip is 8.2 mm long, 8.6 mm wide (Figure 4).

Bioreceptor Immobilization on Microfluidic Biochip. The procedure was modified from Yang et al.[31] for the immobilization of bioreceptors on microfluidic biochip. First, the biochip was rinsed with DI water at a flow rate of 0.5 μL/mL for 10 min. A constant flow rate was used for the injection process using a WPI SP200i syringe pump (World Precision Instruments, Inc., Sarasota, FL) at a flow rate of 0.05 μL/mL for 10 min, and then 0.1 μL/mL for 10 min were used for all immobilization solutions, and a flow rate of 0.5 μL/mL for 10 min was used for all washing steps. B-BSA (0.5 mg/mL in PBS) then was injected into each channel of the biochip and incubated at ambient temperature for 1 h to allow B-BSA absorption onto the SiO_2 surface and washed using PBS. Streptavidin (0.25 mg/mL in PBS) was injected and washed after 1 h of incubation. Then B-Hsp60 and B-mAb-C11E9 (10 μg /mL in PBS) were introduced into each channel and incubated for 1 h. The unbound protein was removed by PBS wash. Concurrently, acridine orange (30 μg/mL, Sigma) was used to stain bacteria at 37 °C for 30 min. The stained bacteria were injected into each channel of the biochip, incubated at ambient temperature for 1 h, and then washed. Captured bacteria were measured on the microfluidic biochip using shareware software Image J (Wayne Rasband, National Institute of Health, Bethesda, MD). Cells retained in each channel were counted and compared to the bacterial concentration before injection and after elution using eq 2.[31]

$$\text{Capture efficiency}(\%) = \frac{cfu/mL_{in} - cfu/mL_{out}}{cfu/mL_{in}} \times 100\% \quad (2)$$

Statistical Analysis. All experiments were repeated at least three times with separate biochips. Quantitative analysis at every biochip was carried out with at least nine random images. Statistical comparisons were carried out using the ANOVA (SAS, Cary, NC), and the Tukey's multiple comparison of means at $P < 0.05$ was used to show significant differences.

RESULTS AND DISCUSSION

Comparative Analysis of *Listeria* Capture by Anti-*Listeria* Antibody vs Receptor Hsp60 under Static Condition. The efficiency of Hsp60 as a receptor for the biosensor was compared to that of anti-*Listeria* monoclonal antibody C11E9 at equivalent conditions on the SiO_2 wafer (Figure 2A). Estimated total net binding for Hsp60 was $1.2 \pm 0.5 \times 10^5$ cells/biochip and $1.3 \pm 1.9 \times 10^3$ cells/biochip for mAb-C11E9 with initial bacteria concentration of 5×10^7 cells/biochip. The capture rate was 83% higher for Hsp60 than mAb-C11E9 ($P = 0.002$). When the total captured bacteria included the background binding, there were still significant differences between Hsp60 and mAb-C11E9 as observed by microscopic images. Increased bacterial capture by Hsp60 over mAb-C11E9 is a function of increased surface expression of Hsp60-specific ligand, higher binding affinity, or reduced chemical modification of bioreceptor during immobilization. Hsp60 interacts with 104 kDa LAP[24,33] while mAb-C11E9 interacts with 66 kDa *N*-acetyl muramidase (NAM)[13,26] on *Listeria* cells. Surface expression level of NAM and LAP was determined previously using mAb-C11E9[34] and monoclonal anti-LAP antibody,[33] respectively. mAb-C11E9 has been used for *L.*

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monocytogenes detection on several biosensor platforms using the biotin-avidin immobilization process.[31,35,36]

Under physiological conditions, LAP[33] expression was at least four times lower than that of the NAM[34] on the cell surface, thus suggesting surface expression may not be responsible for increased capture. Binding affinity may also affect the capture rate; however, in a previous experiment, it was demonstrated that binding affinity (K_D) for LAP to Hsp60 was 1.68×10^{-8} M,[24] which falls within the range (1×10^{-6}–1.68×10^{-9} M) for antigen and antibody interactions.[37] The immobilization process employing biotin-avidin may have interacted with the binding domains in antibody, but not in Hsp60, thus causing reduced bacterial capture by mAb-C11E9. Cho et al.[12] showed biotin randomly binds to the primary amide group in molecules throughout the antibody and hinders the accessibility of ligand to the binding domain. Hsp60 also can have random biotinylation that lowers the binding site; however, the size of the protein (150 kDa for mAb-C11E9 and 58 kDa for Hsp60), the structure of the protein, and the possible lack of an amide group in the binding domain of Hsp60 may account for increased capture of bacteria compared to C11E9.

Specific interaction of Hsp60 to *L. monocytogenes* was compared with E-cadherin, which interacts with Internalin A protein of *L. monocytogenes*. Internalin A is an adhesion/invasion protein that promotes *L. monocytogenes* entry into human intestinal epithelial cells during infection[38] by targeting the receptor E-cadherin.[39] For this experiment, ELISA was used indirectly to measure the binding level of E-cadherin to *L. monocytogenes*. The average net absorbance at 492 nm for E-cadherin was 0.04 ± 0.15 and 1.94 ± 0.31 for Hsp60, which had significantly higher binding to *L. monocytogenes* ($P < 0.0001$) (Figure 2B). The K_D of Internalin A and E-cadherin is 8.00×10^{-6} M,[40] which is 200 times lower than the Hsp60 and LAP interaction 1.68×10^{-8} M.[24] Thus, E-cadherin for *Listeria* detection is less attractive because of its lower binding affinity.

Analysis of Capture Limit for Hsp60 on SiO_2 under Static Condition. To measure the capture limit on the biochip, *L. monocytogenes* cells were serially diluted and the binding rate was observed on the Hsp60-coated biochip (direct method) and data were compared with 96-well microplate-based ELISA. The capture limit was defined when the captured bacteria concentration no longer showed differences at serial diluents of initial bacteria concentration. The capture limit on the biochip (Figure 3A) and ELISA (Figure 3B) was determined to be about 5×10^5 cells/well. To further improve the Hsp60-mediated capture, DEP force was applied (see below).

We also attempted to improve the Hsp60 mediated capture on the SiO_2 surface without DEP by using two other assay

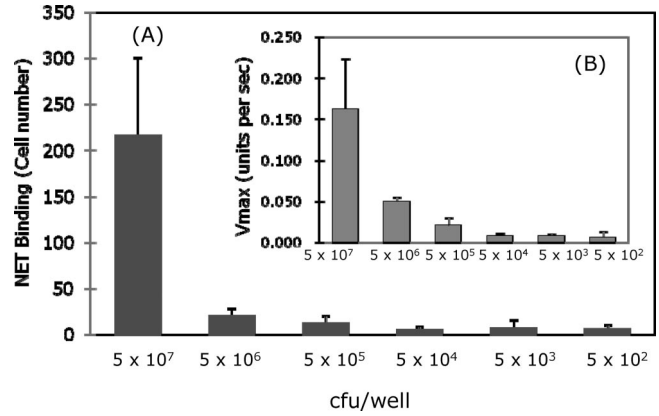

Figure 3. Determination capture limit for Hsp60 and *L. monocytogenes* interaction using the (A) biochip and an (B) ELISA. The average net binding of the bacteria on biochip was counted by fluorescent microscopic analysis.

configurations; indirect and direct-mix method. In the indirect method, immobilized biotinylated anti-Hsp60 antibody was first reacted with Hsp60 and then allowed to capture bacteria. In the direct-mix method, first biotinylated Hsp60 was allowed to react with bacteria freely in solution in a separate tube before adding to the streptavidin coated SiO_2 surface. None of these two methods provided satisfactory results (data not shown).

Specific Capture Analysis of Hsp60 for Pathogenic *Listeria* Detection under Static Condition. To observe specific binding efficiency of Hsp60 to pathogenic *Listeria* species, all *Listeria* species were incubated on the Hsp60 immobilized biochip (Figure 1). As shown in Table 1, *L. monocytogenes* cells were captured at $2.0 \pm 0.5 \times 10^5$ cells and *L. ivanovii* at $1.4 \pm 1.1 \times 10^5$ cells on each SiO_2 wafer. The concentrations were significantly higher than other *Listeria* species having about 10^3–10^4 cells for each SiO_2 chip ($P = 0.030$). LAP deletion mutant *L. monocytogenes* KB208 as a negative control also showed significantly decreased number of binding to Hsp60 as compared to *L. monocytogenes* wild type (WT) strain (Table 1). Supporting Information, Figure S1 shows the microscopic images of propidium iodide stained *Listeria* spp. on a SiO_2 wafer.

Indirect ELISA was also performed to verify the result of capture on the wafers. The interaction between Hsp60 and bacteria was detected, and the result was equivalent to the reaction on the biochip (Table 1). *L. monocytogenes* had 5.5-fold higher reaction levels compared with KB208. There was no difference between *L. monocytogenes* and *L. ivanovii* for binding to Hsp60. This result is expected since *L. ivanovii* is an animal pathogen and behaves like *L. monocytogenes*.[1] *L. innocua*, *L. seeligeri*, *L. welshimeri*, and LAP deletion mutant KB208 had 2.9 to 5.6-fold lower binding level compared to *L. monocytogenes* WT, and the results were significantly different from *L. monocytogenes* WT ($P < 0.0001$). *L. grayi* showed higher binding than other three non-pathogenic *Listeria*; however, the binding level was still significantly lower than that for both pathogenic *Listeria* (*monocytogenes* and *ivanovii*).

LAP is an enzyme (alcohol acetaldehyde dehydrogenase) and also acts as an adhesion factor for *L. monocytogenes* that interacts with host cell receptor, Hsp60[25] during infection.[33] Although LAP is present in all *Listeria* species,[41] Hsp60 mediated capture was markedly specific for pathogenic *Listeria* over non-pathogenic stains. Surface expression of LAP is high in pathogenic *Listeria*

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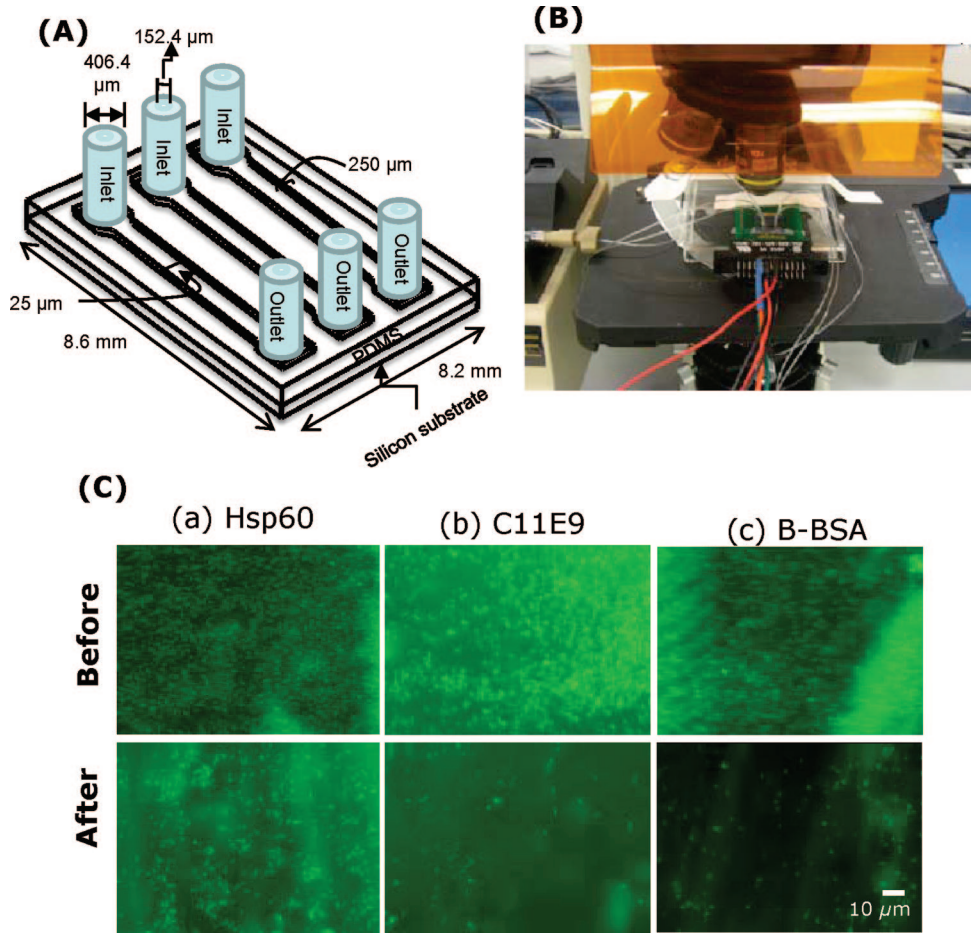

Figure 4. Microfluidic biochip setup for capture of *Listeria* using bioreceptors. (A) Schematic drawing of the microfluidic chip (not drawn to scale), (B) Actual prototype chip connected with microfluidic tubings is placed on the microscope stage, and (C) Fluorescent images of captured bacteria on a microfluidic biochip: Acridine orange stained bacteria were captured on each channel with Hsp60, mAb-C11E9, and no receptor (B-BSA in PBS). The results were compared with images from before washing and after washing with PBS.

Table 1. Specific Hsp60-Mediated Capture of *L. monocytogenes* on the SiO_2 Surface by Comparing with Different *Listeria* spp

bacteria	initial CFU/well	estimated total cell number/well[a]	relative capture percentage[b] (%)	ELISA (Abs490)[c]
L. monocytogenes	5.8×10^7	$2.01 \pm 0.48 \times 10^5$	100.00	0.619 ± 0.179^{A}
L. ivanovii	4.1×10^7	$1.44 \pm 1.09 \times 10^5$	71.57	0.499 ± 0.048^{A}
L. innocua	3.3×10^7	$8.89 \pm 6.22 \times 10^3$	4.49	0.212 ± 0.067^{C}
L. welshimeri	2.2×10^7	$3.46 \pm 7.71 \times 10^3$	1.75	0.203 ± 0.087^{C}
L. seeligeri	3.3×10^7	$8.52 \pm 5.35 \times 10^3$	4.24	0.111 ± 0.072^{C}
L. grayi	2.5×10^7	$4.55 \pm 5.65 \times 10^3$	2.24	0.348 ± 0.054^{B}
Lm KB208[d]	4.4×10^7	$2.17 \pm 1.13 \times 10^4$	10.72	0.112 ± 0.012^{C}

[a] The cell number was calculated based on the net binding level for each image multiplied by the total area of the SiO_2surface. [b] Relative binding percentage of each *Listeria* spp. was calculated as the ratio of concentration of the captured bacteria to wild type *L. monocytogenes*. [c] Indirect ELISA was applied to determine the interaction between Hsp60 and *Listeria* spp. Different *Listeria* spp. were coated on 96 microtiter plate and the binding level of Hsp60 was compared by a microplate reader at 490 nm. Mean values in a column with different superscript letters were significantly different using Tukey's test at $P < 0.05$. [d] KB208 is the LAP deletion mutant of *L. monocytogenes* F4244.

over non-pathogenic *Listeria* possibly because of evolutionary changes in the protein translocation system. A similar observation was made for malate synthase (MS) enzyme of *Myocobacterium* species, which is responsible for interaction with host cell laminin and fibronectin. MS is present on the surface of pathogenic *M. tuberculosis*, while it is absent on the surface but present in the cytosol of non-pathogenic *M. smegmatis* bacteria.[42]

Specificity of Hsp60 to *L. monocytogenes* was compared with other bacteria including common foodborne pathogens. Since bacteria mostly exist in a complex environment, it is difficult to capture target bacteria in the presence of other background microflora in contaminated food. Thus, the detection system must have high selectivity in the heterogeneous mixture of pathogenic and spoilage bacteria. When compared the estimated total bound

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Table 2. Specific Capture of *L. monocytogenes* in the Presence of Pathogenic or Non-Pathogenic Bacteria[a]

	L. monocytogenes:*S. Enteritidis*:*E. coli* O157:H7				*L. monocytogenes*:*E. coli* K-12:*Lac. acidophilus*			
	1: 1: 1	1: 1: 0	1: 0: 1	0: 1: 1	1: 1: 1	1: 1: 0	1: 0: 1	0: 1: 1
mixed cell number/image	15.4 ± 13.0[b]	18.9 ± 11.8	17.2 ± 11.1	5.8 ± 1.5	16.1 ± 11.6	15.0 ± 5.3	10.5 ± 3.7	7.5 ± 3.4
GFP expressing cell number/image	11.3 ± 10.3	11 ± 6.3	13.2 ± 10.9	0	11.1 ± 11.7	10.5 ± 4.6	7.6 ± 3.4	0
% bound of target organism	73.5	58.2	76.7	0	69.2	70	71.9	0

[a] The selectivity test was done using the mixture of pathogenic or non-pathogenic bacteria at the same concentration of 7×10^7 CFU/well and each bacterium was taken out to observe the % bound as the negative control. Total cell number for each image was observed under the fluorescent microscope following staining with propidium iodide, and the GFP expressing *L. monocytogenes* was counted among total bacteria. [b] Cell number of each image was counted under the microscope, and at least ten images were assessed for each assay. The experiment was duplicated.

cell number, one to two log lower numbers of cells were captured for other bacteria by Hsp60 compared to *L. monocytogenes* (Supporting Information, Table S1). Only *Sta. aureus* did not show statistical difference in binding as *L. monocytogenes* ($P = 0.129$). *E. coli* O157:H7, *Pro. vulgaris*, *Pse. aeruginosa*, and *Ser. marcensens* had zero net binding. The overall cell numbers for other bacteria were less than 10^4 cells including the background binding for each SiO_2 wafer with a significantly lower level of binding than for *L. monocytogenes* (data not shown). To observe the specific capture of *L. monocytogenes* by Hsp60 in the presence of other bacteria, GFP expressing *L. monocytogenes* was added to the Hsp60 immobilized SiO_2 wafer with the cocktail of pathogenic or non-pathogenic bacteria (Table 2). Each set of experiments including *L. monocytogenes* had a similar level of bacterial count, and both assays with pathogenic and non-pathogenic bacteria had 58−77% of average capture rate of GFP expressing bacteria. There were no GFP expressing bacteria in the absence of *L. monocytogenes*. Overall, the result suggests that Hsp60 specifically interacts with *L. monocytogenes* compared to other *Listeria* spp. and other foodborne bacteria except for *Sta. aureus*, which expresses the fibronectin binding protein (FnBP) that is known to interact with Hsp60.[43]

Microfluidic-Biochip Detection of *L. monocytogenes* using Hsp60. A flow-through microfluidic biochip was set up to capture bacteria from flowing fluid (panels A and B of Figure 4). Three channels were designed on single SiO_2 wafer and Hsp60, mAb-C11E9, and B-BSA as negative control were immobilized, respectively. Captured bacteria were observed before and after the washing step on each channel (Figure 4C). More acridine orange-labeled bacteria were captured in the Hsp60 immobilized channel than in the mAb-C11E9 and in the B-BSA immobilized channels. The capture rate was 58.6% for Hsp60, 16.6% for mAb-C11E9, and 5.6% for control (B-BSA only) by quantification based on Image J analysis. These results were similar to the unbounded cell count from the outlet of the tubes, 57.8, 33.9, and 17.9% for Hsp60, C11E9, and control, respectively. The highest capture of *L. monocytogenes* was observed with Hsp60 as bioreceptor.

Application of DEP on Hsp60-based Capture on Biochip. The efficiency of Hsp60-based capture was enhanced by applying DEP force with a $10V_{p\text{-}p}$ ($7.07\ V_{rms}$), at frequency of 1 KHz at the final incubation step through embedded Au/Ti electrodes. This condition has been optimized to minimize cell damage and to avoid electrolysis on electrodes previously.[30,31,44] Table 3 shows the results of the application of the DEP signal for 0, 5, 30 min, and 1 h at the final 1 h incubation step. An average increase in capture efficiency between results of DEP for 5 min and no DEP was 60% at a bacterial concentration of 10^7 cfu/mL. With extended application of DEP from 5 min, 30 min, and 1 h, the capture efficiency increased from 60%, three times the captured cells to 8 times the captured cells when compared to cases without DEP, respectively, showing a tremendous enhancement by combining DEP with Hsp60. Lowering the initial cell concentration to 10^6 cfu/mL decreased the capture efficiency enhancement. No enhancement was found at 10^6 cfu/mL with 5 min of DEP application. Increasing the DEP exposure time from 5 to 30 min, the capture rate increased twice the number of captured cells in no DEP experiment (Table 3). However, the capture sites were saturated in cases beyond 30 min of DEP application since counts for 30 and 60 min were equivalent. This result suggests that for lower cell concentration, diffusion of cells into effective range of DEP forces limited the capture efficiency of Hsp60 when combined with DEP.

Table 3. Enhancement of Capture Efficiency by Coupling DEP with Hsp60

	avg. cell counts ($500 \times 400\ \mu m^2$)	estimated total cell counts/cm^2
Sample Concentration: 10^7 cfu/mL		
No DEP	$3.84 \pm 0.60 \times 10^2$	$1.92 \pm 0.30 \times 10^5$
DEP 5 min	$6.15 \pm 0.80 \times 10^2$	$3.08 \pm 0.40 \times 10^5$
DEP 30 min	$1.68 \pm 0.21 \times 10^3$	$8.38 \pm 1.05 \times 10^5$
DEP 60 min	$3.79 \pm 0.00 \times 10^3$	$1.90 \pm 0.00 \times 10^6$
Sample Concentration: 10^6 cfu/mL		
No DEP	$2.44 \pm 0.20 \times 10^2$	$1.22 \pm 0.10 \times 10^5$
DEP 5 min	$2.36 \pm 0.36 \times 10^2$	$1.18 \pm 0.18 \times 10^5$
DEP 30 min	$6.84 \pm 1.42 \times 10^2$	$3.42 \pm 0.71 \times 10^5$
DEP 60 min	$6.05 \pm 1.72 \times 10^2$	$3.02 \pm 0.86 \times 10^5$

Application of DEP has been proven to improve the capture efficiency (as high as 90% increase) of the target entity in the microfluidic device[31] and in the electrode-embedded single well device as well. The DEP forces attract the bacteria to the surface of the electrodes (at the edges where the electric field gradient is the maximum) when the field was turned on.[32] DEP applications can concentrate the *Listeria* cells by the localization of the electrodes and helping close contact to the bioreceptor.[31] DEP may cause the structural changes of cell wall (membrane) because of the applied electrical field and allow LAP to be more exposed and accessible to the Hsp60. Yang et al.[30] demonstrated increased immuno-reactivity of different antibodies with *L. monocytogenes* following exposure to DEP. They also recommended applying 1 h or less of DEP to ensure that no adverse effects are imparted on

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the bacterial cells. Longer exposure of DEP can cause irreversible damage to the cells. In the case of single well electrode-embedded device, because of sedimentation of cells in static solution, the higher the initial sample concentration, the better the enhanced capture efficiency was found in DEP mediated Hsp60 capture. With the lower initial cell concentration, improvement in capture efficiency can be expected by applying longer DEP. Integration of DEP mediated Hsp60 capture on microfluidic device can be useful and will be our future research goal for the simultaneous and continuous mode of detection.

CONCLUSION

The specificity and capture efficiency of Hsp60 over the monoclonal antibody C11E9 and E-cadherin provided preliminary results of utilizing DEP for enhanced capture and detection of pathogens for future application. The use of Hsp60 as a novel molecular recognition element made it possible to detect specifically the pathogenic *Listeria* with a higher level of sensitivity as compared to other antibodies. Enhanced specificity of capture of pathogenic *Listeria* will minimize the false-positive detection of *Listeria* in food. Moreover, biotinylation of Hsp60 for the immobilization of the protein can broaden the application to other platforms of biosensors and detection by real time monitoring of the receptor−ligand interaction. As well as antibodies, eukaryotic Hsp60 can be produced by recombination cDNA into *E. coli,* which would reduce the cost[45] of this protein.

ACKNOWLEDGMENT

This research was supported through a cooperative agreement with the Agricultural Research Service of the U.S. Department of Agriculture project number 1935-42000-035 and the Center for Food Safety and Engineering at Purdue University. Sincere thanks to Brad Reuhs for critical reading of the manuscript. We thank Richard Linton, Director of the Center for Food Safety Engineering for his leadership of the Center and his support of this research, and Dr. Shu-I Tu, of USDA ERRC for his inputs and support.

SUPPORTING INFORMATION AVAILABLE

Further details are given in Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review January 13, 2009. Accepted March 6, 2009.

AC9000833

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