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Smart phone based immunosensor coupled with nanoflower signal amplification for rapid detection of *Salmonella* Enteritidis in milk, cheese and water

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ABSTRACT

Salmonella is a standout amongst the most foodborne pathogens causing harmful disease. To protect consumers from food poisoning due to *Salmonella* infection, it is important to develop a quick, simple, reliable and sensitive method, which can detect *Salmonella* in foods at low concentration in a timely manner. We have effectively established a novel magnetic nano biosensor with high sensitivity for the visual and quantitative detection of *S.* Enteritidis from milk, cheese and water. Milk, cheese and water samples inoculated with different concentrations of *S.* Enteritidis have been tested using anti *S.* Enteritidis streptavidin magnetic beads and biotin labeled antibody as capture platform and coupled with nanocomposite (detecting antibody-HRP enzyme and inorganic nanoflower), where the signal amplification based on HRP enzyme which is enhanced by the action of nanoflower and produce visual color easily detected by the smartphone device at a very low concentration. The developed assay was able to detect *S.* Enteritidis in tap water, milk and cheese, with a detection limit of 1.0 CFU mL^{-1} and 1.0 CFU/g, respectively. Recoveries percentages of spiked milk, cheese and tap water samples with10^2, 10^3 and 10^4 CFU mL^{-1} from live *Salmonella* were 98.2, 96.1 and 95.4 (in milk), 94.3, 98.6 and 99.5 (in cheese) and 95.8, 101.2 and 97.8 (in water) using designed device, respectively. The effective application of this innovation in milk and cheese indicates the possibility of its application in various food products.

1. Introduction

Salmonella is one of the major foodborne pathogens that cause food poisoning and affects human health [1,2], causing fever, abdominal cramps, vomiting, diarrhea and weakness. Humans usually infected from eating undercooked poultry meat, milk and eggs, fresh produce [3,4], or foods cross-contaminated with *Salmonella* [5,6].

Different techniques have been developed to detect the presence of low levels of *Salmonella* in foods. Conventional methods for *Salmonella* detection involved different steps of pre-enrichment, selective enrichment and selective plating, which is time consuming, tedious and arduous [7]. Other methods such as PCR and ELISA are developed for *Salmonella* detection but these methods require costly hardware and considerable technical expertise to perform and furthermore lack of specificity and sensitivity [8,9]. The improvement of new systems with quicker reaction time, better affectability and selectivity and no requirement for pre-enrichment remains a test of research intrigue.

Biosensors have demonstrated awesome potential for quick detection of foodborne pathogens. Additionally, nanotechnology shows an extraordinary chance to develop quick, accurate and cost-effective diagnostics for the detection of food borne pathogens [10,11]. The nanomaterials are characterized by their small size (1–100 nm) and large surface area, resulting in enhanced surface reactivity, improved electrical conductivity and enhanced magnetic properties, among others [12]. Above all, adjustments of the nanostructures' surface can change significantly some of their properties [13,14]. Numerous nanoparticles have been developed to detect particular molecular targets in biodiagnostic applications, including pathogen detection. Among them, gold nanoparticles, silver nanoparticles, alloy nanoparticles, inorganic nanoflower nanoparticles and magnetic nanoparticles [15–17].

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Magnetic nanoparticles connected to antibodies have been utilized for the immunomagnetic separation of nucleic acids, proteins, viruses and bacteria [10]. Immunomagnetic separation (IMS) was used to successfully isolate or enrich of target organisms from complex matrices in food, clinical, and environmental samples [18–20]. IMS reduces the total assay time and provides higher sensitivity for pathogen detection in complex food or environmental samples [21–23]. Magnetic beads (MBs) can not only improve the efficiency of immobilization of capture antibody, but also enhance the sensitivity of detection. Because of the large specific surface area of MBs, more signal molecules can be captured by MBs thus the detection signal will be amplified. Besides, the magnetism separation process makes the separation more effective, therefore, less signal molecules will be lost during the washing procedure. Through the utilizing of the MB, the platform shows good advantages over other traditional method like using the microplate as the platform for capture antibody immobilization.

Recently, protein-inorganic hybrid nanoflowers made of protein and Cu_3 $(PO_4)_2$ have attracted much consideration since being initially portrayed by Richard Zare and co-workers [24]. The antibody-enzyme-inorganic nanoflower showed incredibly improved catalytic activity and stability [25,26]. The previous work in our group published by Wei utilized the three-in-one copper antibody-enzyme-nanoflower to detect *Escherichia coli* O157:H7. By taking advantage of nanoflower's high capture capability and catalytic activity, ultrasensitive detection of targets was realized [25].

In recent years, smartphone based portable and low-cost devices have been used in bio sensing and point of care diagnostics. Smartphone, a multifunctional platform with good memory system and high-quality camera lenses, now not only worked as a communication tool, but also as a sensing platform for research studying or detection with signal output. It is more accessible and cheaper than other huge laboratory instrument to everyone, without limitations among users [27].

Researchers recently approve the possibility of using smartphone in bioanalytical sciences including smartphone colorimetric reader [28]. We therefore constructed a smartphone based immunosensor coupled with nanoflower signal amplification for rapid detection of *Salmonella* Enteritidis in dairy foods and water. We conjugated the streptavidin magnetic beads with biotin labeled antibody and combined them with the prepared enzyme-inorganic calcium nanoflower composite. The combination of the magnetic-antibody and the nanocomposite facilitates the specific capture of the antigen, i.e. pathogenic bacteria, and enhances the enzymatic activity and stability for producing an amplified signal that could be detected and quantified by the developed smartphone based device. Besides using the magnetic-antibody based system for immobilizing capture antibody for target *Salmonella* detection, for the detection method, we took advantage of the 3D printed integrated smartphone based device for optical detection. Instead of using the microplate reader which is bulky, expensive and heavy, the platform we utilized is more handy, low cost and can be used for onsite detection. Comparing with the previous paper using the three-in-one nanoflower for pathogen detection, the method and platform we used are novel and reliable results can be obtained from this platform. Our data indicated that this approach resulted in a highly sensitive detection method, which is minimally affected by other components in the food.

2. Material and methods

2.1. Chemicals and materials

Salmonella Enteritidis PT30 was from American Tissue Culture Collection (ATCC, Manassas, VA). Mouse monoclonal anti-*Salmonella* Enteritidis antibody was purchased from Abcam (Cambridge, MA). Goat polyclonal biotin-labeled anti- *Salmonella* antibody was purchased from KPL Inc. (Gaithersburg, MD). Streptavidin magnetic beads were purchased from ThermoFisher Scientific Inc. (Eugene, OR, USA). Horseradish peroxidase (HRP), dibasic sodium phosphate (Na_2HPO_4), monobasic sodium phosphate (NaH_2PO_4), calcium chloride anhydrous ($CaCl_2$), 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for ELISA, bovine serum albumin (BSA), phosphate-buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C), Tween 20 and Greiner 96-well V-bottom plates were purchased from Sigma-Aldrich (St. Louis, Mo, USA).

2.2. S. *enteritidis* preparation

S. Enteritidis PT30 (ATCC BAA-1045) was maintained at −80 °C in Trypticase Soy Broth (Becton, Dickinson and Company, Sparks, MD) supplied with 0.6% Yeast Extract (Fisher Scientific, Fair Lawn, NJ) (TSBYE) and 15% (v/v) glycerol. Bacteria were first activated in TSBYE at 37 °C for 8 h statically, 1:1000 transferred to TSBYE, and incubated at 37 °C statically for additional 14 h. The overnight culture was washed once and re-suspend in sterile PBS (pH7.4). The resulting bacterial suspension was serially diluted to appropriate concentrations for live cell experiment. For the dead cell experiment, the bacterial suspension was heat-inactivated in boiling water for 30 min followed addition of formalin (J.T. Baker, Phillipsburg, NJ) to a final concentration of 0.5% (v/v).

2.3. Preparation of magnetic- antibody and antibody-enzyme-inorganic nanoflowers

To synthesize streptavidin magnetic beads- biotin labeled antibody conjugate, Goat polyclonal biotin-labeled anti- *Salmonella* antibody (1 μg/mL) was added to streptavidin magnetic beads (50 μg/ ml) at a ratio of 1:1, mixed thoroughly and then incubated at room temperature for 2 h in a shaker incubator (Barnstead/Lab-Line Instrument, USA).

To synthesize antibody-enzyme-inorganic nanoflowers, 15 μL of 200 mM NaH_2PO_4, 15 μL 200 mM Na_2HPO_4, 29.5 μL HRP (10 mg/mL), 5 μL of monoclonal anti- *S.* Enteritidis antibody were added to 920 μL distill water. Then, 20 μL of 200 mM $CaCl_2$ was added to the solution. Mix and incubate at room temperature for 12 h. The products (synthesized nanoflowers) were obtained by centrifugation at 10 000 rpm for 5 min and washing twice with distill water.

The synthesized nanoflowers were dried on a filter membrane (pore size: 0.1 μm) and then sputter coated with gold for SEM observation. For TEM observation, the synthesized nanoflowers were dried on a carbon grid at room temperature.

2.4. Sandwich magnetic-antibody and three-in-one nanoflower-based ELISA of S. *Enteritidis*

On a 96 well plate that is fitted firmly over a 96 well plate magnetic separator, add 50 μL of conjugated streptavidin magnetic beads and biotin labeled anti-*Salmonella* antibody followed by addition of 200 μL of PBSA (1% BSA in 0.01 M PBS) and incubated at 37 °C for 1.5 h. The corresponding wells of plate on its 96 well plate magnetic separator was aspirated and washed with 300 μL of PBST (0.05% Tween 20 in 0.01 M PBS) four to five times to remove unbounded antibodies. Then 50 μL of *S.* Enteritidis PT30 diluted in PBS at different concentrations was added to each well and incubated at 37 °C for 1 h, then followed by washing for four to five times. Afterwards, 50 μL of antibody-enzyme-inorganic nanoflowers diluted five times was added to each well and incubated at 37 °C for 40 min. The plate was washed five times with PBST to