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Scano-magneto immunoassay based on carbon nanotubes/gold nanoparticles nanocomposite for *Salmonella enterica* serovar Typhimurium detection

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ABSTRACT

To improve sensitivity of *S. enterica* serovar Typhimurium detection, multiwalled carbon nanotubes (MWCNTs) and gold nanoparticles (AuNPs) were combined and used as a label to amplify signal in a scanometric based assay. In this study, the MWCNTs/AuNPs nanocomposite was fabricated by directly assemble of Au^{3+} to MWCNTs and allowed growing of AuNPs along the MWCNTs surface. This MWCNTs/AuNPs nanocomposite was then attached to anti-*S. typhimurium* antibody (MWCNTs/AuNPs/Ab_1) and used as a detecting molecule. Upon binding to *Salmonella,* they were pre-concentrated by magenetic beads/antibody (MBs/Ab_2) forming a sandwich immuno-complex which is later spotted on a nitrocellulose membrane coated slide. Silver reduction was applied to amplify signal. The detection limit of 42 CFU/ml was achieved when 2% BSA was used as a blocking agent. Given different types of real samples testing, chicken broth was found to give lowest detection limit, followed by orange juice low fat and whole milk. Selectivity testing was performed by using *Escherichia coli* as interference and found slightly cross-reactivity which could be due to specificity of the Ab used. By virtue of using a slide for multi-samples spotting and a flatbed scanner for signal-read out acquisition, this scano-magneto immunoassay could enable low-cost detection as well as high throughput screening.

1. Introduction

Gram negative bacteria are often a major cause of foodborne illness outbreak worldwide. *Salmonella spp*., in particular, always came in the first rank because they inhabit in the gastrointestinal track of animals or animal feeds which can easily be transmitted to human through undercooked meat, dairy products, vegetables and fruits (Maciorowski et al., 2006). *Salmonella spp*. can cause serious health threatening diseases and can eventually lead to death. Several methods employed to detect *Salmonella spp*. include conventional biochemical method (De Zutter et al., 1991; Huang et al., 1999; Kumar et al., 2008a, 2008b), enzyme link immunosorbent assay (ELISA) (Fukuda et al., 2005; Jouy et al., 2005), polymerase chain reaction (PCR) (de Freitas et al., 2010; Ellingson et al., 2004; Kawasaki et al., 2009; Pathmanathan et al., 2003), and biosensors (Fan et al., 2005; Gehring et al., 1996; Guntupalli et al., 2007; Ivnitski et al., 1999; Liébana et al., 2009; Liu et al., 2001, 2008; Taylor et al., 2006; Zhang et al., 2005).

Typically, the immunoassay is based on the specific interaction between antigen and antibody. In the early day, most immunoassay relies on ELISA where signal read out is easily monitored through the color change of enzymatic product. In general, ELISA possesses sensitivity in pM range (Gupta et al., 2007). It may be, however, insufficient for the determination of pathogenic bacteria in food. Meanwhile, shorter assay time and low cost are also desirable. Mirkin and coworkers have introduced a scanometric assay to overcome these pitfalls. In this case, the enzymes were replaced with gold nanoparticles for signal amplification, experiment was miniaturized on a glass slide and a flatbed scanner was used as a signal read-out instrument (Taton et al., 2000). This scanometric assay has been successfully applied for both DNA and protein detection (Gupta et al., 2007; Hill et al., 2007; Jaffrezic-Renault et al., 2007; Kim et al., 2009; Lei and Butt, 2010; Lia et al., 2010).

To achieve high assay sensitivity, both carbon nanotubes (CNTs) and gold nanoparticles (AuNPs) have been used and are gaining popularity as a label because of their high surface to volume ratios and their chemical, electrical and optical properties (Ambrosi et al., 2009; Bi et al., 2009; Cao et al., 2005; Chunglok et al., 2011; Dai et al., 2002; Elkin et al., 2005; Gupta et al., 2007; Merkoçi et al., 2005; Sanvicens et al., 2009; Sinha et al., 2006; Wang 2005; Yang et al., 2008; Yun et al., 2007). A variety of

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immunoassay based on CNTs has been proposed by using CNT itself (Lee et al., 2011; Qi et al., 2011; Villamizar et al., 2008), or CNTs coupled with enzymes (Bi et al., 2009; Chunglok et al., 2011; Liu et al., 2011; Piao et al., 2011), conducting polymer (Villamizar et al., 2008; Wang et al., 2010), redox mediator (Chen et al., 2009; Jiang et al., 2010; Qiu et al., 2009; Zhang et al., 2012) and metal particles (Lerner et al., 2011; Zarei et al., 2011) to enhance assay sensitivity. Recently, combination of CNTs and AuNPs has provoked great interest in the area of sensors with respect to the combination of their unique properties. Typically, either direct or indirect method has been used to deposit AuNPs to CNTs (Zhang et al., 2009). The CNTs/AuNPs nanocomposite was successfully exploited to amplify electrochemical signal for detection of a variety of analytes (Chauhan and Pundir, 2011; Du et al., 2010; Huang et al., 2011; Lin et al., 2009; Mubeen et al., 2009; Pundir et al., 2011; Xiao et al., 2007). Herein, we wish to present a simple and sensitive method to detect *Salmonella enterica* serovar Typhimurium which combines unique properties of CNTs, AuNPs and magnetic bead (MBs) and specificity of antibody to construct a new type of immunoassay based on scanometric detection. To the best of our knowledge, such platform has yet been reported. AuNPs were attached on the surface of multiwalled carbon nanotubes (MWCNTs) forming a MWCNTs/AuNPs nanocomposite where antibody (Ab) was attached to. The high surface areas of MWCNTs would allow a large number of AuNPs and Ab depositions which could results in improvement of the assay sensitivity whereas MBs aided the separation process. Signal amplification was finally carried out with silver reduction.

2. Materials and methods

2.1. Reagents

Goat anti *Salmonella* (Ab_1) and goat anti *Salmonella* functionalized with biotin (Ab_2) were purchased from Genway (USA), Multiwalled carbon nanotubes (MWCNTs) carboxylic acid functionalized (MWCNTs-COOH) was purchased from Cheap Tube Inc, (USA). Streptavidin functionalized magenetic beads were from Invitrogen (USA). Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$), sodium citrate dihydrate, silver enhancer solution (A and B) and *N*-hydroxysuccinimide (NHS) were obtained from Sigma–Aldrich (USA). 1-ethyl-3(-3dimethylaminopropyl carbodimide (EDC) was from Fluka (Switzerland). All reagents were of analytical grade and were used as received. Solutions were prepared with Milli-*Q* water.

2.2. Instruments

Field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) analyses were conducted on a Hitachi S-4700 system and a JOEL model JAM-2100, respectively.

2.3. Preparation of multiwall carbon nanotubes/gold nanoparticles (MWCNTs/AuNPs) nanocomposite

Three milligrams of multiwall carbon nanotubes (MWCNTs) was added into 4 ml of sodium citrate solution (1% w/v). The mixture was ultrasonicated for 5 min before being transferred to a flask containing 96 ml of milli-*Q* water. It was then stirred and heated to the boiling point. An aliquot of 0.5 ml of $HAuCl_4$ (1% w/v) was added and kept boiling for 5 min. After that the MWCNTs/AuNPs nanocomposites were separated from the solution by membrane filter and washed 3 times with milli-*Q* water. The MWCNTs/AuNPs nanocomposites were then resuspended in 500 μL of milli-*Q* water for further uses.

2.4. Conjugation of antibody to MWCNTs/AuNPs nanocomposite by covalent attachment

One milliliter of MWCNTs/AuNPs nanocomposite (2 mg/ml in phosphate buffer (PB), pH 7.0) was mixed with 1 ml of EDC and NHS at 400 mM and 100 mM, respectively, in the same buffer and rolling at RT for 2 h to activate carboxylic acid groups on the surface of MWCNTs. Then, the nanocomposite was washed by centrifugation at 10,000 rpm for 5 min and the pellet was resuspended in PB, pH 7.4. Ab_1 was added to the final concentration of 100 ng/ml and the mixture was shaken overnight at 4 °C. The MWCNTs/AuNPs/Ab_1 was separated out from the solution by centrifugation and washed 5 times with PB, pH 7.4. The pellet was dispersed in the same buffer and stored at 4 °C until used.

2.5. Immunoassay of Salmonella enterica serovar Typhimurium

2.5.1. Preparation of reaction I (Magentic beads/Ab_2 complex: MBs/Ab_2)

Ten μl of streptavidin functionalized MBs (0.1 mg/ml) were mixed with 100 ng/ml of biotinylated antibody (Ab_2) in PBS pH 7.4. The mixture was incubated by rolling for 1 h at RT and washed 3 times with PBS pH 7.4+0.05%v/v Tween 20 (PBST). MBs/Ab_2 was resuspended in 50 μl of PBST pH 7.4.

2.5.2. Preparation of reaction II (MWCNTs/AuNPs/Ab_1 complexed with Salmonella)

Heat-killed *S. enterica* serovar Typhimurium was 10-fold serially diluted in 0.125 mg/ml of MWCNTs/AuNPs/Ab_1 in PBST pH 7.4. The mixture was incubated by rolling for 1 h at RT and washed 3 times with PBST pH 7.4. The complex was resuspended in 50 μl of PBST pH 7.4.

2.5.3. Immunoassay

Reaction I and II were mixed together in PBST pH 7.4 for 1 h at RT. The whole complex was then separated out from the solution under a magnetic field and washed 3 times with milli-*Q* water. After that, it was resuspended in 10 μl milli-*Q* water and 1 μl-aliquot was spotted onto a nitrocellulose membrane coated slide. Silver enhancer solution was then added onto the slide and incubated for 5 min. Afterwards, the slide was washed with milli-*Q* water and scanned by using a flatbed scanner. The obtained image was analyzed by an image processing software ImageJ. Graphpad Prism software was used to analyze data by fitting to a dose-response curve equation:

$$\%\frac{B}{B_0} = \left(B_0 + \frac{B - B_0}{1 + 10^{\log EC50 - x}}\right) x100$$

where B and B_0 were intensities of spots in the presence (B) and absence (B_0) of *S. enterica* serovar Typhimurium and EC50 was the concentration of bacteria that gives half-way response between B and B_0. From the sigmoid plot, the limit of detection (LOD) was then interpolated based on background signal plus 3 standard deviations (3SD).

2.6. Real samples detection

Commercial UHT whole and low fat milk and freshly squeezed orange juice were spiked with heat-killed *S. enterica* serovar Typhimurium, *Escherichia coli* and the mix of both to obtain the final concentration of 10^7 CFU/ml. For chicken breast sample, 25 g of finely chopped chicken breast was mixed with 225 ml of PBS