Biosensors and Bioelectronics 26 (2011) 3584–3589

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Immunoassay based on carbon nanotubes-enhanced ELISA for *Salmonella enterica* serovar *Typhimurium*

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ARTICLE INFO

Article history:
Received 30 December 2010
Received in revised form 1 February 2011
Accepted 4 February 2011
Available online 16 February 2011

Keywords:
Carbon nanotubes
Immunoassay
ELISA
Salmonella enterica serovar *Typhimurium*

ABSTRACT

Among the methods used to detect pathogenic bacteria, enzyme linked immunosorbent assay (ELISA) is one of the most widely used techniques in routine sample analysis. For *Salmonella enterica* serovar *Typhimurium* detection, a typical ELISA yields a sensitivity of 10^6–10^7 CFU/ml. To enhance the detection sensitivity, single-walled carbon nanotubes (SWCNTs) was employed in this study as a labelling platform for antibody and horseradish peroxidase (HRP) co-immobilizing. With high proteins recovery after the coupling process, the resulting Ab/SWCNTs/HRP bioconjugate was used in the proof-of-concept ELISA experiments. Limit of detection (LOD) was found to be 10^3 and 10^4 CFU/ml for direct and sandwich ELISA, respectively, when Ab/HRP at 1:400 ratio was used. This figure accounts for 1000-time greater in detection sensitivity when compared to a commercial Ab-HRP conjugate. The Ab/SWCNTs/HRP bioconjugate was tested further in real samples and found a superior activity over the commercial Ab-HRP by showing 100-time greater detection limit.

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1. Introduction

Salmonella enterica serovar *Typhimurium* have long time been recognized as a serious life threatening pathogen. To detect the existence of *S. enterica* serovar *Typhimurium*, several methods have been employed including polymerase chain reaction (PCR) (Courtney et al., 2006; de Freitas et al., 2010; Kumar et al., 2008b), real-time PCR (Ellingson et al., 2004; Perelle et al., 2004), surface plasmon resonance (Jongerius-Gortemaker et al., 2002; Mazumdar et al., 2007; Oh et al., 2004) and enzyme-linked immunosorbent assay (ELISA) (Huang et al., 1999; Kumar et al., 2008a; Lazcka et al., 2007; Poucke, 1990; Tan et al., 1997; Tsen et al., 1994). Among these well-known techniques, ELISA has been widely used for routine analysis due to its ease of use, ability to handle a large number of samples and automation. However, despite having good merits, a better sensitivity of the ELISA is still required for the pathogen detection.

Several attempts were made to lowering detection limits for *Salmonella* spp. based on ELISA method. For example, paramagnetic bead coated antibody was used in combination with ELISA (IMS-ELISA) with aiming to pre-concentrate cells from mixed cultures (Mansfield and Forsythe, 2000). Nevertheless, the detection sensitivity was considered close to that of a conventional ELISA (10^{-5}–10^{-6} CFU/ml). In addition, the firefly luciferase was also used to replace enzyme in a technique called bioluminescent enzyme immunoassay (BEIA) (Fukuda et al., 2005). In this case, in order to lower the detection limit, a specific type of culture media must be used. Similarly, a chemiluminescent-based ELISA system was exploited in an array format to detect foodborne pathogens, though detection sensitivity was as low as the conventional ELISA (Karoonuthaisiri et al., 2009). Besides, the assay performance was pushed further through the combination between PCR and ELISA (PCR-ELISA) and that gave a comparable signal to a light cycler PCR (LC-PCR) method (Perelle et al., 2004).

Alternatively, the pathogen detection sensitivity could be improved through the use of nanomaterials as described elsewhere (Yang et al., 2008a). Carbon nanotubes (CNTs), in particular, have become one of the most promising nanomaterials to be used in biodiagnostic applications (Katz and Willner, 2004; Lin et al., 2009; Lynama et al., 2009; Veetil and Ye, 2007; Yang et al., 2008a, 2007). Unlike other nanomaterials, CNTs have a large surface area to volume ratio which makes them an ideal candidate for immobilization applications. Additionally, the ability of CNTs to be easily

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doi:10.1016/j.bios.2011.02.005

introduced new functional groups such as carboxyl and amine has extended another dimension to their uses by being able to integrate with biological elements. On the other hand, these biomolecules can be coupled with CNTs directly with no further modification required. In this respect, the use of CNTs as a carrier to incorporate biomolecules such as enzymes and antibodies to use in bioanalytical applications could help promoting signal and therefore detection sensitivity. This approach has been successfully applied in several applications to enhance assay sensitivity both in electrochemical (Wang et al., 2004; Yu et al., 2006) and optical biosensors (Bi et al., 2009; Yang et al., 2008b,c).

In this study, we wish to provoke on the use of CNTs as a labelling platform to enhance detection sensitivity of *S. enterica* serovar *Typhimurium* in ELISA. Single-walled CNTs (SWCNTs) were used to co-immobilize antibody to *S. enterica* serovar *Typhimurium* (Ab) and horseradish peroxidase (HRP). The sensitivity of the resulting bioconjugate (Ab/SWCNTs/HRP) was compared with a commercial HRP-labelled antibody (Ab-HRP) through direct and sandwich ELISA.

2. Materials and methods

2.1. Instruments

Maxisorp 96 well ELISA microplates were from Nunc (Thermo Fisher Scientific, Denmark). The spectrophotometric measurements were performed using an Infinite® 200 Pro Multi-Mode Microplate Reader (Tecan, Austria). Field emission scanning electron microscopy (FESEM) analysis was conducted on a Hitachi S-4700 system (Japan).

2.2. Reagents and buffers

Single-walled carbon nanotubes functionalized with carboxylic acid (SWCNTs-COOH, purity > 90%, diameter 1–2 nm, length 0.5–2.0 μm) was purchased from Cheap Tubes Inc. Co. Ltd. (USA). Rabbit anti *Salmonella* sp. with and without horseradish peroxidase conjugated and goat anti rabbit IgG (H&L) horseradish peroxidase conjugated were purchased from Biodesign International (USA). Peroxidase type II from horseradish and 2-(N-Morpholino)ethanesulfonic acid (MES) were purchased from Sigma–Aldrich (USA). 3,3′,5,5′-tetramethylbenzidine (TMB) was from Invitrogen (USA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC) was from Fluka (USA). All buffers and other chemicals were obtained from Sigma–Aldrich and used without further purification. Milli-Q water and doubly distilled water were used throughout the experiment.

Carbonate/bicarbonate buffer pH 9.6 was used as coating buffer. Phosphate buffered saline (PBS) pH 7.4 containing 2% (w/v) BSA was used as a blocking buffer and that containing 0.05% (v/v) Tween 20 (PBST) was used as a washing buffer.

2.3. Preparation of Ab/SWCNTs/HRP bioconjugate

Ab/SWCNTs/HRP bioconjugate was prepared according to Yu et al. and with a slight modification. A 1.5 mg of carboxylated single-walled carbon nanotubes (SWCNTs-COOH) was sonicated in 100 ml of 1 M NaOH solution for 5 min to achieve net negative charge carboxyl groups and then washed repeatedly with water until the pH of solution was around 7. After washing, the SWCNTs-COOH was mixed with 1 ml of 200 mM EDAC in MES buffer (pH 6.0). The mixture was sonicated for 10 min at room temperature and centrifuged at 10,000 rpm for 5 min. The pellet was then redispersed in 1 ml of MES buffer (pH 6.0). The rabbit anti *Salmonella* antibody (Ab) and HRP were then added into the reaction mixture using different combinations i.e. 1:200, 1:400 and 1:800 (used as microgram of Ab or HRP per milligram of SWCNTs-COOH) by adding HRP 30 min following the Ab. The reaction mixture was stirred for 2 h at room temperature and centrifuged at 10,000 rpm for 10 min. The pellet was washed 3 times with PBS containing 0.05% Tween-20 (PBST) and kept in PBS (pH 7.4) containing 1% (w/v) BSA at 4 °C. The Ab/SWCNTs/HRP bioconjugate was diluted with PBS buffer containing 0.05% (w/v) Tween-20 and 1% (w/v) BSA before use.

2.4. Determination of S. typhimurium using direct ELISA

A 100 μl aliquot of various concentrations of heat-killed *S. enterica* serovar *Typhimurium* in carbonate coating buffer were added into polystyrene microplate and incubated at 4 °C overnight. After washing 3 times with PBST, the plate was blocked with 100 μl of PBS (pH 7.4) containing 2% (w/v) BSA and incubated for 1 h at room temperature, followed by washing 3 times with PBST. Then, either Ab/SWCNTs/HRP bioconjugate or commercial antibody (HRP conjugated rabbit anti *S. enterica* serovar *Typhimurium*, Ab-HRP) in PBST containing 1% BSA was added and incubated for 2 h at room temperature. After washing with PBST, a 100 μl aliquot of TMB substrate was added into each well and incubated for 10 min. The reaction was stopped by adding 50 μl of 0.5 M H_2SO_4 and the absorbance of the reaction was measured at 450 nm with a microplate reader. The schematic diagram of direct ELISA is shown in Fig. 1.

2.5. Determination of S. enterica serovar Typhimurium using sandwich ELISA

A 96-well polystyrene microplate was first coated with rabbit anti-*Salmonella* antibody overnight at 4 °C. After washing 3 times with PBST, the plate was blocked with 100 μl of blocking buffer and left incubated at room temperature for 1 h. The washing step was repeated. Then, a 100 μl aliquot of various 10-fold serial dilutions of heat-killed *S. enterica* serovar *Typhimurium* in PBST containing 1% BSA was added into each well and left incubated for 2 h at room temperature. The plate was washed 3 times with PBST. Afterwards, either Ab/SWCNTs/HRP bioconjugate or Ab-HRP in PBST containing 1% BSA was added and incubated for 2 h. After washing with PBST, the TMB substrate was added into each well and the remaining steps were carried out as in the direct ELISA. The schematic diagram of sandwich ELISA is shown in Fig. 1.

2.6. Detection of S. enterica serovar Typhimurium in food sample

Commercial UHT milk, fat-free (0% fat) and plain milk (9% fat), was spiked with heat-killed *S. enterica* serovar *Typhimurium*. For direct ELISA, a 100 μl aliquot of spiked UHT milk serially diluted with coating buffer was added into polystyrene microplate and incubated at 4 °C overnight. After washing, the plate was blocked with blocking buffer and the remaining step was carried out as in direct ELISA. For sandwich ELISA, serially diluted spiked UHT milk in PBST + 1% BSA was added into the polystyrene microplate coated with rabbit anti-*Salmonella* antibody and incubated at room temperature for 1 h. After washing, the remaining process was carried out as aforementioned.

3. Results and discussion

In this work, we propose to replace the HRP labelled Ab (Ab-HRP) used in a typical ELISA with an Ab/SWCNTs/HRP bioconjugate where SWCNTs acts as carriers to accommodate more HRP loading per Ab. The combination of HRP with Ab and SWCNTs could provide a mean of improving sensitivity by a simple co-immobilization process that could overcome the single enzyme labelled used in a

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