Biosensors and Bioelectronics 64 (2015) 69–73

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Highly sensitive detection of a bio-threat pathogen by gold nanoparticle-based oligonucleotide-linked immunosorbent assay

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ARTICLE INFO

Article history:
Received 25 May 2014
Received in revised form
7 August 2014
Accepted 19 August 2014
Available online 23 August 2014

Keywords:
Francisella tularensis
Bacteria
ELISA
OLISA
Detection sensitivity
Gold-nanoparticle

ABSTRACT

Francisella (*F.*) *tularensis* causes the zoonotic disease tularemia and categorized as one of the highest-priority biological agents. The sensing approaches utilized by conventional detection methods, including enzyme-linked immunosorbent assay (ELISA), are not sensitive enough to identify an infectious dose of this high-risk pathogen due to its low infective dose. As an attempt to detect *F. tularensis* with high sensitivity, we utilized the highly sensitive immunoassay system named gold nanoparticle-based oligonucleotide-linked immunosorbent assay (GNP-OLISA) which uses antibody-gold nanoparticles conjugated with DNA strands as a signal generator and RNA oligonucleotides appended with a fluorophore as a quencher for signal amplification. We modified the GNP-OLISA for the detection *F. tularensis* to utilize one antibody for both the capture of the target and for signal generation instead of using two different antibodies, which are usually employed to construct the antibody sandwich in the ELISA. The GNP-OLISA showed 37-fold higher sensitivity compared with ELISA and generated very consistent detection results in the sera. In addition, the detection specificity was not affected by the presence of non-target bacteria, suggesting that GNP-OLISA can be used as a sensitive detection platform for monitoring high-risk pathogens thereby overcoming the limit of the conventional assay system.
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1. Introduction

Francisella (*F.*) *tularensis*, the causative agent of the zoonotic disease tularemia, is categorized as one of the highest-priority biological agents of concern by the U.S. Center for Disease Control and Prevention (CDC) because it has a very low infectious dose (10 colony-forming units (CFU) of *F. tularensis*) (Ellis et al., 2002; Froude et al., 2011; Franz et al., 2001). Vaccination is the most efficient weapon against *F. tularensis* infection, but no vaccine has been officially approved by the U.S. Food and Drug Administration to date. Treatment of patients with proper antibiotics during the early stages of infection and decontamination of pathogen-containing environments with selected agents are the only ways to the control outbreak to the disease and to reduce risk of public health. Identification of *F. tularensis* bacteria in biological and environmental samples is essential for efficient clinical treatment and appropriate decontamination by public health official. Hence, a sensitive and specific detection method to detect tiny amounts of *F. tularensis* is required.

The conventional detection method for *F. tularensis*is requires at least three days of bacterial cultivation and identification using specific media (Pohanka et al., 2008). As an alternative, real-time polymerase chain reaction (RT-PCR) has been utilized due to its selectivity, speed, and sensitivity. However, the limit of detection (LOD) using RT-PCR is 10^3 CFU/mL for *F. tularensis* and it does not meet clinical demand (Dauphin et al., 2011). In addition, RT-PCR can generate false-positive signals if contaminated or false-negative signals if the target gene is mutated or if the sample contains *Taq* polymerase inhibitors (Alaeddini, 2012). ELISA is another widely used detection method; however, ELISA has a detection sensitivity of only 10^3–10^4 CFU/mL for *F. tularensis* (Grounow et al., 2000).

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http://dx.doi.org/10.1016/j.bios.2014.08.038
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Several studies have reported higher detection sensitivity for bacterial pathogens using ELISA techniques, but there are additional steps and accessories required. Immuno-magnetic microbeads (Liu et al., 2001), electrode-based technologies (Liebana et al., 2009), and polyacrylonitrile coated with antibody (Chattopadhyay et al., 2013) have all been suggested as alternative methods for rapid detection of pathogens. Single-walled carbon nanotubes conjugated with horseradish peroxidase (HRP) have also been reported to be used as a labeling platform in ELISA for the sensitive detection of pathogen (Chunglok et al., 2011). Since the detection sensitivity of conventional methods is not sufficient to allow for the detection of the bacteria and diagnosis of infected patients in the early stage; therefore, improvement upon conventional methods or development of a novel sensing method with high sensitivity is urgently needed.

Previously, we introduced a novel detection system called oligonucleotide-linked immunosorbent assay (OLISA). Instead of HRP producing the absorbance signal as in ELISA, OLISA uses DNA strands to generate the fluorescent detection signal (Han et al., 2013). In addition, we found that gold nanoparticles coated with multiple strands of the signal generator lead to additional amplification of the fluorescence signal. We also demonstrated that this gold nanoparticle-based oligonucleotide-linked immunosorbent assay (GNP-OLISA) could be used for quantitative analysis of cancer biomarkers with 70 to 100-fold higher detection sensitivity than ELISA (Han et al., 2012). In the present study, we modified the GNP-OLISA procedure to utilize a single antibody instead of the antibody sandwich requiring two different antibodies to detect a bio-threat bacterial strain, *F. tularensis.* The adjusted GNP-OLISA was used for detection of the bacteria not only in buffer but also in rabbit serum samples, which closely approximate clinical samples. The performance of GNP-OLISA was compared with ELISA to evaluate the detection sensitivity of GNP-OLISA. Finally, the specificity of the assay was examined by detecting the target bacteria in the presence of other non-target bacterial species a competitors.

2. Materials and methods

2.1. Bacteria

F. tularensis (subspecies *holarctica* live vaccine strain) was cultured in 250 mL of LB broth containing IsoVitaleX (BD Biosciences, USA) or brain heart infusion broth (BD Bioscience, USA) for 72 h at 37 °C with shaking (200 rpm). To determine the CFUs of *F. tularensis* present, bacteria were diluted with phosphate buffed saline (PBS) and spread onto chocolate agar plates (HANIL, Korea). Plates were incubated at 37 °C for 72 h and colonies were counted. *Bacillus anthracis* (Sterne) was cultured in 25 mL of Leighton-Doi broth at 37 °C for 24 h with shaking (200 rpm). Stock preparation and calculations were performed as above, except that LB media instead of chocolate agar plates was used.

2.2. Preparation of F. tularensis LPS

After culturing *F. tularensis*, the lipopolysaccharide (LPS) on the surface of *F. tularensis* was extracted and purified using a commercially available kit following the manufacturer′s recommendation (Intron Biotechnology, Korea).

2.3. ELISA

PBS-diluted *F. tularensis* LPS, *F. tularensis*, or PBS were used to coat a 96-well plate (Nunc, Denmark), which was incubated at room temperature for 1 h. The plate was washed with 300 μL of PBS containing 0.05% Tween-20 3 times and 200 μL of 1% BSA in PBS was then added. After 1-h incubation at room temperature, the plate was washed and 100 μL of anti-*F. tularensis* LPS antibody (Abcam, ab2033, UK) was added to the wells and incubated for 1 h. After washing, 100 μL of HRP-conjugated rat anti-mouse IgG2a antibody was added to each well and the plate was incubated for another hour. After rinsing with PBS containing 0.05% Tween-20, 100 μL of TMB solution was added and the development reactions were stopped using 100 μL of 2 N H_2SO_4 solution. The optical density of each well was measured at 450 nm using a SUNRISE absorbance plate reader (TECAN, Austria). For fluorescence-based ELISA, 20 μL of Ampliflu™ Red (10 mM, Sigma-Aldrich, USA) was added instead of the TMB solution. Fluorescence intensity was measured using a SpectraMax® M2e (Molecular Devices, USA), with excitation at 530 nm and emission at 585 nm, respectively.

2.4. Preparation of Ab-GNP-DNA complex

An Ab-GNP-DNA probe complex was prepared as described previously (Han et al., 2012). Briefly, 2 mL of 10-nm gold colloid (British Biocell International, UK) was incubated for 15 min at room temperature with 20 μg anti-*F. tularensis* LPS (Abcam, ab2033, UK). After adding 5 μL of 10% Tween-20, 50 μL of 50 μM 5′-thiolated DNA oligonucleotide probe (5′-AACCACAGTG-3′, Bioneer, Korea) and 20 μL of 0.5 M phosphate buffer, pH 8.0, the solution was incubated overnight at 4°C. Then, the solution was salted by adding 30 μL of 5 M NaCl, and incubated at room temperature for 1 h. The Ab-GNP-DNA probe was stabilized by adding 500 μL of 1% BSA. The solution was centrifuged for 10 min at 18,000*g*, the supernatant was removed, and the pellet was resuspended with 1 mL of 1% BSA in PBS. All incubation procedures were conducted under gentle agitation.

2.5. Transmission electron microscope (TEM) analysis

To prepare the negative-staining control samples, 5 μL of emulsion (*F. tularensis*+Ab-GNP-DNA probe) were applied to a formvar-coated 200 mesh copper grid for 1 min; the emulsion was then removed using filter paper. The grid was allowed to dry for 10 min and then stained with 2% uranyl acetate for 10 s; the staining solution was removed using filter paper and dried for a further 10 min. To make microtome sections of each sample, pellets (*F. tularensis*+Ab-GNP-DNA probe) were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer at 4 °C for 2 h, washed with 0.1 M phosphate buffer (3 times), and fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h at 4 °C. After washing, the pellets were dehydrated by soaking with 50%, 70%, 80%, 90%, and 95% ethanol, sequentially, for 5 min (2 times each concentration) and 100% ethanol for 10 min (3 times). The residual ethanol in the pellets was removed by rinsing with propylene oxide for 20 min (3 times). For embedment, the pellets were dipped in prophylene-Epon 812 solutions (prophylene:Epon=3:1 and 1:1 for 30 min; prophylene:Epon=1:2 overnight) and pure Epon 812 resin for 2 h. The sample was moved to a silicon plate mold and polymerized in an oven at 60 °C for 48 h incubation. The sample was then trimmed and sectioned using ultra microtome (UC7, Leica Microsystems, Germany). The sectioned sample was moved onto 100 mesh copper grid and double stained with 2% uranyl acetate and lead citrate. Both of the negative stained and sectioned samples were analyzed using transmission electron microscope (Libra 120, Carl Zeiss, Germany) at 120 kV.

2.6. GNP-OLISA

GNP-OLISAs were performed using a protocol modified from our previous report (Han et al., 2012). Briefly, *F. tularensis* LPS and

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