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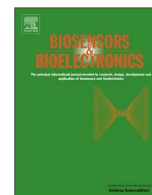

An easy and sensitive sandwich assay for detection of *Mycobacterium tuberculosis* Ag85B antigen using quantum dots and gold nanorods

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ABSTRACT

Mycobacterium tuberculosis is a serious global infectious pathogen causing tuberculosis (TB). The development of an easy and sensitive method for the detection of *M. tuberculosis* is in urgent need due to complex and low specificity of the current assays. Herein, we present a novel method for *M. tuberculosis* detection based on a sandwich assay via antigen-antibody interaction using silica-coated quantum dots (SiQDs) and gold nanorods (AuNRs). A genetically engineered recombinant antibody (GBP-50B14 and SiBP-8B3) was bound to surfaces of AuNRs and SiQDs respectively, without any surface modification. The antigen-antibody interaction was revealed using *M. tuberculosis*-specific secretory antigen, Ag85B. Two biocomplexes showed a quenching effect in the presence of the target antigen through a sandwich assay. The assay response was in the range of 1×10^{-3}–1×10^{-10} μg mL^{-1} (R=0.969) and the limit of detection for Ag85B was 13.0 pg mL^{-1}. The Ag85B was selectively detected using three different proteins (CFP10, and BSA), and further specifically confirmed by the use of spiked samples. Compared with existing methods, a highly sensitive and selective method for Ag85B-expressing *M. tuberculosis* detection has been developed for better diagnosis of TB.

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1. Introduction

Mycobacterium tuberculosis, the main causative agent of tuberculosis (TB), is responsible for more deaths per year than any other bacterial pathogen. According to the World Health Organization (WHO, Global *Tuberculosis* Control Report, 2014), about 9.6 million cases were reported in 2014 globally; of these, 1.5 million died from the disease, and 5.7 million were newly diagnosed. Therefore, there is a strong need for fast and precise diagnostic methods, with higher sensitivity and specificity than existing assays.

At present, a diagnosis of active TB requires confirmed clinical samples from patients and is based on bacteriological methods, such as tuberculin skin tests (TSTs) and interferon gamma release assays (IGRAs) (Wu et al., 2014). These tests are limited by low specificity, low sensitivity, and costly reagents. TST is not an appropriate method to diagnose active TB, as it only indicates whether the patient has been infected or not. Moreover, TST may provide false-positive and false-negative results in people who have been vaccinated with *Bacillus Calmette-Guerin*. Moreover, TST might give false-positive results for those who have been infected with TB in the past as their memory T cells still secrete interferon-γ. These methods are indirect measures of an induced immune reaction against antigen exposure by a recent infection and reinfection. Furthermore, TSTs and IGRAs cannot distinguish between latent TB, active TB, and complete recovery from TB. To overcome these shortcomings, we have focused on *M. tuberculosis* secretory proteins suitable for early detection of TB. One of these is Ag85B, a major secretory antigen from active *M. tuberculosis*.

The aim of this study was to use a combination of gold nanorods (AuNRs) and silica-coated quantum dots (SiQDs) to enhance the sensitivity of sandwich assays. AuNRs are excellent candidates for biosensors because they present an expandable visible spectrum and a larger surface area than gold nanoparticles (Nikoobakgt and El-Sayed, 2003; Sau and Murphy, 2004; Wang et al., 2010; Chen et al., 2011; Wang et al., 2011). Owing to their anisotropic rod shape, AuNRs are characterized by biocompatibility, chemical stability, and rapid electron transfer (Jorge et al.,

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2005; Vigderman et al., 2012; Vigderman and Zubarev, 2013; Huang et al., 2014). This makes them interesting for biomedical and plasmonic applications involving the study of nucleic acids, metal ions, amino acids, and proteins (Hu et al., 2006; Huang et al., 2008; 2009; Alkilany et al., 2012; Warren et al., 2012). Quantum dots (QDs) are small light-emitting particles of nanometer size. They represent a new class of fluorescent probes for biological and medical applications (Marcel et al., 1998; Sondi et al., 2000; Taylor et al., 2000; Mamedova and Kotov, 2001; Chan et al., 2002; Wang et al., 2002; Alivisatos, 2004; Pinaud et al., 2006). Compared with conventional organic dyes and fluorescent proteins, QDs have unique optical and electronic properties, such as high luminosity, simultaneous excitation of different fluorescent colors, and high stability against photobleaching (Marcel et al., 1998; Wu et al., 2003). The silica-shell coated QD forms a hybrid material (Rogach et al., 2000; Gerion et al., 2001). The silica nanoparticles are of great interest for their dimensional similarity to biomolecules (e.g., DNA and proteins) and for applications in biological imaging and bioconjugation.

AuNRs have been used previously in fluorescence-based assays for biological analysis, mostly through fluorescence resonance energy transfer (FRET). Accordingly, the excitation energy of the donor (SiQDs) is transferred to an acceptor (AuNRs) through an induced-dipole/induced-dipole interaction. FRET technology is very convenient and can be applied routinely at a single-molecule detection level. In addition, FRET is very sensitive due to the short distance between donor and acceptor (Maxwell et al., 2002; Lee et al., 2008; Griffin et al., 2009). Previously, a recombinant antibody immobilized on AuNRs was conjugated to SiQDs-SiBP-8B3, whereby 8B3 was another TB secretory antibody. When this complex was bound to Ag85B, FRET between SiQDs and AuNRs was observed and the intensity of SiQDs red fluorescence decreased (Nie and Emory, 1997; Krug et al., 1999).

Herein, we studied the bioconjugation of AuNRs and SiQDs with proteins to create complex structures. We report the preparation of bioconjugates of antigen and antibody to AuNRs and SiQDs of different size. Moreover, we demonstrated that such immunocomplexes can be used to improve the detection of *M. tuberculosis* in a sandwich assay via antigen-antibody interactions using AuNRs and SiQDs. This can be accomplished by taking advantages of the competitive inhibition of FRET in antigen-antibody immunocomplexes (Fig. 1). We have developed a highly sensitive, selective, specific, fast and economical assay for the detection of TB secretory antigen in infected individuals. To this end, we employed the secreted antigen Ag85B and fused recombinant antibodies GBP-50B14 and SiBP-8B3 for the detection of the active *M. tuberculosis*.

2. Materials and methods

2.1. Materials

Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), cadmium perchlorate hydrate ($Cd(ClO_4)_2$), sodium silicate ($Na_2O_7Si_3$), thioglycolic acid ($HSCH_2COOH$, TGA), bovine serum albumin (BSA), ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG) and phosphate-buffered saline (PBS, pH 7.4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-ascorbic acid (99.5%) and hydrochloric acid (HCl) were from Samchun Chemical (Pyeongtaek, Korea). The secretory antigen, Ag85B, from *M. tuberculosis* was obtained from Chungnam National University (Daejeon, Korea). Cetyltrimethylammonium bromide (CTAB), sodium borohydride ($NaBH_4$), and silver nitrate ($AgNO_3$) were purchased from Daejung Chemicals (Siheung, Korea). Sulfuric acid (98%) was from Junsei (Tokyo, Japan). (3-mercaptopropyl)trimethoxysilane ($HS(CH_2)_3Si(OCH_3)_3$, MPS, 95%) was purchased from Alfa Aesar (Ward Hill, MA, USA). Spectra/Por dialysis membrane (6000–8000 kDa molecular weight cut-off) was supplied by Spectrum Labs (Rancho Dominguez, CA, USA). Aluminum telluride (Al_2Te_3) was purchased from Advanced Chemical Company (Warwick, RI, USA). Deionized (DI) water at 18.2 MΩ cm was purified using a Milli-Q system (Millipore, Billerica, MA, USA). All chemicals were used without further purification.

2.2. Synthesis of AuNRs

AuNRs were synthesized by a chemical reduction process using a seeding growth method as previously reported (Huang et al., 2014). Briefly, gold seed particles were prepared by mixing 5 mL of 0.5 mM $HAuCl_4$ with 5 mL of 0.2 M CTAB, followed by addition of 0.6 mL of 10 mM ice-cold $NaBH_4$ and vigorous stirring. The seed solution was prepared at least 2 h prior to use. For AuNR synthesis, 18 mL of 5 mM $HAuCl_4$ and 180 μL of 0.1 M $AgNO_3$ were added to 90 mL of 0.2 M CTAB. Upon addition of 180 μL of 1.2 M HCl and 10.5 mL of 10 mM ascorbic acid the mixture was gently stirred as the color turned from dark orange to transparent. After the color change, 150 μL CTAB-stabilized gold seed solution was rapidly injected, gently mixed for 10 s, and left undisturbed overnight at 37 °C.

In order to change the size of AuNRs, we varied the seeding concentration of $AgNO_3$ (0.01–0.1 M in aqueous solution), since metal ions are important for the nanorod growth. To remove CTAB, the resulting solution was centrifuged three times at 15,110 × *g* for 10 min, and the pellet was resuspended in DI water. The color change of the resulting solution depended on the concentration of silver ions.

2.3. Synthesis of thiol-capped CdTe QDs and SiQDs

The aqueous synthesis of thiol-capped CdTe QDs followed previous reports (Gaponik et al., 2002; Rogach et al., 2007). Typically, 0.985 g (2.35 mM) $Cd(ClO_4)_2 \cdot 6H_2O$ was dissolved in 125 mL DI water inside a three-necked flask and 5.7 mM TGA, a thiol stabilizer, was added under stirring. To adjust the pH, 1.0 M NaOH was added drop by drop until a clear solution was obtained. TGA was replaced by CdTe nanocrystals by refluxing the reaction mixture at 100 °C under stirring and open-air conditions with a condenser attached. The mixture was placed in a three-necked flask and was deaerated by N_2 bubbling for 20 min. The tellurium precursor H_2Te was prepared by adding 15–20 mL of 0.5 M sulfuric acid dropwise to a 0.2 g (0.46 mM) lump of Al_2Te_3 under constant stirring, followed by N_2 bubbling for 20 min. The product was stored at 4 °C.

To prepare SiQDs (Wolcott et al., 2006), 1.5 mL MPS (48 mM in pure ethanol) was added to a 10 mL solution of CdTe QDs. To produce a homogeneous dispersion of MPS, the mixture was stirred during the 2 h required for surface priming. Next, the solution was dialyzed against 2.0 L DI water for 1 h. In order to ensure whole surface coverage, an additional 1.5 mL MPS was added and continued dialysis for 1 h. After surface priming, 2 mL sodium silicate was added to the solution under stirring to begin silica polymerization around the surface of the particles. This was continued for 12 h to ensure shell growth around CdTe QDs. To potentialize silica shells, silicate solubility was decreased by addition of 2 mL ethanol followed by vortexing to ensure suitable mixing until the solution was slightly turbid. SiQDs were centrifuged at 15,110 × *g* for 2 min and the supernatant was discarded. The QD pellet was resuspended in 1 mL DI water and then sonicated for 30 min. In order to determine the concentration of SiQDs in solution, absorption spectrum method was used as described in a previous report (Yu et al., 2003).

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