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Plasmonic ELISA for the ultrasensitive detection of *Treponema pallidum*

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ABSTRACT

In this report, we have developed a plasmonic ELISA strategy for the detection of syphilis. Plasmonic ELISA is an enzyme-linked immunoassay combined with enzyme-mediated surface plasmon resonance (SPR) of gold nanoparticles (AuNPs). Immune response of the *Treponema pallidum* (*T. pallidum*) antibodies triggers the acetylcholinesterase-catalyzed hydrolysis of acetylthiocholine to produce abundant thiocholine. The positive charged thiol, in turn, alters the surface charge distribution the AuNPs and leads to the agglomeration of the AuNPs. The induced strong localized SPR effect of the agglomerate AuNPs can, thus, allow the quantitative assay of *T. pallidum* antibodies due to the remarkable color and absorption spectral response changes of the reaction system. The plasmonic ELISA exhibited a quasilinear response to the logarithmic *T. pallidum* antibody concentrations in the range of 1 pg/mL–10 ng/mL with a detection limit of 0.98 pg/mL. Such a low detection limit was 1000-fold improvements in sensitivity over a conventional ELISA. The results of plasmonic ELISA in syphilis assays of serum specimens from 60 patients agreed with those obtained using a conventional ELISA method. The plasmonic ELISA has characteristics (analyte specific, cost-effective, ease of automatic, low limit of detection) that provide potential for diagnosis and therapeutic monitoring of syphilis.

1. Introduction

Syphilis, a sexually transmitted infection of considerable public health importance, is on the increase again (Chakraborty and Luck, 2008). The health effects resulted by syphilis infection are overwhelming. The dissemination of *Treponema pallidum* (*T. pallidum*), the causative organisms of syphilis, begins at the early infection until the pathogens throughout the infector's body, including skin, bones, central nervous and cardiovascular systems (Tipple et al., 2011; Tantalo et al., 2005; Martin et al., 2009). For untreated infected women, *T. pallidum* may be transmitted from mother to fetus during pregnancy or at birth (Nahmias et al., 2011). The systemic syphilis incursion also increases the risk of other infections, such as human immunodeficiency virus (HIV) (Park et al., 2011a, 2011b). Hitherto, syphilis remains an important global health problem and continues to challenge clinicians in diagnosis and therapeutic monitoring.

Syphilis diagnosis mainly relies on serologic assays (Jiang et al., 2013; Herremans et al., 2010; Wellinghausen and Dietenberger, 2011), because the causative organisms cannot be cultured in vitro (Jantzen et al., 2012) and the infection is characterized by long periods of latency in excess of twenty years (Zoni et al., 2013). Serologic assays include two types, nontreponemal and treponemal tests (Lipinsky et al., 2012; Castro et al., 2013, 2010; Zhang et al., 2012).The early tests are nontreponemal ones using nontreponemal lipoidal antigens to react with the antibodies to *T. pallidum*, such as toluidine red unheated serum test (TRUST) (Zhuang et al., 2012) and rapid plasma reagin test (RPR) (Sweene et al., 2013). Advantages of these tests are that they are inexpensive and simple to perform. However, the results of nontreponemal tests must be further confirmed by treponemal tests because of their low specificity and limited sensitivity (Seña et al., 2010). Overall, treponemal tests using *T. Pallidum* antigens have higher sensitivity and specificity than nontreponemal ones (Seña et al., 2010). Of all the treponemal tests cleared by the U.S. Food and Drug Administration (FDA) for diagnostic, confirmatory, and blood donor screening test purposes (Kania, et al. 2009), enzyme-linked immunosorbent assay (ELISA) is one of the most sensitive and specific techniques (Seña et al., 2010; Cheow et al., 2010). Enzyme-catalyzed signal transformation and amplification greatly promote the analytical performance. ELISA combined with recombinant *T. pallidum* antigens shows sensitivities of 94.7–99.1% and specificities more than 99% in clinical syphilis diagnosis (Seña et al., 2010; Cheow et al., 2010). It is also cost-effective and ease of automatic compared with other

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treponemal tests, such as treponema pallidum gelatin particle agglutination (TPPA) (Maple et al., 2010) and fluorescent treponemal antibody absorption assay (FTA-ABS) (Park et al., 2011a, 2011b). The problem of current ELISA strategies is that the detection limit of ~1 ng/mL (Engvall and Perlmann, 1979; Lems-Van Kan et al., 1983; Fortin et al., 2009) is relatively high. It means these methods may be incapable if the concentrations of *T. pallidum* antibodies in serum specimens are quite low (Seña et al., 2010), for example, early primary infections. Yet, early diagnosis is of considerable importance in disease treatment and preventing the transmission of infection. In this context, sensitive and reliable detection of *T. pallidum* is of paramount importance for clinic diagnostics and therapeutics.

Plasmonic ELISA has emerged as an ultrasensitive strategy enabling the detection of a few molecules of analytes with the naked eye (de la Rica and Stevens, 2012). Rather than the most common ELISA catalyzing a color change reaction of organic molecules, plasmonic ELISA performs an enzyme-mediated localized surface plasmon resonance (LSPR) (Wang et al., 2010) of metallic nanoparticles. LSPR is a special optical phenomenon conferred by the interaction of light with electrons on the metallic nanoparticle surfaces (Guo and Kim, 2012). The light, under which LSPR occurs, is strongly dependent on the metallic nanoparticle size, shape, surface and agglomeration state (Wang et al., 2010; Ogiso et al., 2013). Minor changes of the state of metallic nanoparticles will lead to immense optical property changes of them. In plasmonic ELISA, such LSPR can be quantitatively controlled and amplified by enzyme-mediated surrounding alteration of metallic nanoparticles (de la Rica and Stevens, 2012; Liu et al., 2013). It, thus, offers a potential approach for ultrasensitive assays of target molecules.

We reported the development of an ultrasensitive plasmonic ELISA strategy based on an enzyme-linked immunoassay format with enzyme-mediated SPR of gold nanoparticles (AuNPs) for the detection of total antibodies to *T. pallidum*. The performance of plasmonic syphilis ELISA strategy was demonstrated using the serum specimens from sixty patients and compared to commercial TP-ELISA.

2. Materials and methods

2.1. Materials and reagents

Acetylcholinesterase (AChE) (Type VI-S, 200–1000 U/mg), acetylthiocholine (ATC), streptavidin (SA) and (+)-Biotin N-hydroxysuccinimide ester (BNHS) were obtained from Sigma Aldrich Chemical Co. Biotinylated mouse anti-human IgG was purchased from Jackson Immuno Research Laboratories Inc. The ELISA kits of *T. pallidum* antibodies for immunoassay and the 96-well polystyrene plates were purchased from Zhuhai Livzon Diagnostic Inc. When obtained, the 96-well polystyrene plates have been treated using 1 μg/mL *T. pallidum* antigens (TpN15, TpN17 and TpN47) in PBS buffer (10 mM, pH 7.4) at 4 °C over night and the wells were blocked with 5% fetal bovine serum for 1 h followed by washed three times using wash buffer. All other chemicals were of analytical grade and obtained from Beijing Dingguo Changsheng Biotechnology Co. Ltd. All solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system. Peripheral blood samples from 60 patients were collected from the Third Xiangya Hospital via venipuncture in tubes without anticoagulant. After centrifuged at 3000 rpm for 5 min, the plasma was stored at −20 °C and thawed immediately before analysis. These 60 patients were 34 male and 26 female, aged between 20 and 70 years old.

2.2. Preparation of biotinylated AChE and citrate-stabilized AuNPs

Biotinylated AChE was prepared using NHS-activated biotins to react efficiently with amino groups ($-NH_2$) of AChE in PBS buffer. Briefly, freshly prepared 150 μL of 10 mM NHS-biotin in dimethylformamide (DMF) was mixed with 2 mg AChE in 1 mL of 10 mM phosphate buffered saline (PBS, pH 7.4), and incubated for 2 h at room temperature. The solution was then desalted by Microspin G-50 columns (GE Healthcare, UK) to remove excess NHS-biotin.

AuNPs were synthesized by citrate reduction of $HAuCl_4$ according to reported protocols (Liu and Lu, 2006; Hill and Mirkin, 2006): 10 mL 38.8 mM trisodium citrate was rapidly added to a stirred boiling solution of $HAuCl_4$ (100 mL, 1 mM), of which the color changed from pale yellow to deep red within several minutes. Then, the solution was heated under reflux for another 30 min to ensure complete reduction followed by slow cooling to room temperature. The average size of AuNPs was 13 ± 2 nm as calculated from the transmission electron microscopy (TEM) image, with a concentration of ~13 nM determined based on an extinction coefficient of $2.7 \times 10^8\ M^{-1}\ cm^{-1}$ at 520 nm for 13 nm AuNPs (Hill and Mirkin, 2006). The final AuNPs solution was stored at 4 °C for future use.

2.3. Analytical protocol of plasmonic TP-ELISA

Commercial 96-well polystyrene microtiter plates modified with *T. pallidum* antigens were directly used. In typical assays, 100 μL of serum samples containing certain concentration of *T. pallidum* antibodies were added on the wells of the microtiter plates and incubated for 1 h. Then, the left liquid in the wells were poured out and biotinylated anti-human IgG (100 μL, 1.5 μg/mL) was added, for 1 h. After pouring out the liquid, streptavidin (SA) (100 μL, 4 μg/mL) was added and incubated for 30 min followed by adding biotinylated AChE (100 μL, 20 μg/mL) and incubating for another 30 min. Then, the plates were carefully washed five times with wash buffer and twice with distilled water. AChE-catalyzed hydrolysis reaction was performed by adding freshly prepared ATC (100 μL, 20 μM in hepes buffer) to each well and incubating 15 min at 37 °C. After that, 100 μL 1.6 nM citrate-stabilized AuNPs were added to each well.

We measured the surface plasmon absorption spectra of AuNPs in the wavelength range from 400 nm to 800 nm in a 50 μL quartz cuvette on a UV-2450 UV–vis absorption spectrophotometer (Shimadzu, Japan) at room temperature.

Dynamic light scattering analysis was performed using a Zetasizer 3000 HS particle size analyzer (Malvern Instruments, U.K.) to determine the hydrodynamic sizes of the AuNPs. Zeta potential (ζ) analysis was performed on a Zeta Sizer Nano ZS (Malvern Zetasizer Nano ZS90) to determine the surface charge distributions of the AuNPs. The TEM images were obtained on a field-emission high-resolution 2100F TEM (JEOL, Japan) at an acceleration voltage of 200 kV.

Clinical serum specimens from 60 donors were analyzed by following the above mentioned protocol, but the optical density (OD) of the reaction solutions at 520 nm was measured using a RT6100 Microplate Spectrophotometer (Rayto, U.S.A.). Conventional ELISA was also performed by strictly following the introduction of the kit, to demonstrate the reliability of plasmonic ELISA.

3. Results and discussion

3.1. Analytical principle of plasmonic TP-ELISA

The plasmonic TP-ELISA was based on an enzyme-linked sandwiched immunoassay format with enzymatic LSPR control of AuNPs. The analytical principle is illustrated in Scheme 1. Instead of an enzymatic-catalyzed color change of organic molecule in conventional TP-ELISA, plasmonic TP-ELISA utilizes an enzymatic reaction to alter the surroundings of AuNPs and control the LSPR effect of AuNPs. First, like common ELISA for *T. pallidum*