



A label-free biosensor based on localized surface plasmon resonance for diagnosis of tuberculosis

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ABSTRACT

A biosensor based on localized surface plasmon resonance (LSPR) was developed to detect the antibody of *Mycobacterium tuberculosis* using the fusion protein CFP10-ESAT6 as an antigen. To explore the diagnostic potential of the biosensor for tuberculosis (TB), the fusion protein CFP10-ESAT6 was immobilized on gold nanorods (Au NRs) by chemical modification, and the functionalized Au NRs were subsequently incubated with serums collected from TB patients, non-tuberculous pulmonary disease patients or healthy individuals. The change in the LSPR properties of Au NRs from the specific interaction between the antigen and antibody was monitored, and detection of the target antibody was completed based on the proposed biosensor. Serum analysis showed that the sensitivity of the biosensor was 79% and the specificity was 92%. Therefore, the LSPR biosensor is a valuable tool for serodiagnosis of TB.

1. Introduction

Tuberculosis (TB) is one of the world's deadliest infectious diseases mainly caused by infection with *Mycobacterium tuberculosis*. It remains a major public health problem worldwide, as one-third of the world's population is estimated to be infected with *Mycobacterium tuberculosis* and more than 9 million new cases occur annually (Getahun et al., 2015). The major reasons for the high prevalence rate of TB include poor access to effective diagnostic methods and treatments. Furthermore, it is still challenging to detect TB patients with drug-resistant strains of *Mycobacterium tuberculosis* and patients who are co-infected with human immunodeficiency virus (HIV). Therefore, the development of rapid, reliable and effective diagnostic methods for TB is of vital importance for disease control.

Localized surface plasmon resonance (LSPR) technology is a new bio-sensing technology with high sensitivity (Unser et al., 2015) that can be used to monitor the interaction between bio-molecules, such as antigens and antibodies, in real-time and in situ without using any labels (Kim et al., 2007; Raghu et al., 2015). LSPR is a phenomenon caused by the resonant excitation of noble (e.g., Ag and Au) metal nanoparticles' free electrons (Jia et al., 2014; Mayer et al., 2010). Gold nanorods (Au NRs) possess an anisotropic configuration and unique optical properties that exhibit transverse and longitudinal plasmon

bands. The longitudinal plasmon band is extremely sensitive to changes in the dielectric properties of the surroundings, producing strong, characteristic absorption bands in the visible to infrared spectrum (Xiang et al., 2010). A significant change in the plasmon spectra in response to a change in the refractive index in the vicinity of the Au NRs arises from LSPR properties and can be effectively utilized to detect specific target binding events. Therefore, we can use the wavelength shift of the LSPR spectrum to detect molecular adsorption to Au NRs.

The fusion protein CFP10-ESAT6 has been shown to be a great candidate antigen for the immunodiagnosis of TB due to its high specificity in enzyme linked immunosorbent assay (ELISA) (Zhang et al., 2010). However, some disadvantages, including tedious washing procedures and antibody labeling, may limit wide use of the method in the detection of TB. In the present study, we established a simple and rapid method for determination of the anti-CFP10-ESAT6 antibody for TB diagnosis based on a LSPR biosensor, and our results demonstrated that the specific CFP10-ESAT6-based LSPR biosensor could potentially be used for TB diagnosis.

2. Materials and methods

2.1. Regents

Cetyltrimethylammonium bromide (CTAB), hydrogen tetrachloroaurate

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(III) trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), sodium borohydride (NaBH_4), silver nitrate (AgNO_3) and L-ascorbic acid (AA) were purchased from Sinopharm Chemical Reagent Co., Ltd. 11-mercaptoundecanoic acid (MUA) was purchased from Sigma. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Aladdin. Chemiluminescence detection kit was purchased from Auragene Bioscience Inc., China. Double distilled water was used in all experiments.

2.2. Antigen

The fusion protein CFP10-ESAT6 was prepared as the antigen in this study. The genes encoding CFP10 and ESAT6 proteins were amplified by PCR using the CFP10 primers (forward primer P1: 5'-CCGGA TCCATGGCAGAGATGAAGAC-3'; reverse primer P2: 5'-TTCCACTGC TGCTCTGTGAAGCCATTGCGAGG-3') and the ESAT6 primers (forward primer P3: 5'-CCTCGCAAAATGGGCTTCACAGAGCAGCAGTGGA-3'; reverse primer P4: 5'-ATCGTCGACCTATGCGAACATCCAG-3') from the genomic DNA of *Mycobacterium tuberculosis* reference strain H37Rv, and the CFP10-ESAT6 fusion gene was subsequently amplified by Gene SOEing. After sequence analysis, the fusion gene was further subcloned into expression vector pQE-30 in the *Bam*H I/*Sal* I site. The fusion protein CFP10-ESAT6 was expressed in *E. coli* JM109 and purified with affinity chromatography, and can be stored with 20 M Tris-HCl buffer (pH 8.0) at -20°C for six months. The antigenicity of the fusion protein was analyzed by Western blotting.

2.3. Serums

Serum samples from 52 clinically diagnosed TB patients were provided by the clinical laboratory of Hunan Province Chest Hospital following informed consent and approval by the Ethics Committee of Hunan Province Chest Hospital. Serums from 29 non-tuberculous pulmonary disease patients and 39 healthy individuals were provided by the clinical laboratory of Hunan Province People's Hospital following informed consent and approval by the Ethics Committee of Hunan Province People's Hospital. All subjects in the study were aged between 18 and 70 years. Serums were stored at -20°C before use.

2.4. Synthesis of Au NRs

The seed-mediated growth methods were described previously (Garcia-Ramirez et al., 2016; Orendorff et al., 2006). In the present study, some experimental parameters were slightly modified as follows: 10 ml of 0.10 M CTAB solution was mixed with 0.24 ml of 0.02 M HAuCl_4 . 5 ml of double distilled water was added, followed by the addition of 1.2 ml of ice-cold 0.01 M NaBH_4 . All the reagents were added with stirring, resulting in the formation of a light-brown solution. The seed solution was vigorously stirred for 2 min and then kept at 25°C for 2 h before use.

To create the growth solution, 0.8 ml of 0.01 M AgNO_3 and 1.5 ml of 0.02 M AuCl_3 were added to 30 ml of 0.10 M CTAB solution in a separate conical flask at 25°C , followed by the addition of 350 μl of 0.10 M ascorbic acid with stirring for 2 min to make the orange solution fade. Subsequently, 70 μl of the seed solution was added to the growth solution. The resulting mixture was left undisturbed and aged for approximately 2 h at 25°C . The as-synthesized Au NRs were stored in a refrigerator at 4°C until they were used for analysis within 3 months.

2.5. Chemical modification of Au NRs

Au NRs were interacted with 0.005 M MUA overnight at 25°C , centrifuged at 14,000 rpm and dispersed with 0.01 M CATB. Next, 0.075 M EDC and 0.015 M NHS were added to the solution for 40 min at 25°C to activate the Au NRs by linking amidogen to the Au NRs. Then, Au NRs were centrifuged at 8000 rpm and dispersed with double distilled water.

2.6. Conjugating antigen to Au NRs

To conjugate the fusion protein CFP10-ESAT6 to the particles, the activated Au NR solution was mixed with CFP10-ESAT6 (0.456 mg/ml) at a volume ratio of 50:1 and allowed to react for 1 h at 25°C . The carboxyl group of the protein was connected with the amidogen of the Au NRs to complete the conjugation between the antigen and Au NRs. After attachment of CFP10-ESAT6 onto the Au NRs, the modified Au NRs were collected by centrifugation at 14,000 rpm for 10 min and then resuspended in a 10 mM PBS buffer solution (pH 7.4) containing 0.005 M CTAB. The solution could be stably stored for 3 to 4 weeks at 4°C .

2.7. Seroanalysis of the biosensor

Seroanalysis was performed to explore the diagnostic potential of the CFP10-ESAT6-based LSPR biosensor for TB. Serum samples from TB patients, non-tuberculous pulmonary disease patients and healthy individuals were diluted at a ratio of 1:10⁴ in double distilled water. Serums were incubated with CFP10-ESAT6-conjugated Au NRs at 25°C for 2 min.

2.8. UV-Visible absorbance

The absorption spectra were collected using a Lambda 35 UV-Visible spectrophotometer over the range from 400 to 1100 nm. The samples were measured against water as a reference. All samples were loaded into a quartz cell for measurements.

3. Results

To establish a biosensor by detecting the antibody of *Mycobacterium tuberculosis* with the fusion protein CFP10-ESAT6 as an antigen, the first step was to express and purify the CFP10-ESAT6 fusion protein. We cloned the CFP10-ESAT6 fusion gene into the pQE30 vector, named the plasmid pQE30-CFP10-ESAT6, and validated the recombinant plasmid by PCR and restriction enzyme digestion with *Bam*HI and *Sal*I. Consistent with the predicted band size, the PCR and restriction enzyme digested products were approximately 600 bp (Fig. 1A). The sequence of the CFP10-ESAT6 fusion gene in pQE30-CFP10-ESAT6 was also confirmed by direct sequencing (data not shown). Moreover, pQE30-CFP10-ESAT6 was transformed into *E. coli* JM109, and CFP10-ESAT6 fusion protein was heterologously expressed and purified using a Ni-NTA column. Subsequently, SDS-PAGE was used to detect the His-CFP10-ESAT6 fusion protein. The Coomassie brilliant blue staining results showed that His-CFP10-ESAT6 fusion protein was specifically purified (Fig. 1B).

To further determine whether the fusion protein His-CFP10-ESAT6 is a detectable antigen for establishment of the biosensor, Western blotting analysis was further performed. The results showed that the expressed fusion protein His-CFP10-ESAT6 can be specifically detected using anti-His antibodies and that the protein's molecular weight is approximately 25 KD (Fig. 1C). Moreover, Western blotting analysis showed that the His-CFP10-ESAT6 fusion protein can be detected using the serums of TB patients (Fig. 1D), but not the serums of normal subjects (Fig. 1E), indicating that His-CEP10-ESAT6 acted as the detectable antigen, reacting with the corresponding antibodies in the serums of TB patients.

When the antigen was prepared, the key steps for the preparation and serological reaction of the CFP10-ESAT6 LSPR biosensor included the synthesis and chemical modification of Au NRs, conjugation of the fusion protein CFP10-ESAT6 to the Au NRs and seroanalysis of the biosensor (Fig. 2). Serum analysis was performed to explore the diagnostic potential of the CFP10-ESAT6 LSPR biosensor for TB. The as-prepared Au NRs were first characterized by transmission electron microscopy (TEM) image as shown in Fig. 3A, and the corresponding

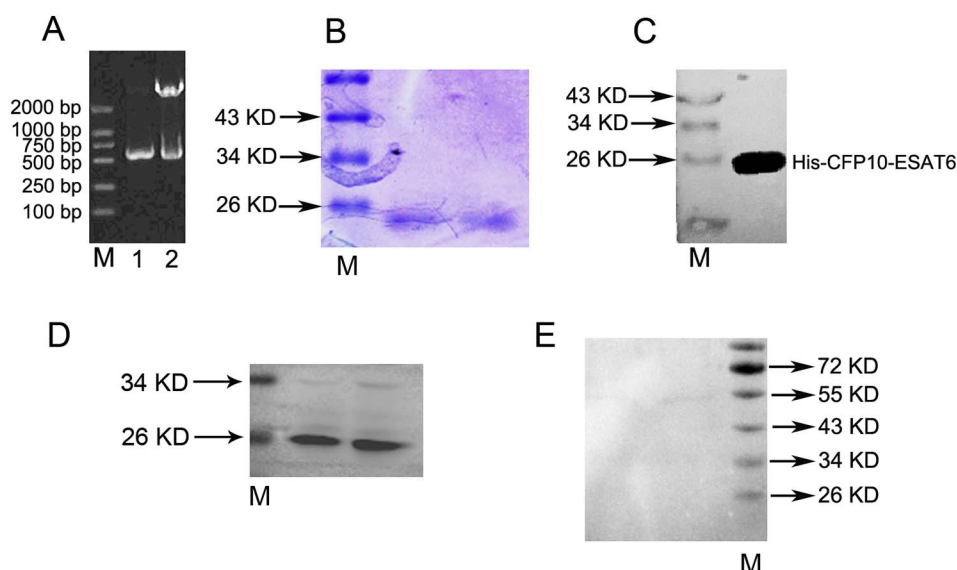


Fig. 1. Expression, purification and analysis of the His-CFP10-ESAT6 fusion protein. A. PCR and restriction enzyme digestion of plasmid pQE30-cfp10-esat6. The predicted coding region of *cfp10-esat6* is 600 bp long. Lane M: DNA marker. Lane 1: PCR product of *cfp10-esat6*; Lane 2: enzyme-digested products of *Bam*HI and *Sall*. B. SDS-PAGE of the fusion protein His-CFP10-ESAT6 stained with Coomassie brilliant blue. His-CFP10-ESAT6 was expressed in JM-109 bacterial cells and purified using a Ni-NTA column. The molecular weight of His-CFP10-ESAT6 is 25 KD. Lane M: protein marker. C. Purified His-CFP10-ESAT6 was detected by Western blotting analysis by using anti-His antibodies. The molecular weight of His-CFP10-ESAT6 is 25 KD. Lane M: protein marker. D. Purified His-CFP10-ESAT6 was detected by Western blotting analysis using the sera of tuberculosis patients (lanes 1 and 2). Lane M: protein marker. E. Purified His-CFP10-ESAT6 was not detected by Western blotting analysis using the sera of normal humans (lanes 1 and 2).

absorption spectrum shows a transverse plasmon peak (TPP) at 520 nm and a longitudinal plasmon peak (LPP) at 700 nm, respectively, indicating the typical LSPR spectrum of Au NRs (Fig. 3B). A significant redshift of LPP was seen after the Au NRs reacted with serum samples from TB patients. However, no detectable change of LPP wavelength was observed with the serum from healthy controls or non-tuberculous pulmonary disease (Fig. 3B).

Lastly, we further investigated the efficacy of the CFP10-ESAT6 LSPR biosensor. It is worth noting the positive reaction rates of the CFP10-ESAT6 LSPR biosensor for the diagnosis of groups, including TB patients, non-tuberculous pulmonary disease patients and healthy controls. Among the sera of TB analyzed by the proposed LSPR biosensor, a redshift of the LPP wavelength occurred for most of them. According to the healthy controls, we choose redshift of 4 nm as the cut-off value, as shown in Fig. 4. Table 1 shows that the positive reactions of the TB, non-tuberculous pulmonary disease and healthy controls are 79%, 9% and 8%, respectively, demonstrating the sensitivity and specificity of the biosensor.

4. Discussion

The Asian Pacific region is the most prevalent area of TB in the world with approximately 1 billion patients or carriers (Desikan et al., 2013). Due to the rapid increase in TB patients and high prevalence rate, there is an urgent need to develop rapid, simple and reliable methods for TB diagnosis. TB is a chronic infections disease caused by *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium africanum* etc. Among them, the main pathogens of TB are *Mycobacterium tuberculosis*. In the bacteriological examination of tuberculosis, *Mycobacterium tuberculosis* is usually divided into *Mycobacterium tuberculosis* complex (MTC) and Nontuberculosis mycobacteria (NTM). The MTC includes *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum* and *Mycobacterium microti*. ESAT-6 and CFP-10 are encoded by the RD1 region, this which only exists in the MTC and some pathogenic NTM. In the present study, we created a label-free CFP10-ESAT6 LSPR biosensor that can be used to specifically and sensitively detect *Mycobacterium tuberculosis*.

It is well known that Au NRs have two distinct plasmon resonance absorption bands: a longitudinal band corresponding to electron motion along the long axis of the particle and a transverse band corresponding to motion along the width of the particle (Chang et al., 2012). The absorption spectrum of these Au NRs was closely related to their size and shape. The peak location was highly dependent upon the local refractive index, and the wavelength of LPP changed significantly when the refractive index of the Au NRs' surrounding environment was slightly altered (Haes et al., 2006). As molecular adsorption to Au NRs caused by the interaction between antigens and antibodies increased the local refractive index, it led to a redshift of LPP; thus, we used the degree of redshift to analyze the interaction between serum samples and the CFP10-ESAT6-based biosensor.

LSPR technology has been previously used to detect proteins (Jo et al., 2016), DNA (Soares et al., 2014), and polymers (Kim et al., 2008). Moreover, previous studies indicated that the LSPR biosensor could be used for diagnosis of pancreatic cancer (Joshi et al., 2014), Alzheimer's disease (Kang et al., 2015) and hepatitis B virus (HBV) (Zheng et al., 2010). However, LSPR technology had rarely been applied to detect *Mycobacterium tuberculosis*. For development of new detection technology, it is important to evaluate sensitivity and specificity. To this end, we used the CFP10-ESAT6-conjugated Au NRs as a platform to analyze 120 serum samples. Our results showed that the sensitivity of the biosensor was 79% and the specificity was 92%, indicating that the CFP10-ESAT6 LSPR biosensor was capable of specifically detecting *Mycobacterium tuberculosis*. It is worth noting that PCR related methods have much higher sensitivity and specificity to detect

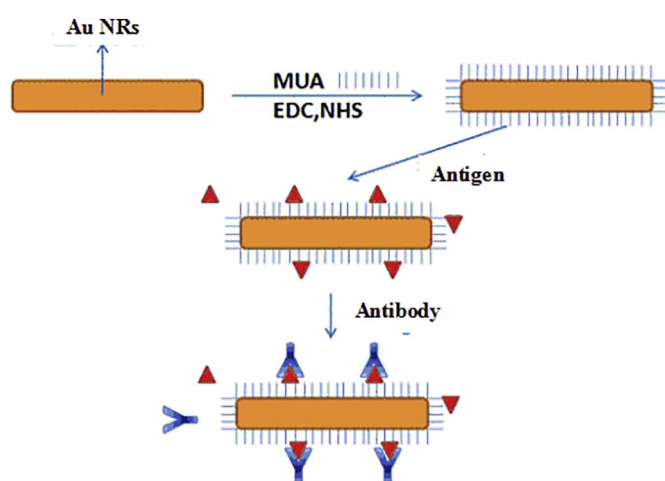


Fig. 2. Scheme of the preparation and serological reaction of the CFP10-ESAT6 based-LSPR biosensor. Schematic of the key steps in the preparation and serological reaction of the CFP10-ESAT6 based-LSPR biosensor include the following: (1) synthesis of gold nanorods (Au NRs); (2) chemical modification of Au NRs; (3) conjugation of the antigen to Au NRs; (4) seroanalysis of the biosensor. During these steps, Au-SH is used to fix the MUA to the surface of the Au NRs, then the -NH₂ of the protein was connected to the -COOH of the Au NRs by the actions of NH-CO to complete the conjugation between the antigen and Au NRs.

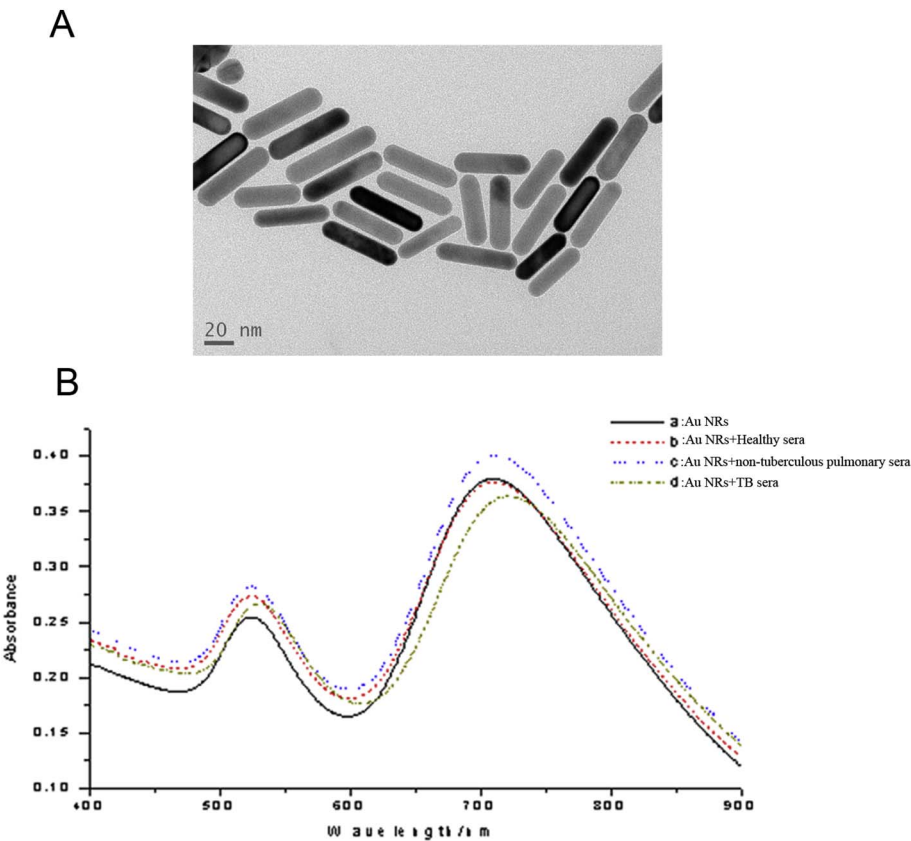


Fig. 3. Characterization of synthesized Au NRs. A. Representative transmission electron microscopy (TEM) image of the as-synthesized Au NRs. B. The LSPR spectra of Au NRs after reacting with serum. Two distinct plasmon resonance absorption peaks of Au NRs, the transverse plasmon peak (TPP) and the longitudinal plasmon peak (LPP), are typical spectral characteristics of Au NRs. The wavelength of TPP is approximately 520 nm, and the wavelength of LPP is approximately 700 nm. (a) The CFP10-ESAT6-conjugated Au NRs; (b) Au NRs react with sera from healthy controls; (c) Au NRs react with sera from non-tuberculous pulmonary disease patients; (d) Au NRs react with sera from TB patients.

Mycobacterium tuberculosis. For example, the sensitivity of nested polymerase chain reaction was 100% (*Mycobacterium* culture positive) and 95% (acid fast bacilli smear positive) with a specificity of 93.1% (Dubey et al., 2013). However, the CFP10-ESAT6 LSPR biosensor has similar sensitivity and specificity with ELISA related methods, as a previous study indicated that ELISA with antigen 5 can achieve a sensitivity of 81.4% and a specificity of 93.4% for tuberculosis (Balestrino et al., 1984). Although PCR related methods have higher sensitivity and specificity, these methods may be expensive and time-consuming. The CFP10-ESAT6 based-LSPR biosensor is a simple and easy method that may provide a cost-effective alternative method for the potential diagnosis of TB. In addition, there was one caveat that we did not analyze the efficacy and specificity of the biosensor in samples from subjects who were co-infected with *Mycobacterium tuberculosis* and other pathogenic microorganisms in this study. Future studies will use more samples to further evaluate the diagnostic potential of the biosensor.

Table 1
Analysis of seroreactivity of the CFP10-ESAT6 LSPR biosensor for diagnosis of TB.

Group	Serum screened	Positive reaction, n (%)
Tuberculosis	52	41 (79%)
Non-tuberculous pulmonary disease	34	3 (9%)
Healthy controls	39	3 (8%)

The sensitivity and specificity of the biosensor were 79% and 92%, respectively.

In conclusion, we have developed a rapid, simple and inexpensive method based on an LSPR biosensor for the diagnosis of TB. Our study provided a scientific basis for the application of this new method. We believe that the biosensor will serve as a new technology for the diagnosis of TB.

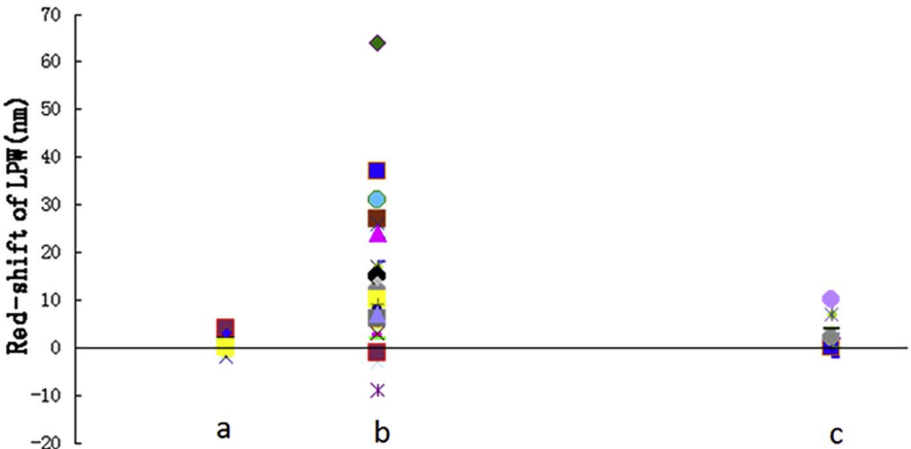


Fig. 4. Seroanalysis of the CFP10-ESAT6 LSPR biosensor. This figure is a scatter diagram of serological reactions. The scatter diagram shows a redshift of LPP wavelength after reacting with serum. Consistent with the statistical results, it was apparent that the TB patient group had higher values than the healthy group and the non-tuberculous pulmonary disease group. a. Au NRs react with sera from healthy controls; b. Au NRs react with sera from TB patients; c. Au NRs react with sera from non-tuberculous pulmonary disease patients.

Conflict of interest statement

None declared.

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