



## Sensitive and simultaneous detection of different disease markers using multiplexed gold nanorods

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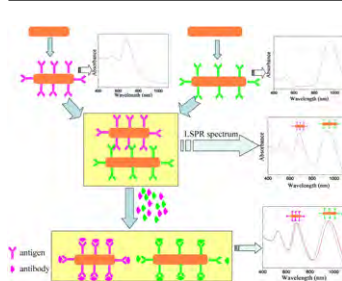
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### HIGHLIGHTS

- ▶ A rapid and sensitive multiplex bioassay based on gold nanorods has been developed.
- ▶ This bioanalytical assay can simultaneously detect different acceptor–ligand pairs in a wide detection wavelength range.
- ▶ This assay allows easy detection of human serum specimens infected by different diseases without sample pretreatment.

### GRAPHICAL ABSTRACT



### ARTICLE INFO

#### Article history:

Received 17 August 2012

Received in revised form 8 October 2012

Accepted 11 October 2012

Available online 23 October 2012

#### Keywords:

Multiplex bioanalytical assay

Gold nanorod

*Schistosoma japonicum*

Tuberculosis

### ABSTRACT

A multiplexed bioanalytical assay is produced by incorporating two types of gold nanorods (GNRs). Besides retaining the desirable features of common GNRs LSPR sensors, this sensor is easy to fabricate and requires only a visible–NIR spectrometer for detection. This assay can simultaneously detect different acceptor–ligand pairs by choosing the proper GNRs possessing various LPWs in a wide detection wavelength range and can be developed into a high-throughput detection method. This bioanalytical assay allows easy detection of human serum specimens infected by *S. japonicum* and tuberculosis (TB) from human serum specimens (human serum/Tris–HCl buffer ratio = 1:10<sup>4</sup>) without the need for sample pretreatment. The technique is very sensitive compared to other standard methods such as indirect hemagglutination assays (IHA) that require a serum concentration ratio of larger than 1:20 and enzyme-linked immunosorbent assays (ELISA) requiring a ratio larger than 1:100. This methodology can be readily extended to other immunoassays to realize wider diagnostic applications.

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### 1. Introduction

In life sciences, there has been a continuously growing interest to find new methods and devices that can provide easy, highly

reproducible, and sensitive sensing assays for biomolecular reactions [1–4]. Localized surface plasmon resonance (LSPR) based on gold nanorods (GNRs) is one of powerful candidates for biotechnology and biosensors. GNRs possess two plasmon bands: a transverse plasmon wavelength (TPW) and another longitudinal plasmon wavelength (LPW) in the visible or NIR range. These anisotropic GNRs have potential applications as molecular probes since significant changes occur in the plasmon spectra as a result of changes in the refractive index in the vicinity of GNRs. This is a useful property that enables sensing of specific target binding events.

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Because the LPW of GNR strongly depends on the length-to-diameter aspect ratio of the nanorod, the corresponding LPW can be fine-tuned by adjusting the length and diameter of the nanorod [5–7]. If GNRs with different LPWs are designed carefully and combined properly, a possible multiplex assay suitable for the simultaneous detection of multiple targets can be produced. This multiplex optical analytical assay can be fabricated based on the tunable LPW of GNRs and has advantages such as label-free detection, low cost and broad tunability. Moreover, the multiplex assay provides a possibility to diagnose or detect different diseases simultaneously.

Schistosomiasis is an important disease posing serious health threats especially in Asian, African, and Latin American countries [8–11]. There have been extensive efforts to develop effective methods to diagnose, control, and prevent the associated diseases. Currently, microscopic identification of eggs in stools is the most practical diagnostic approach. Serological diagnosis methods such as enzyme-linked immunosorbent assays (ELISA) [12–14], indirect hemagglutination assays (IHA) [15,16], circumoval precipitin tests (COPT) [17,18], and dot immuno-gold silver staining (dot-IGSS) [19] are also available and highly specific. These serological diagnostic methods deal with complicated serum samples containing the parasitic antigen and are usually quite time consuming. In addition, conventional serological diagnostic methods still do not satisfy the requirements for clinical analysis for schistosomiasis related diseases, especially in the early stage of infection when the antibody is less than 1:20 (IHA) [20,21]. Therefore, there is an urgent need to develop rapid, simple, and inexpensive alternatives, particularly with regard to on-the-spot epidemic screening of schistosomiasis.

Recent development of materials with improved bioanalytical properties has led to a resurgence of interest in the associated detection of *Schistosoma japonicum* (*S. japonicum*) [20,22,23]. As their optical properties can be tuned by modulating the aspect ratio of the GNR, the materials provide the possibility of fabricating a high throughput assay to detect multiplex targets [24–26]. A disease may induce a plethora of complications, for instance, diabetic complications, and myocardial disease complications. Therefore, a high-throughput method is very useful in the effective diagnostics of these complications. In the work reported here, we develop and demonstrate a rapid and sensitive multiplex bioanalytical assay by taking advantage of the LSPR properties of GNR. Two different diseases, *S. japonicum* and tuberculosis (TB), are studied to demonstrate the feasibility of this multiplex assay, which explore viable and stable method for further diagnosing complications induced by a disease. TB is one of the leading causes of death of humans from a single infectious agent worldwide. In our work, two kinds of GNRs are functionalized with *S. japonicum* antigen (SjAg) and tuberculosis antigen (TBAG), respectively, and the sensing capability of *S. japonicum* and TB is evaluated simultaneously. Combination of the functionalized GNRs produces a multiplex assay to determine human serum specimens without pretreatment and specimens infected by *S. japonicum* or TB can be easily identified whereas healthy human serum specimens show no significant positive response.

## 2. Experimental

### 2.1. Materials

H<sub>2</sub>AuCl<sub>4</sub>·3H<sub>2</sub>O, cetyltrimethylammonium bromide (CTAB), ascorbic acid, silver nitrate, and Tris(hydroxymethyl) aminomethane were purchased from Sinopharm Chemical Reagent Co., Limited (Shanghai, China). *N*-Hydroxysuccinimide (NHS) was purchased from ACROS (New Jersey, USA), *N*-ethyl-*N*-[(dimethylamino) propyl]carbodiimide (EDC) from Avocado

Research Chemicals, Ltd. (Lancashire, UK), and mercaptoundecanoic acid (MUA) from Aldrich (Milwaukee, USA). All the chemicals, unless mentioned otherwise, were all of analytical reagent grade and used as received. Aqueous solutions were prepared in doubly distilled water.

### 2.2. Preparation of GNRs and instruments

The seed solution of the GNRs was prepared according to the technique reported previously [27]. The CTAB solution (1.5 mL, 0.1 M) was mixed with 100 μL of 0.02 M HAuCl<sub>4</sub>, and 100 μL of ice cold 0.01 M NaBH<sub>4</sub> was added to the solution to form a brownish yellow solution. Vigorous stirring was continued for 2 min and then the seed solution was kept at room temperature (25 °C) and used at least 2 h after preparation.

To synthesize the GNRs, 1.5 mL of 0.02 M HAuCl<sub>4</sub> and 1.0 mL of 0.01 M AgNO<sub>3</sub> were added to 30 mL of 0.1 M CTAB, followed by addition of 0.8 mL of 0.08 M ascorbic acid. Ascorbic acid served as a mild reducing agent and changed the solution from dark yellow to colorless. Afterwards, 70 μL of the seed solution was added and the color of the solution changed gradually within 15 min.

The yield and aspect ratios of the GNRs were determined using transmission electron microscopy (TEM, JEM-2010) at 80 kV. Absorption spectra were acquired from the GNR samples for each stage of the experiments on a Lambda 35 UV–visible spectrophotometer (PerkinElmer, USA) in the wavelength range between 400 and 1100 nm.

### 2.3. Procedures

#### 2.3.1. Expression and purification of rSjGST

The DNA sequence encoding *S. japonica* 26 kDa glutathione S-transferase (GST) was contained in the prokaryotic expression vector pGEX-5X-3. rSjGST was expressed in the JM109 strain of *Escherichia coli* containing the plasmid pGEX-5X-3 when induced with 0.1 mM IPTG at 37 °C for 2 h. The cells were harvested by centrifugation, suspended in a phosphate buffered saline (PBS) containing 0.1% Triton-X100, and sonicated (400 W) using an ultrasonic cell disruption apparatus. The cell debris was removed by centrifugation at 12,000 × *g* for 20 min at 4 °C and the rSjGST-32 was purified from the supernatant of bacterial lysate by glutathione affinity chromatography using a 1 mL GSTrap column (GE Healthcare) according to the operation guide and was analyzed by 12% SDS–PAGE under reduction conditions. The protein concentration was determined by the Bradford assay using BSA as the standard.

#### 2.3.2. Expression and purification of TBAG

The fusion protein CFP10–ESAT6 was prepared as the antigen in this study. The genes encoding CFP10 and ESAT6 protein were amplified by PCR from the genomic DNA of the *Mycobacterium tuberculosis* reference strain H37Rv and then the *cfp10-esat6* fusion gene was amplified by Gene SOEing. After cloning and sequence analysis, the fusion gene was subcloned into the expression vector pQE-30. The fusion protein CFP10–ESAT6 was expressed in *E. coli* JM109 and purified by affinity chromatography. The antigenicity of the fusion protein was analyzed by Western blot.

#### 2.3.3. GNRs functionalized with SjAg and TBAG

Functionalization of the GNRs was carried by taking advantage of the well-known affinity between gold and thiol compounds. The GNRs were easily modified by alkanethiols to form SAMs to facilitate attachment to recognition agents (antigens in this work). Chemical modification of the GNRs was achieved by the following process. 0.5 mL of 20 mM ethanol solution of MUA were added to 5 mL of the GNR suspension and left to stabilize for at least 18 h at room temperature. The nanorods were collected by centrifugation

at 12,000 rpm for 12 min and re-dispersed in a 0.005 M CTAB solution. Afterwards, the activated nanorods were added to the freshly prepared 15 mM EDC and 75 mM NHS solution and sonicated for 30 min. The resulting nanorods were collected by centrifugation at 12,000 rpm for 10 min and incubated in a 10 mM Tris–HCl buffer solution (pH = 7.4) containing  $0.01 \text{ gL}^{-1}$  SjAg and 0.005 M CTAB. After attachment of the SjAg onto the GNRs, the modified GNRs were collected by centrifugation at 12,000 rpm for 10 min. Functionalization of the TBAg on the surface of the GNR was performed by the same method. After fictionalization of antigens, the modified GNRs were blocked by 1 M ethanolamine at pH 8.5 for 10 min. The uncreative ethanolamine was removed by centrifugation.

#### 2.3.4. Serum samples

The serum samples were supplied by Hunan Institute of Parasitic Diseases. 43 human serum samples infected by *S. japonicum* were collected. The eggs of *S. japonicum* in the feces were detected and antibodies to the *S. japonicum* antigen in the serum samples were determined by indirect hemagglutination assays (IHA). At the same time, 38 normal human serum samples were collected from healthy people for the control experiments.

In addition, serum samples of 52 TB patients diagnosed clinically were provided by the clinical laboratory of Hunan Province Chest Hospital. Sera of 39 healthy individuals were provided by the clinical laboratory of Hunan Province People's Hospital. All the study population was aged between 18 and 70 years. The sera were stored at  $-20^\circ\text{C}$ .

#### 2.3.5. Evaluation of human serum specimens

The LSPR sensing experiments were performed using the Lambda 35 UV–visible spectrophotometer. Generally, in a regular LSPR apparatus, the SjAg and TBAg conjugating GNRs in the cuvette were mounted vertically on an optical bench, and the transmitted light was detected by a single-element detector. The as-prepared GNR suspension was put into the cuvette and the absorption spectrum was recorded. Similarly, another absorption spectrum was obtained from the GNRs functionalized with SjAg and a significant red-shift in the longitudinal plasmon wavelengths was observed. The human serum sample infected by *S. japonicum* was added to the conjugated nanorod suspension and incubated for 10 min before the absorption spectra were recorded. At the same time, the control experiments were carried out on the normal human serum samples collected from healthy people. Thus, our experiments compared the changes in the transmission through the samples as a result of the SjAg conjugated GNRs reacting with the *S. japonicum* antibody (SjAb). The same method was conducted to evaluate the human serum specimens infected by TB.

### 3. Results and discussion

#### 3.1. GNRs functionalized with SjAg

Fig. 1a shows the TEM image of the GNRs produced by the protocol discussed earlier [27]. The corresponding absorption spectrum in Fig. 1b shows a transverse LSPR peak at 520 nm and longitudinal LSPR peak at 765 nm. Functionalization of the GNRs takes advantage of the well-known affinity between gold and thiol compounds. The nanorods react easily with alkanethiols to form a self-assembled monolayer (SAM) for attachment of the recognition agents [28]. Here, the SAM of MUA is formed to covalently link biomolecules via the  $\text{NH}_2$  and  $\text{COOH}$  terminal groups.

The GNRs are highly sensitive to changes in the refractive index of the surrounding medium. Thus, slight variation in the surrounding medium alters the LSPR property making the technique suitable for the detection of biological macromolecules. When the MUA SAM is formed, SjAg can be covalently attached by the  $\text{NH}_2$  of the protein

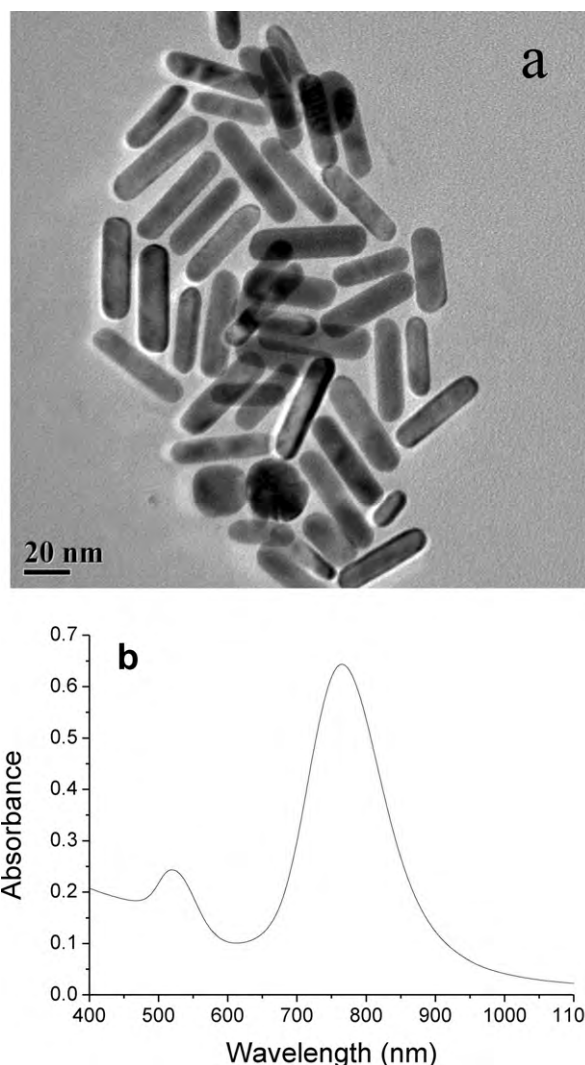
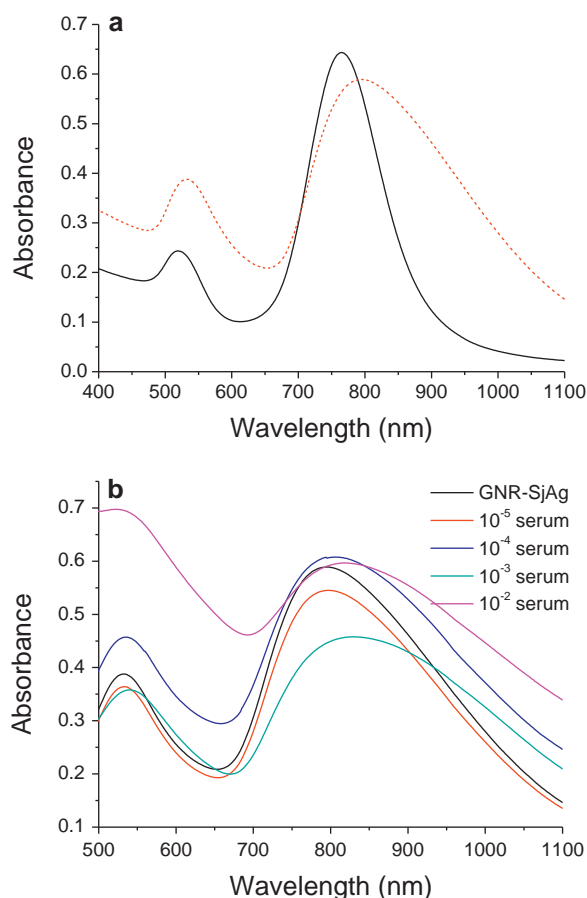


Fig. 1. TEM image of (a) GNRs and (b) corresponding LSPR absorption spectrum.

to the  $\text{COOH}$  side of the MUA SAM. Subsequently, there is a red-shift in the plasmon peak because of SjAg attachment. To eliminate possible non-specific adsorption on the functionalized nanorods, the samples are rinsed with a 10 mM Tris–HCl buffer solution (pH = 7.4). The two types of rods after SjAg attachment show a significant shift compared to the unmodified rods, as shown in Fig. 2a. A 25 nm red-shift is measured from the longitudinal plasmon peak indicating successful attachment of SjAg onto the surface of the GNRs. The sensitivity revealed by the plasmon spectra forms the basis of molecular biosensing.

Although the SjAg can only covalently attach to the MUA-activated sites, physisorption of SjAg to the CTAB capped faces is possible. The isoelectric point of SjAg is 5.7 [29] and hence, during the functionalization reaction (pH = 7.4), the SjAg molecules are negatively charged and bind to the positively charged CTAB cap electrostatically. However, the physisorbed SjAg molecules are not as strongly attached to the GNR surface as covalently bound ones. Under vigorous washing after functionalization, a significant portion of them is removed. In order to obtain the functionalized GNRs that have the consistent SjAg coating, the MUA activation route is preferred especially for a small SjAg/nanorod ratio. Actually, ultrasonic and vigorous washing after functionalization and centrifugation removes the adsorbed antibody. Thus, functionalization of GNRs is chemical modification rather than physisorption in the study.

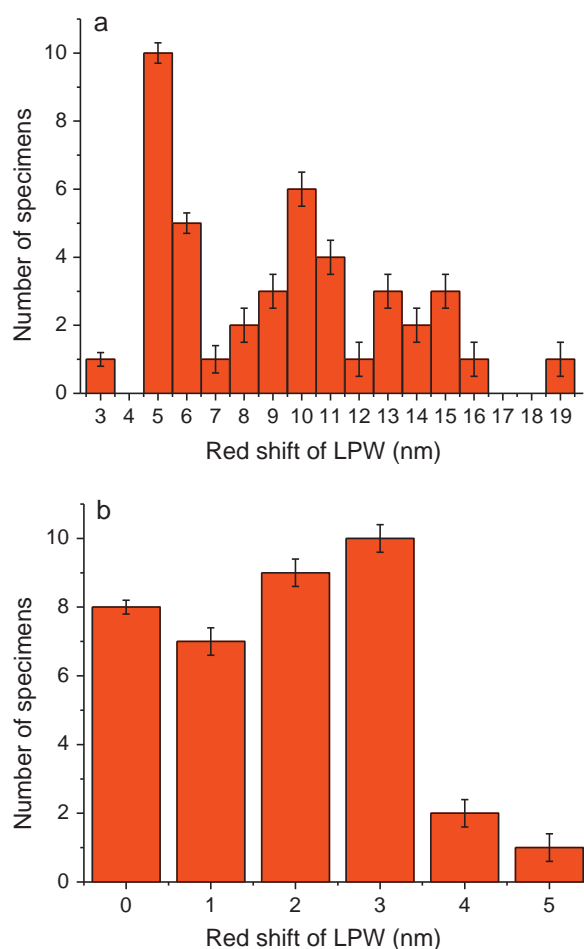


**Fig. 2.** (a) Absorption spectra acquired from the GNR (solid line) before and (dot line) after functionalization with SjAg. (b) Concentration dependence of the human serum infected by *S. japonicum* on the SjAg-attached GNRs.

### 3.2. Optimization condition for the assay of *S. japonicum*

The SjAg-derivatized GNR dispersion with various particle concentrations is employed to investigate the effects of the functionalized GNR particle dosage on the *S. japonicum* antibody (SjAb) serum samples. At a fixed concentration of human serum infected by *S. japonicum* (ratio of serum/Tris–HCl buffer = 1:5000), the LSPR response with concentrations of functionalized GNRs is investigated. The particle concentrations that are too high or too low may lead to a serious imbalance in the antigen–antibody ratio thus decreasing the sensitivity of this assay. Therefore, the absorption intensity of the functionalized GNRs is selected to be in the range of 0.3–0.8 in the subsequent experiments, and the corresponding concentration of GNRs is around  $(5.17\text{--}12.1) \times 10^{-9}$  M [30].

The concentration of the human serum specimens in this assay is also very important and so the optimal concentration is investigated. Fig. 2b shows the detection of human serum infected by *S. japonicum* using the functionalized GNRs by monitoring the successive plasmon shifts. The total time of the specific immuno-reaction process is around 20 min which may slightly vary with the target SjAb concentrations in the samples. Generally, the magnitude of the red-shift in the LPW is enhanced with increasing human serum concentration. The plasmon peak near 520 nm is not very sensitive to the refractive index change induced by target binding, thus offering no opportunity for the detection of specific target binding. However, the longitudinal peak of the nanorods is extremely sensitive to the refractive index variation. As shown in Fig. 2b, when the human serum is diluted by adding Tris–HCl buffer solution to 1:10<sup>5</sup> (human serum/Tris–HCl buffer = 1:10<sup>5</sup>), only a 2 nm



**Fig. 3.** Evaluation of human serum samples by the biosensor based on functionalized GNRs with (a) representing the specimens infected with *S. japonicum* and (b) the healthy human serum specimens.

red-shift is observed. When the concentration is increased to 1:10<sup>4</sup>, an 11 nm red-shift is observed, and the LSPR response increases with the human serum concentration. However, a high concentration of human serum, for example 1:10<sup>2</sup>, may produce a broad LSPR peak. Therefore, the concentration is chosen to be 1:10<sup>4</sup> in the subsequent specimen evaluation. In the standard methods such as IHA and ELISA for real samples, the IHA test requires the serum concentration be larger than 1:20, and that of the ELISA is larger than 1:100. These results indicate that the GNR-based assay has very high sensitivity compared to the standard methods of IHA and ELISA.

### 3.3. Single human serum specimen evaluation

To investigate the practicality of this biosensor, 43 serum samples infected by *S. japonicum* identified by IHA are analyzed under the optimized conditions and the results are shown in Fig. 3a. All the specimens show a positive response. With the exception of one sample showing a red-shift of 3 nm in the LPW, shifts larger than 5 nm are observed from the other samples and the largest is 19 nm. In the experiments, the human serum is diluted by a buffer solution to 1:10<sup>4</sup>. Gradual increase of the infected serum concentration leads to a further red-shift of the LPW. The significant red-shift in the LPW enables identification of *S. japonicum*. Our results reveal different response from various specimens and it may be attributed to the different degree of infection.

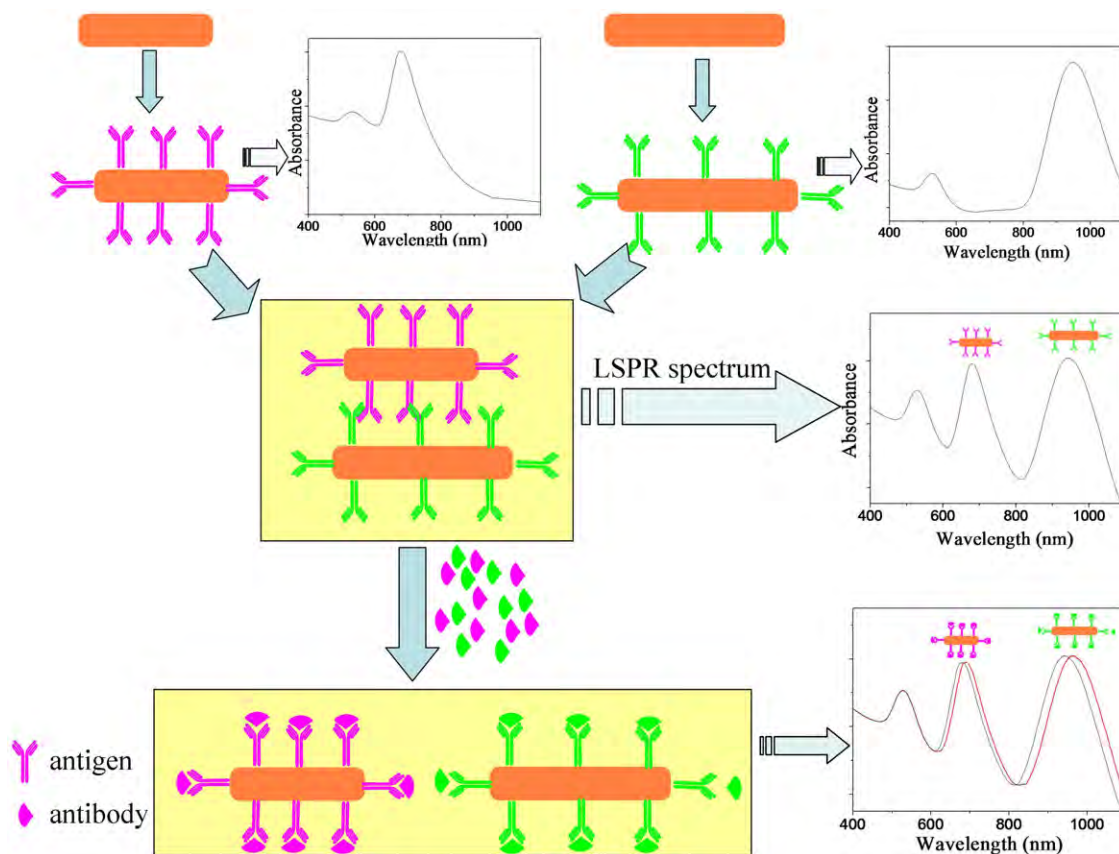


Fig. 4. Schematic illustration of multiplex LSPR biosensor based on GNRs.

The LPW of the GNRs is highly sensitive to changes in the refractive index of the surrounding medium, non-specific adsorption, or aggregation of GNRs. Control experiments are needed to verify that such effects are indeed negligible. In the control experiments, 38 healthy human serum samples were studied under the same experimental conditions and the corresponding results are presented in Fig. 3b. Some samples have slight response (average red-shift of 1.8 nm) and 8 specimens do not exhibit any changes in the LPW at all. With increase in the concentration of healthy human serum, however, there is no significant further red-shift of the LPW. All in all, the overall response observed from the control specimens is much smaller than that from the infected samples. These control experiments further demonstrate that the LSPR signals arise from the specific reaction between the antigen-antibody and functionalized GNRs in the serum samples infected by *S. japonicum* instead of non-specific adsorption or aggregation of GNRs. From the medical statistical evaluation of this assay shown in Fig. 3 obtained by the concentration of serum maintains at  $1:10^4$ , if the red-shift in the peak wavelength is larger than 5 nm, it is reasonably conferred that the serum is infected by *S. japonicum*. Similar to the results of IHA, The GNR-based assay indicates that the specimens infected by *S. japonicum* exhibit significantly positive response, boding well for regular clinical diagnostics. The GNR-based biosensor reported here can easily distinguish between uninfected samples and positive (infected) samples semi-quantitatively and shows clinical potential.

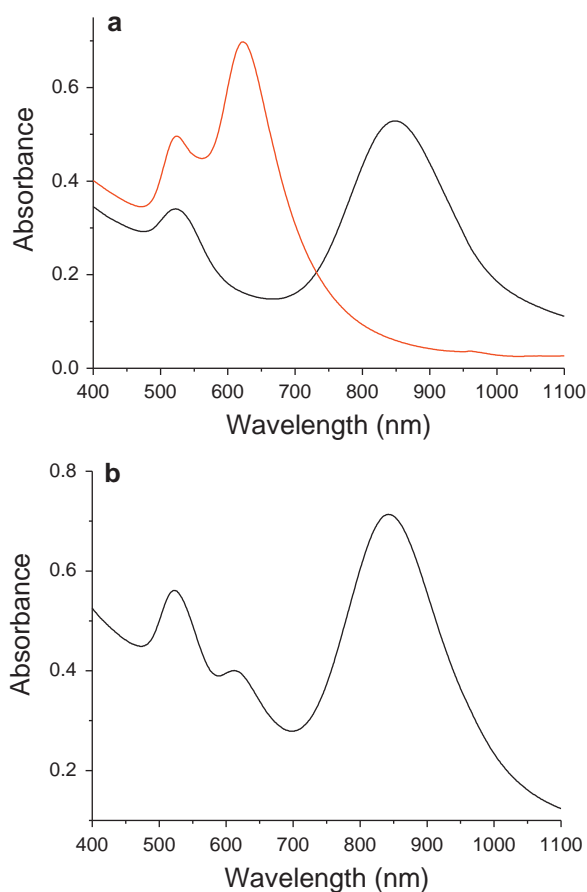
#### 3.4. Fabrication of multiplex LSPR bioanalytical assay

For an ideal multiplex SPR bioanalytical assay, the GNRs should be able to display LSPR peaks in a wide range, with the neighboring LPWs being distinctly separated. Therefore, it is crucial to choose

the appropriate GNRs. Each kind of GNRs has an intrinsic LSPR wavelength and corresponding peak width, but only those showing small peak widths can deliver the qualified peak resolution essential to the construction of multiplex SPR sensors that are capable of detecting acceptor–ligand pairs within a certain wavelength range. Since the small peak widths of the nanorods result from a narrow distribution of their shape and size, the preparation of GNRs should focus on the particle size consistency. A scheme of the multiplex LSPR optical sensor is illustrated in Fig. 4 in which different GNRs are attached by different biomolecules to act as probes.

GNRs of various aspect ratios which display accurate LPWs can be obtained by the methods developed described in a previous study [31]. The TEM images of two types of GNRs are also shown in Fig. S1. Fig. 5a shows the spectra that display the corresponding LSPR peaks of two kinds of nanorods and Fig. 5b exhibits the multiplex SPR spectrum comprising the two types of nanorods. Obviously, a proper ratio of the two kinds of nanorods can distinguish the plasmon peaks in the multiplex SPR spectrum from each other, and the ratio of GNR1 to GNR2 is about 3:5 in this study. It can be clearly seen that these LPWs are distinguished from each other, implying the potential for multiplex detection and possibility of a bio-sensing system to detect two acceptor–ligand pairs simultaneously.

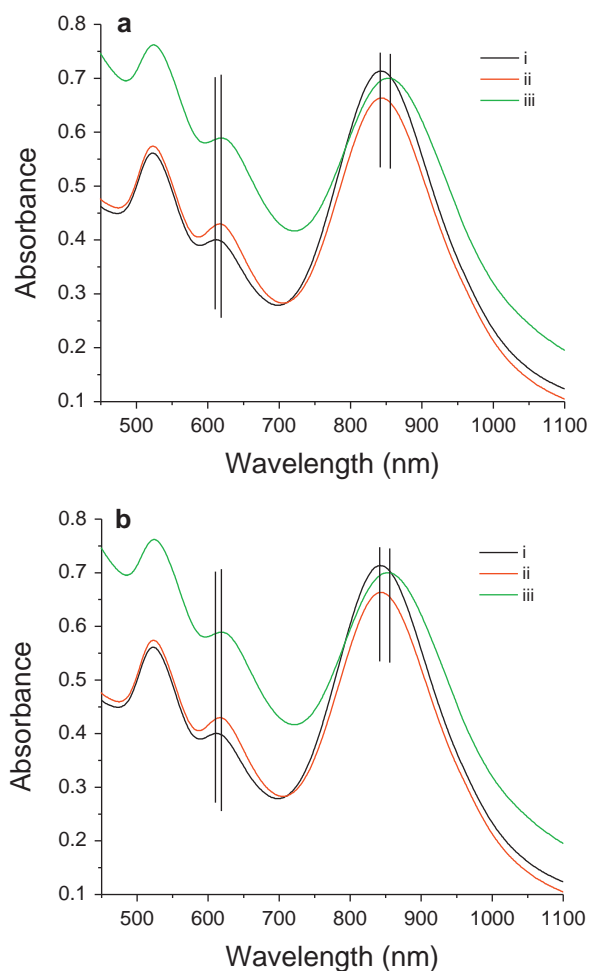
With regard to the regular LSPR apparatus, the LSPR signal is detected on a general UV–visible spectrophotometer equipped with a single-element detector. In the same way, the multiplex LSPR can measure the change in the transmission across the sample and different modified GNRs are added to the detection cell. No special detector is needed in the fabrication of the multiplex LSPR sensor and more importantly, the multiplex points associated with various acceptor–ligand pairs can be measured simultaneously.



**Fig. 5.** (a) Absorption spectra of gold nanorods with two kinds of LPWs (612 nm and 842 nm); (b) multiplex LSPR spectrum comprises two LSPR peaks resulted from GNRs related to (a).

### 3.5. Evaluation of human serum specimens infected by *S. japonicum* and TB using the multiplex LSPR bioanalytical assay

The optical evaluation of *S. japonicum* and TB is carried out using the SjAg and TBAG attached on the multiplex biosensors. The GNRs attached by SjAg dispersed in Tris–HCl buffer solution are firstly added to a cuvette, and subsequently, another type of GNRs modified with TBAG is added. The absorption spectrum of the multiplex LSPR is recorded. Besides the transverse plasmon peak, two other peaks appear in the spectrum, one at 612 nm corresponding to SjAg and another at 842 nm related to TBAG. Fig. 6a shows the wavelength change of the LSPR peaks with the addition of human serum sample infected by *S. japonicum*. It results in a 6 nm red-shift due to the interaction between the acceptor and ligand. There is no significant red-shift in the peak wavelength (842 nm) related to TB, indicating that there is no binding event between the human serum infected by *japonicum* and TBAG. Binding of human serum infected by TB to the gold nanorods functionalized with TBAG is investigated. Curve iii in Fig. 6a shows the representative absorption spectra acquired from the antigen–antibody binding experiment. Successful binding induces a 10 nm red-shift in the LSPR peak, indicating that the TB antibody (TBAb) in the human serum binds to the antigen attached onto the nanorod. Interestingly, no significant wavelength change is observed from another peak, suggesting that the TBAb in the human serum reacts specifically with TBAG chemically tethered on the GNRs. In our study, 10 pairs of specimens including 10 human serum species infected by TB and 10 human serum species infected by *S. japonicum* are analyzed by this multiplex biosensor system and they all exhibit positive responses.



**Fig. 6.** Multiplex SPR spectra comprises two LSPR peaks resulted from GNRs. (a) LSPR spectra acquired by the addition of human serum specimens: curve (ii) represents the addition of human serum infected by TB and curve (iii) is *S. japonicum*. (b) Curve (ii) represents the addition of human serum infected by *S. japonicum* and curve (iii) is TB.

Moreover, the exchange of the two kinds of GNRs functionalized with antigens is performed. Namely, SjAg is chemically tethered on the GNR related to the LPW of 842 nm and the GNR with the LPW of 612 nm is modified by TBAG. Evaluation of the other 10 pairs of specimens yields the same results, as shown in Fig. 6b. In addition, there are no detectable signals after the addition of healthy people sera. These results indicate that this multiplex biosensor can simultaneously detect different diseases.

Separate experiments are conducted to investigate the mutual influence of the two types of GNRs in this assay. Firstly, only the GNRs attached by TBAG are placed in the cuvette with the buffer solution. The LSPR peak is observed at 615 nm is slightly different from the multiplex system at 612 nm in which the spectrum is the sum of the two of absorption spectra of the two types of nanorods. A gradual red-shift of the LPW appears after addition of the human serum infected by TB. Fig. S2 shows the change in the LSPR peaks after addition of the human serum infected with TB, indicating that the TBAb in the human serum reacts specifically with TBAG attached on GNRs. Although the LSPR position changes slightly compared to the multiplex LSPR system, the two systems exhibit the similar behavior.

A series of human samples infected by *S. japonicum* is added to isolated GNRs attached by SjAg. The similar results displayed in Fig. S3 show the representative changes in the absorption spectra before and after addition of the human serum infected by

*S. japonicum*. The peak position of the LSPR spectrum at 845 nm is slightly different from that of the multiplex system too. A red-shift of the LPW occurs after addition of the human serum infected by *S. japonicum*, indicating that the nanorods in the separate experiments or the multiplex system exhibit the similar behavior in the analytical process. However, the multiplex LSPR system takes advantage of that the two pairs of acceptor–ligand can be detected simultaneously. Moreover, this method may be further refined to deliver even higher throughput if various nanorods with the proper LPW are carefully designed and fabricated.

#### 4. Conclusion

A bioanalytical assay is developed by incorporating two types of GNRs. In addition to preserving the desirable features of common GNR LSPR sensors, the sensor is easy to fabricate and only requires a visible–NIR spectrometer for detection. This assay provides simultaneous detection of different acceptor–ligand pairs by properly choosing GNRs possessing various LPWs in a wide detection wavelength range and has large potential as a high-throughput detection device. The bioanalytical assay allows easy detection of human serum specimens infected by *S. japonicum* and TB without sample pretreatment. Moreover, the GNR-based assay boasts high sensitivity compared to standard methods such as IHA and ELISA. This sensing approach is faster, easier to perform, and less expensive compared to conditional standard methods and can be readily extended to other immunoassays to enable wider diagnostic applications.

#### Acknowledgments

This work was supported by Natural Science Foundation of China (21075035), Hunan Provincial Natural Science Foundation of China (10JJ5004), and Hong Kong Research Grants Council (RGC) General Research Funds (GRF) Nos. CityU 112510 and 112212.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aca.2012.10.020>.

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