Real-time monitoring of T-cell-secreted interferon-\(\gamma\) for the diagnosis of tuberculosis

Changlin Wu\(^a\), Jianan He\(^b\,c\), Boan Li\(^d\), YunQing Xu\(^b\,c\), Dayong Gu\(^b\,c\), Houming Liu\(^e\), Dan Zhao\(^e\) and Chaopeng Shao\(^a\)

\(^a\)Clinical Transfusion Laboratory, Second People’s Hospital of Shenzhen, First Affiliated Hospital of Shenzhen University, Shenzhen, PR China; \(^b\) Central Laboratory, Shenzhen Academy of Inspection and Quarantine, Shenzhen Entry-Exit Inspection and Quarantine Bureau, Shenzhen, PR China; \(^c\) Central Laboratory of Health Quarantine, International Travel Health Care Center, Shenzhen Entry-Exit Inspection and Quarantine Bureau, Shenzhen, PR China; \(^d\) Clinical Laboratory, 302 Military Hospital of China, Beijing, PR China; \(^e\) Clinical Laboratory, Third People’s Hospital of Shenzhen, Shenzhen, PR China

ABSTRACT

In this paper, we report the development of a label-free biosensor, based on surface plasmon resonance, for real-time monitoring of captured human CD4\(^+\) T-cells, and their dynamic cytokine production upon specific antigen stimulation. Microarrays of CD4\(^+\)T-cells, and interferon gamma (IFN-\(\gamma\)) specific antibody (Ab) spots were printed side by side onto the poly(OEGMA-co-HEMA) matrix. The placement of CD4\(^+\) T-cells near the anti IFN-\(\gamma\) Ab-coated spots ensured a high local concentration of secreted IFN-\(\gamma\) for detection. We have demonstrated that this approach enables the detection of tuberculosis (TB) infection in clinical samples, with an overall sensitivity of 85.5% and an overall specificity of 97.7%.

Introduction

Tuberculosis (TB) continues to be a serious public health problem; TB was the cause of approximately 1.5 million deaths in 2014 [1,2]. Laboratory confirmation of TB is key to ensuring that any individuals with TB signs and symptoms are correctly diagnosed and treated [3]. Stain sputum smear microscopy is the primary laboratory tool for TB diagnosis because it is inexpensive and compatible with the majority of laboratory work conditions. However, the direct smear microscopy can be used to detect the specimens containing more than 10,000 AFB/mL, with sensitivity varying from 20% to 80%. Most of the discrepancies are due to poor sensitivity and specificity of direct smear microscopy, which may cause many TB cases to remain missed [4]. In addition, the bacterial culture method is considered most accurate; however, this technique is time-consuming and labour-intensive, due to the fact that Mycobacterium tuberculosis is a slow growing bacterial species. Early detection of TB is critical to reducing the TB-related morbidity and mortality [5]. Therefore, a fast and inexpensive detection method is indispensable for controlling the disease.

Interferon gamma (IFN-\(\gamma\)) is an important cytokine released by T-cells upon stimulation with pathogenic antigens. Therefore, it is often used to evaluate cellular immune responses to infectious diseases [6], and is employed clinically to diagnose TB. IFN-\(\gamma\) release assays (IGRAs) have been recently developed for measurement of T-cell release of IFN-\(\gamma\) following stimulation by antigens. Although these immunoassays are sensitive and specific, they are often expensive and time-consuming, involving multiple washing steps, and do not allow real-time detection [7–9].

Surface plasmon resonance (SPR) is an established technique for label-free bio-molecular interaction analysis in the field of life-science and pharmaceutical research. It can provide a label-free and real-time format to investigate biomolecular interaction and operates with high-throughput analysis [10]. While SPR has been successfully applied to measure the binding in a large number of bio-molecular interactions, such as protein–protein, peptide–protein, DNA–DNA [11] and protein–drug [12], there have been just a few attempts to conduct analysis in complex media, such as serum or cell lysates, due to the challenges in controlling non-specific adsorption [13,14]. To address these issues, we have developed a three-dimensional (3D) surface matrix based on copolymers of oligo(ethylene glycol) methacrylate(OEGMA) and 2-hydroxyethyl methacrylate (HEMA),...
namely poly(OEGMA-co-HEMA), via surface initiated polymerization [15]. In addition to the excellent non-fouling properties, the poly(OEGMA-co-HEMA)-based brush has abundant functional groups for the immobilization of biological ligands. Potential applications of this surface chemistry platform enable us to detect specific targets in a complex environment.

In this study, we developed a new biosensor based on this 3D surface chemistry platform for indirect detection of TB in clinical samples. The SPR sensor was modified with poly(OEGMA-co-HEMA) brush, so as to reduce non-specific protein adsorption, and provided a large number of sites for capture antibody immobilization. CD4+ T-cells and IFN-γ Ab were printed side by side onto the poly(OEGMA-co-HEMA) brush. Upon injecting blood, free of erythrocytes, into the SPR sensor cell, CD4+ T-cells were captured by anti-CD4 antibody on the surface and then stimulated with TB specific antigen (e.g. ESAT-6, CFP-10) to release IFN-γ. IFN-γ released by CD4+ T-cells was then detected by anti IFN-γ antibody at the neighbouring site. Importantly, IFN-γ released by CD4+ T-cells could be monitored in a real-time format by SPR, and provides an opportunity to determine whether the CD4+ T-cells have been infected with TB previously. The SPR based biosensor was finally used to evaluate the clinical samples and the results were compared to those from the PCR assay, smear microscopy and culture methods. The SPR assay may offer new directions for fast TB detection, and may be used for routine clinical TB diagnostics in the future.

**Materials and methods**

**Materials**

The initiator thiol (ω-mercaptopundecyl bromoisobutyrate) was purchased from HRBio (Beijing, China), 11- (mercaptopoundecyl)tri(ethylene glycol), OEGMA, HEMA, anhydrous N, N′-dimethylformamide (DMF), succinic anhydride, and 4-(dimethylamino)pyridine (DMAP), N-ethyl-N′-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Aldrich. ESAT-6, CFP-10 and ethanolamine were purchased from Sigma. Mouse anti-human CD4 antibody, goat anti-human IFN-γ antibody and human recombinant IFN-γ were purchased from Biolegend Company.

**Sample processing**

Blood samples were obtained from 83 patients (46 males and 37 females) and 43 healthy donors (24 males and 19 females), through the Second People’s Hospital of Shenzhen (Guangdong Shenzhen, China). These patients had an average age of 34.3 years (range: 17–68 years). Two millilitres of peripheral blood were collected from TB infected patients and non-TB infected people; the erythrocytes were removed according to protocols described previously [15,16]. The peripheral blood mononuclear cells (PBMC) were collected and diluted to a final concentration of 1 × 10^6/mL with phosphate buffered saline (PBS) for the SPR assay.

**Preparation of the 3D matrix and fabrication of the protein array**

The SPR coated with 3D surface matrix was prepared as previously described [15]. Briefly, the fresh clean SPR chip was immersed into 1 mmol/L mixture of initiator and 11-(mercaptoundecyl) tri(ethylene glycol) solution for 15 h at room temperature to form self-assembled monolayers (SAM). The reaction solution was prepared by mixing well with MilliQ-water (5 mL), methanol (5 mL), OEGMAS26 (2.62 g, 5 mmol/L), HEMA (0.65 g, 5 mmol/L), bipyridine (12.5 mg, 0.8 mmol/L) and CuCl₂ solution (1 mL, 0.04 mmol/L). The mixture was deoxygenated with N₂ for 15 min; then, 1 mL ascorbic acid (AscA) (0.04 mmol/L) was injected with a syringe. This mixture was deoxygenated with N₂ for 15 min, then put into a reaction setup in Inert Gas Glove Box. Surface initiated polymerization (SIP) was done at room temperature for 8 h. The SPR chips were subsequently rinsed with methanol, MilliQ-water and dried with N₂. To introduce carboxyl groups in the polymer, the SPR chips were immersed in a DMF solution, which contains succinic anhydride (10 mg/mL) and DMAP (15 mg/mL) for 12 h under room temperature.

The 3D matrix was activated with an aqueous fresh mixture of NHS (0.4 mol/L) and EDC (0.1 mol/L) for 30 min. Rabbit IgG, anti-human IFN-γ antibody and anti-human CD4 antibody were diluted with PBS buffers at 50 μg/mL concentration, then printed onto the SPR chip with a Microarray Printing System, respectively.

**Specificity and sensitivity for IFN-γ detection**

The specificity and sensitivity for IFN-γ detection were confirmed by SPR technology. The IFN-γ Ab and rabbit IgG Ab (negative control) were printed on the SPR chip. Each antibody was printed at the same concentration of 100 μg/mL. After the chip was treated with 1 mL of ethanolamine (1 mol/L, pH 8.0) to deactivate the activate groups in the matrix, a sample containing IFN-γ was injected, to display the specific interaction and non-specific adsorption. To investigate the detection limit of IFN-γ, 1.0 mL of different concentrations of IFN-γ standard
(50, 100, 200, 400, 800 and 1200 ng/mL) were diluted with PBS buffer and were injected over the chip at a flow rate of 3 \( \mu L/s \). The limit of detection (LOD) of the SPR biosensor for IFN-\( \gamma \) was calculated.

**Capture of CD4 T-cells on the SPR chip**

The PBMCs were isolated from whole blood and diluted with PBS buffer at a concentration of \( 1 \times 10^6/\text{mL} \). PBS (\( \text{pH} = 7.4, 10 \text{ mmol/L} \)) was injected into the flow chamber to establish a baseline, then 800 \( \mu L \) of PBMC solution was injected at a flow rate of 3 \( \mu L/s \) over the anti-CD4 antibody coated surface. To wash away any non-specific blood cells, the sample was replaced by PBS at a rate of 6 \( \mu L/s \). The captured CD4\(^+\) T-cells were further identified with immunofluorescent staining by injecting the diluted FITC-CD4\(^+\) antibodies onto the SPR chip. The captured CD4\(^+\) T-cells were imaged with a fluorescence microscopy. The purity of the captured CD4\(^+\) T-cells on the Ab-modified SPR chip was analysed by FCM, and the activity of these CD4\(^+\) T-cells was determined by trypan blue staining.

**Real-time detection of T-cell secreted IFN-\( \gamma \)**

According to the preceding study, SPR was used for real-time monitoring of CD4+ T-cell secreted IFN-\( \gamma \). PBMCs solution collected from TB patients and healthy donors was introduced into the SPR chip coated with anti-CD4 Abs and IFN-\( \gamma \) Abs. The CD4\(^+\)-cells were then captured by anti-CD4 Abs. After that, RPMI1640 phenol red-free cell culture media was used as a running buffer to obtain a steady baseline. In order to stimulate the captured CD4\(^+\) T cells to release IFN-\( \gamma \), culture media containing the TB-specific proteins, ESAT-6 and CFP-10 (final concentration 2 \( \mu g/mL \)) was injected. SPR signals were recorded in real-time during the addition of the TB-specific proteins, which allowed to measure how many IFN-\( \gamma \) molecules secreted by CD4+ cells were bound to the surface. The sensing mechanism is shown in Figure 1.

**Results and discussion**

**Properties of the novel matrix**

In order to enhance the performance of the biosensor, the sensor surface had to be modified to prevent protein non-specific adsorption when exposed to a complex sample. For this purpose, we employed a surface initiated polymerization technique to prepare a 3D poly [oligo(ethylene glycol) methyl ether methacrylate] (POEGMA) matrix. The matrix served a dual function: PEG groups helped to reduce protein non-specific adsorption in complex samples, and the carboxyl groups provided sites for the covalent attachment of biomolecules while retaining biological activity. The polymer was characterized with AFM and ellipsometer, and the thickness of the polymer was \( \sim 10 \text{ nm} \) \[15\]. To measure the non-specific protein adsorption level, PEG SAM and POEGMA matrix coated SPR chips were fixed onto the SPR sensor respectively. Complex samples of 10% human blood plasma (diluted in PBS) were injected for 5 min at a flow rate of 3 \( \mu L/s \). The non-specific adsorption level was calculated from the baseline changes before and after sample injection. Comparison of POEGMA matrix and PEG-SAM was carried out. The results in Figure 2 showed that the POEGMA matrix provided a lower non-specific adsorption level.
could reduce the non-specific protein adsorption to a level below the detection limit of SPR in 10% human serum, while the PEG-SAM matrix has an unacceptable level of non-specific protein adsorption (0.7 Δ% R, corresponding to a protein surface coverage of 110 pg/mm²) [15]. These experimental results demonstrated that the POEGMA surface was suitable for applications in a complex environment.

**Specificity and detection limit of IFN-γ**

For detecting IFN-γ secreted by CD4+ T-cells, microarrays of CD4+ T-cell and IFN-γ specific antibody spots were printed onto POEMGA-coated SPR chips as follows. Prior to printing, POEMGA-coated SPR chips were activated with a mixture of NHS (0.4 mol/L) and EDC (0.1 mol/L) for 30 min, and then anti-human IFN-γ antibody and anti-human CD4 antibody, diluted with spotted buffers (10 mmol/L PBS, pH = 7, containing 10% glycerin) at a concentration of 100 μg/mL, were printed onto the activated surface with a microarray printing system.

To investigate the performance (e.g. sensitivity and specificity) of the SPR biosensor for IFN-γ detection, different concentrations of recombinant IFN-γ (50–800 ng/mL) were diluted with PBS (10 mmol/L PBS, pH = 7) and were injected into the SPR sensor cell at a flow rate of 3 μL/s. Figure 3(a) shows the SPR response to the varying concentrations of IFN-γ, and Figure 3(b) shows the calibration curve constructed according to the SPR response value. It shows that the SPR response values decreased, with decreasing concentrations of IFN-γ. The detection limit of this method for IFN-γ was ~50 ng/mL. For the specificity test, we injected a non-specific model protein (rabbit IgG), with a high concentration (20 μg/mL), into an anti-IFN-γ antibody-coated SPR sensor, which did not result in any detectable signal (data not shown).

**Real-time monitoring of CD4+T cells produce IFN-γ**

For detecting antigen-specific IFN-γ from TB patients by the SPR assay, we first used anti-human CD4 antibody as a ligand to trap CD4+ T-cells from the clinical sample. Two milliliters of peripheral blood were collected from the TB patients and the healthy controls. The erythrocytes were removed according to previously described procedures [16–18]. The PBMCs were isolated and diluted in PBS buffer at a concentration of 1 × 10⁶/mL for the SPR assay. The PBMCs solution was introduced onto the SPR chip immobilized with anti-CD4 Abs for capturing CD4+ T-cells. As shown in Figure 4(a), the specific binding to the anti-CD4 antibody spots resulting in SPR response was significant. In comparison, the SPR response to the other spots was very low, proving that the CD4+ T-cells were successfully captured in the spot coated with anti-CD4 Abs. Further evidence that the CD4+ T-cells were captured, was their cross identification, by incubating with fluorescently-labelled anti-CD4 Ab. The fluorescent image was presented in Figure 5, showing that CD4+ T-cells were specifically captured in the spot coated with anti-CD4 Ab, but not on the POEMGA matrix regions, or on the nonspecific Ab spots. These results demonstrated our ability to isolate CD4+ T-cells in specific locations from a complex environment. To investigate the activity of captured cells, a regeneration solution was injected to release the cells from the SPR surface, which were then collected for a viability test. The viability of these CD4+ T-cells was more than 90% by trypan blue stain.

IFN-γ detection experiments were conducted by injecting a 2 μg/mL antigen-specific solution to stimulate the captured CD4+ T-cells. The antigen solution consisted of 1 μg/mL each of ESAT-6 and CFP-10 in PBS. The IFN-γ release by CD4+ T-cells was then detected by the adjacent anti-IFN Ab spots, and the IFN-γ release levels were monitored in real-time with SPR. A real-time SPR imaging curve for detection of IFN-γ released from CD4+...
T-cells is shown in Figure 4(b). In this study, CD4+ T-cells from TB patients were captured on the chips and activated with ESAT-6 and CFP-10 antigens to induce IFN-γ production. The SPR response value for IFN-γ production was 0.27 Δ%R, which converted into an IFN-γ concentration of approximately 100 ng/mL according to the calibration curve shown in Figure 3(b). Such high local concentration of IFN-γ may be explained by the placement of cells close to the anti-IFN-γ Ab spots. In contrast, CD4+ T-cells from healthy people that were simulated with TB specific antigens showed no change. These results demonstrated that the specific IFN-γ secreted by CD4+ T-cells of TB patients was a direct consequence of TB infection. The patients with TB and healthy controls were confirmed to be TB positive or negative by sputum-smear microscopy after culture or PCR. The detection sensitivity and specificity of this SPR-based platform for the detection of TB in patients was 85.5% (72/83) and 97.7% (42/43), respectively. The results are superior to direct smear microscopy. However, the test result must always be confirmed by culture.

IFN-γ is released from CD4+ T-cells as adaptive immunity against various pathogens. Due to immunological memory, it may be used to determine if a person has been prior exposed to infectious diseases, such as HIV and TB \[19–21\]. In this study, we described a strategy to design a novel surface matrix via surface initiated polymerization, rendering the biosensor non-fouling to proteins and cells in a complex environment. We then evaluated this novel system for its efficacy in the detection of TB infection from clinical blood samples. Using this platform, we have shown a novel approach to trap CD4+ T-cells onto anti-CD4 Ab-coated regions and retaining their biological activity, and then stimulating the captured cells with TB antigen. Compared to some previous reports on TB diagnostics, the IGRA method based on the SPR platform is promising and can be rapidly developed into a reliable diagnostic method, which is simple and rapid, allows dynamic real-time detection and has better specificity. The result is more reliable, but the sensitivity is lower than that of DNA testing. The detection limit of this method can, however, be improved by using a modified SPR chip (such as the graphene modified SPR chip). To detect the DNA of TB by the SPR platform, the process is complex, and has more interfering factors, so it is more easy to produce false-positive or false-negative results \[22–24\].

**Conclusions**

In summary, the objectives of this work were to develop a simple and accessible methodology for TB detection. Although the number of cases involved in this study was limited, our study clearly illustrates that this SPR assay could be utilized to achieve high sensitivity and more convenience in the direct detection of TB patients. The strategy described here offers several advantages: (1) surface chemistry based on POEGMA enables us to
detect specific targets in complex environments without sample preparation. (2) The antibody microarray allows us to perform T-cell capturing and IFN-γ detection in the same chip; such placement of cells near anti-IFN-γ Ab-coated spots ensures high local concentrations of secreted IFN-γ for detection. (3) IFN-γ released by CD4+ T-cells can be monitored in real-time, allowing us to determine the section rate, which is associated with a vigorous immune response to infections. This platform, combining SPR technology with protein array, may be employed for diagnosis of TB infection. This represents an important addition to current TB diagnosis, and in the future, this strategy could be reliably applied to routine laboratory diagnosis.

Disclosure statement
The authors declare no conflict of interest.

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