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A NEW QUANTITATIVE DETECTION METHOD OF RECOMBINANT CFP10-ESAT6 AMALGAMATION PROTEINS FROM MYCOBACTERIUM TUBERCULOSIS BASED ON MICRO-MAGNETIC PROBES STRATEGY

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A new rapid, specific and sensitive method for assay of recombinant CFP10-ESAT6 amalgamation proteins from *Mycobacterium tuberculosis* was proposed. The method used streptavidin-coated magnetic beads to enrich the specific biotinylated anti-CFP10 antibody, then adopted a sandwich-type enzyme linked immunosorbent assay technology with two kinds of monoclonal antibodies: biotinylated anti-CFP10 antibody and HRP-labeled anti-CFP10 antibody to identify the target CFP10-ESAT6 proteins, and finally detected chemiluminescence intensity by a small home-made optical sensor. It was shown that, the corresponding chemiluminescence intensity had a good logarithmic linear response to the concentration of CFP10-ESAT6 proteins when ranging at $1 \sim 1000$ ng/mL, and the correlation coefficient is 0.9937. The proposed method could detect the CFP10-ESAT6 proteins with low detection limit (1 ng/mL) and the detection time could be controlled within 45 min. Compared with commonly used detection methods of *M. tuberculosis*, this method was easy to operate, faster, and of higher sensitivity. The achievement of the quantitative detection of CFP10-ESAT6 proteins has important scientific significance and wide application prospects in tuberculosis control.

Keywords: Enzyme linked immunosorbent assay; chemiluminescence; home-made optical sensor.

1. Introduction

Mycobacterium tuberculosis is an old-aged and increasing public infectious disease in the world.[1] It has been considered as one of the most imperative global infectious disease, promulgated by WHO. It has remained a major challenge for mankind because of the morbidity and mortality it causes.[2] At present, about two billion people, accounting for

nearly 1/3 percent of people on the earth, are infected by *M. tuberculosis*, and 20 million people suffered from it, with about 9 million new ones and 3 million ones die every year.[3] So, it is of great significance to investigate a simple, sensitive and fast detection method, which will achieve early diagnosis and impactful control of *M. tuberculosis*.

The standard diagnosis of tuberculosis still relies on the smear microscopy observation and bacterial cultivation of *M. tuberculosis*. However, these diagnoses have their limits, such as low sensitivity of smear microscopy observation and the long time needed for bacterial cultivation (6−8 weeks).[4] The traditional diagnostic tests for *M. tuberculosis* are the tuberculin skin test, which is also known as purified protein derivative (PPD) test, and the more interferon gamma release assays (IGRAs). The PPD test is unspecific because of cross-reaction to antigens present also in Bacille Calmette−Guérin (BCG) vaccine strains and in environmental mycobacteria. The IGRAs are more specific, but it also cannot discriminate between different clinical entities, such as active tuberculosis or a mere immunological memory of previous tuberculosis disease.[5] Polymerase chain reaction (PCR) is a highly sensitive and specific molecular detection method for *M. tuberculosis* disease.[6] But as the high costs and complex operations in molecular detection, it is not suitable as a means of clinical detection method. Besides, enzyme linked immunosorbent assay (ELISA) is also reported for tuberculosis protein detection with low detection limit (25 ng/mL),[7−9] and the common ELISA method needs more than 4 h.

Assays based on antigen detection display both high specificities and high sensitivities,[10,11] then many studies have focused on the detection of antibodies specific for different *M. tuberculosis* antigens that indicate active disease. 6 KDa early secretory antigenic target (ESAT-6) and 10 KDa culture filtrate protein (CFP-10) are both secretory proteins from *M. tuberculosis*.[12,13] They deserve importance because of their ability to invoke immune responses.[14] ESAT-6 and CFP10 are both immunogenic proteins expressed from region of deletion-1 (RD-1), which is absent in BCG.[15,16] They exist only in deleterious tuberculosis mycobacterium, so as to eliminate the interference of BCG.[17] The amalgamation of these two proteins was investigated by enzyme-linked immunospot assay (ELSPOT) method, testifying that CFP10-ESAT6 amalgamation proteins have higher sensitivity and specific property compared to each of the single protein.[18] Therefore, CFP10-ESAT6 amalgamation proteins are suggested as a much proper antigen protein in *M. tuberculosis* diagnoses. For this antigen protein is always present with low concentration in the early infected body, it is significative to make further study in fast, sensitive and low-concentration detection.

Nowadays, to develop a rapid and highly sensitive method, chemiluminescence (CL) sensors based on magnetic particle and enzyme signal amplification have been widely researched. And it is useful in protein detection area, which is of great importance in diseases diagnosis, food safety, etc. In this study, we applied enzyme-linked immunomagnetic CL to the rapid detection of CFP10-ESAT6 amalgamation proteins by using a home-made optical sensor.[19] This method combined high selectivity of CFP10-ESAT6 proteins, high specification of enzyme-linked immunosorbent assay, high rate and easy operation of magnetic enrichment and high sensitivity of CL detection. The result showed that this method was successful in specific proteins detection.

2. Experimental Procedure

2.1. *Apparatus*

A UV spectrophotometer, to measure the optical absorbency of initial antibody solution and the supernatant at wavelength 280 nm, in order to calculate the rate of anchoring anti-CFP10 antibody onto micro-magnetic particles (MMPs), (Shimadzu, Japan).

A home-made optical sensor, to detect the CL intensity of the immunocomplex (magnetic probes−target CFP10-ESAT6 proteins−horseradish peroxidase (HRP)-conjugated anti-CFP10).

2.2. *Reagents and materials*

The following chemicals and reagents were used in the experiments:

Biotinylated and HRP-conjugated monoclonal mouse anti-Mycobacterium tuberculosis CFP10, purchased from HyTest Ltd. (HyTest, Finland).

Recombinant CFP10-ESAT6 fusion proteins, purchased from Wason Biotech Inc. (Wason Biotech, China).

Streptavidin-coated MMPs with the size of 2.8 μm, purchased from Dynal Biotech (Dynal, Spain).

Max-G HRP CL reacting substance, purchased from Heshangyou Biotech Inc. (Heshangyou Biotech, China). The reacting substance contains two parts: A is luminal and enhancer solution and B is H_2O_2 solution.

All other reagents were in analytical grade.

2.3. *Anchoring CFP10 antibody onto micro-magnetic particles*

As shown in Fig. 1, the binding of the biotinylated anti-CFP10 antibody to streptavidin-coated MMPs was achieved through a streptavidin−biotin bridge. Specific magnetic probes were prepared according to the following steps.

First, 50 μL of streptavidin-coated MMPs (10 mg/mL) was transferred into a 2 mL centrifuge tube. The MMPs were washed for three times with 200 μL of wash buffer (10 mM PBS, pH 7.4, 0.05% Tween-20) while physically retaining them on a magnet.

Second, 100 μL of biotinylated anti-CFP10 antibodies (200 ng/mL) were added to 50 μL of streptavidin-coated MMPs for the preparation of 2.8 μm magnetic probes. Then the mixtures were incubated at 37°C for 30 min with 150 rpm/min, which formed magnetic probes.

Third, wash the magnetic probes for three times with 200 μL of wash buffer (10 mM PBS, pH 7.4, 0.05% Tween-20) while physically retaining them on a magnet. The supernatant fluid and the washed supernatant fluid were carefully transferred into a 2 mL centrifuge tube for the downstream detection of absorbance.

After the washing steps, the probes were dispersed in 500 μL PBS and were stored in a refrigerator at 4°C for the downstream use.

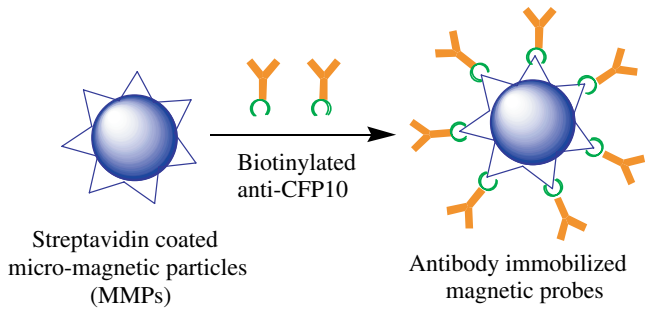

Fig. 1. Preparation of antibody immobilized magnetic probes.

2.4. *Immunoassay for CFP10-ESAT6 fusion proteins*

As shown in Fig. 2, the recombinant CFP10-ESAT6 fusion proteins were detected by a sandwich-type ELISA using two kinds of monoclonal antibodies: Biotinylated anti-CFP10 antibody and HRP-labeled anti-CFP10 antibody, which can recognize different epitopes of CFP10-ESAT6 proteins. The immunoassay procedures are as follows.

First, 50 μL of the above magnetic probes solution, 50 μL of CFP10-ESAT6 proteins solution and 50 μL of HRP-labeled anti-CFP10 antibody solution were mixed in a 2 mL centrifuge tube. The mixtures were incubated at 37°C devoid of light for 20 min. Through this interaction between antibody and antigen, the sandwich immunocomplex (magnetic probes−target CFP10-ESAT6 proteins−HRP-conjugated anti-CFP10) was formed.

Second, the centrifuge tube was placed onto the magnet for 2 min. The formed sandwich complexes were magnetically separated and washed for four times with 200 μL of wash buffer (10 mM PBS, 0.05% Tween-20 included). Then the excess HRP-conjugated antibody was removed.

Third, the immunocomplex was incubated with 100 μL of HRP CL reacting substance (50 μL of A and 50 μL of B) at room temperature devoid of light for 10 min.

Finally, the CL intensity was detected by the small home-made optical sensor.

3. Results and Discussion

3.1. *The ratio of immobilized antibodies on the surface of MMPs*

The ratio of the immobilization that the capture antibody conjugated to MMPs was confirmed by the UV spectrophotometer. The amount of anti-CFP10 antibody modified on the MMPs could be calculated from the different optical absorbency at 280 nm of the anti-CFP10 antibody solution before immobilization and that after immobilization. Assume that the absorbency of the anti-CFP10 antibody solution before and after immobilization is A_0 and A_1, respectively, and R_a indicate the immobility rate, then $R_a = [(A_0 - A_1)/A_0] \times 100\%$.

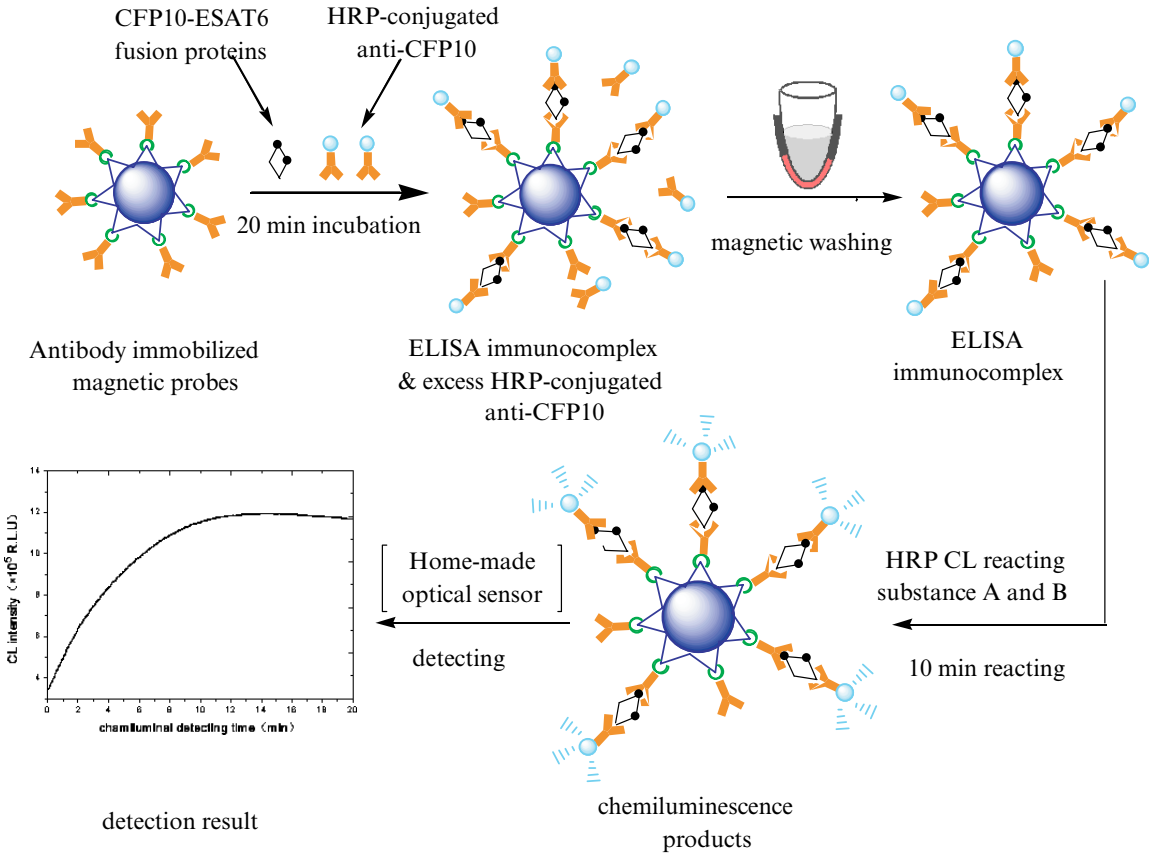

Fig. 2. Procedure for detection based on the magnetic probes.

It was calculated that the average immobility rate of capture antibody was about 90% for the preparation of 2.8 μm magnetic probes. This indicates that the aiti-CFP10 capture antibody was effectively immobilized on the surface of MMPs.

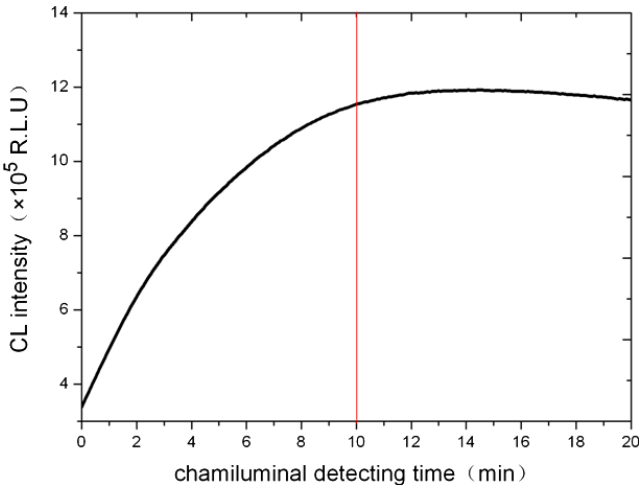

Fig. 3. CL intensity at different time (100 ng/mL CFP10-ESAT6 solution).

3.2. *Determination of CL detecting time*

In order to determine the CL detection time, 100 ng/mL CFP10-ESAT6 solution was investigated by the home-made optical sensor. As shown in Fig. 3, CL intensity increases rapidly in the former 10 min, and almost reach the maximal relative lumen unit (R.L.U) at 10th minute, so the CL detection time was set to be 10 min.

3.3. *CL intensity of various concentrations of CFP10-ESAT6 protein*

The relationship between CFP10-ESAT6 concentrations and CL intensity was investigated by the home-made optical sensor. The CFP10-ESAT6 protein solution was diluted to 1000, 300, 100, 30, 10, 3, 1 and 0 ng/mL in 10 mM PBS buffer and detected. Calibration curve were obtained from the CL intensity for CFP10-ESAT6 concentrations in the range 1 $\sim$ 1000 ng/mL and are shown in Fig. 4. The curve

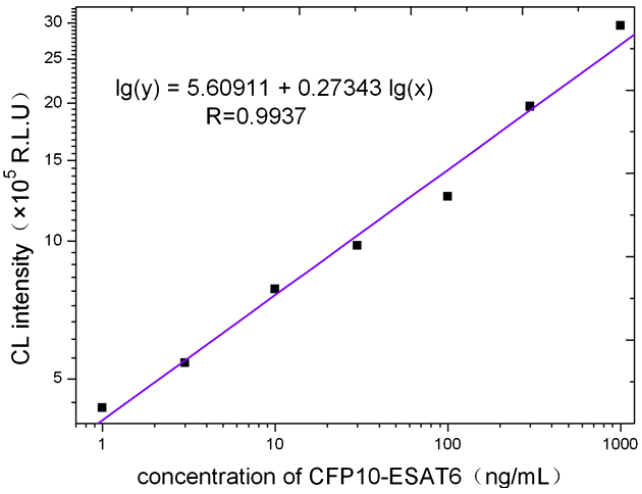

Fig. 4. Detection results of CFP10-ESAT6 protein in different concentration.

Table 1. Result of the sensitivity of the new method.

Concentrations (ng/mL)	CL Intensity (R.L.U)	Slope Factor (R.L.U/(ng/mL))
1000	2955530	
100	1250502	
10	785206	2463
1	436253	
0	113228	

equation is $\lg(y) = 5.60911 + 0.27343\lg(x)$, and the relative coefficient is 0.9937. A detection limit of 1 ng/mL was observed. The sensitivity of the new method was shown in Table 1. The slope factor of this new method is 2463 R.L.U/(ng/mL), which indicated high sensitivity of this new method. Besides, this new method is operated more simply and rapidly with detection time less than 45 min.

Table 2. Results of repetitive experiments (500 ng/mL CFP10-ESAT6 proteins).

No.	CL Intensity (R.L.U.)	Average (R.L.U.)	STDEV (R.L.U.)	CV (%)
1	2,553,496			
2	2,352,168			
3	2,321,035			
4	2,402,809			
5	2,254,081	2329111.3	104113.1	4.47
6	2,230,956			
7	2,208,136			
8	2,331,105			
9	2,308,216			

3.4. *Repetitive characteristic*

The repeatability of this method was researched. The experiment was done for nine times with 500 ng/mL CFP10-ESAT6 proteins solution as detection sample. The result was shown in Table 2. The CV is less than 5% (CV = 4.47%). It reflects that this method is repetitive and reliable, which can be used as a rapid CFP10-ESAT6 or maybe other proteins.

4. Conclusion

In this work, a sensitive, fast and quantitative detection method of recombinant CFP10-ESAT6 fusion proteins from *M. tuberculosis* was carried out by using the magnetic probe strategy. The combination of the sandwich-type ELISA method and micro-magnetic probes strategy made the detection much easier, faster and more sensitive as well. Meanwhile, the adoption of CL detection system further improved the sensitivity. It was also suggested that the method could detect the CFP10-ESAT6 proteins with low detection limit and short detection time, which is helpful to the early diagnosis of *M. tuberculosis* disease.

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