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Using fluorescent nanoparticles and SYBR Green I based two-color flow cytometry to determine *Mycobacterium tuberculosis* avoiding false positives

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ABSTRACT

A method using an improved two-color flow cytometric analysis by a combination of bioconjugated fluorescent silica nanoparticles and SYBR Green I (FSiNP@SG-FCM) has been developed for detection of pathogenic *Mycobacterium tuberculosis*. Antibody-conjugated nanoparticles were prepared by oriented immobilization of the anti-*M. tuberculosis* antibody onto Tris(2,2-bipyridyl)dichlororuthenium(II) hexahydrate-doped fluorescent silica nanoparticles (RuBpy-doped FSiNPs) through Protein A. *M. tuberculosis* was specially labeled with antibody-conjugated RuBpy-doped FSiNPs, then stained with a nucleic acid dye SYBR Green I to exclude background detrital particles, followed by multiparameter determination with flow cytometry. With this method, false positives caused by aggregates of nanoparticle-bioconjugates and nonspecific binding of nanoparticle-bioconjugates to background debris could be significantly decreased. This assay allowed for detection of as low as 3.5×10^3 and 3.0×10^4 cells ml^{-1} *M. tuberculosis* in buffer and spiked urine respectively, with higher sensitivities than the FITC-based conventional flow cytometry. The total assay time including sample pretreatment was within 2 h. This proposed FSiNP@SG-FCM method will be promising for rapid detection of *M. tuberculosis* or other pathogenic bacteria in clinical samples.

1. Introduction

Detection of pathogenic bacteria is vital in clinical diagnosis, infectious diseases prevention and control and public health safety. *Mycobacterium tuberculosis* is a much dangerous pathogenic bacterium that causes tuberculosis (TB)—one of the leading causes of death from infectious diseases (McDade, 2007). Currently, approximately one-third of the human population are infected with TB worldwide (WHO, 2003). The heavy global burden of TB worldwide demands for development of more rapid, automatic and sensitive detection methods. Traditional methods for the detection of pathogenic bacteria involve following basic steps: pre-enrichment, selective enrichment, biochemical screening and serological confirmation (Ivnitski et al., 1999). This process is laborious and time-consuming, especially for the slow-growing bacteria such as *M. tuberculosis*. Many methods have been developed for rapid detection of pathogenic bacteria, including PCR (Rodríguez-Lázaro et al., 2005), ELISA (Brooks et al., 2004), piezoelectric biosensors (Wong et al., 2002), amperometric biosensors (Susmel et al., 2003), potentiometric biosensors (Gehring et al., 1998), surface plasmon resonance (Oh et al., 2005), bioluminescence (Blasco et al., 1998), fluorescent labeling (Pyle et al., 1995), flow cytometry (Hibi et al., 2007) etc. Among these, flow cytometry (FCM) is a promising and developing technique with increasing interest. Due to the capability to provide rapid, automatic and quantitative information, single-cell analysis and high-speed multiparameter data acquisition, FCM has become one of the best options for rapid detection and quantification of a variety of bacteria in environmental, food and clinical samples (Lenaerts et al., 2007; Yamaguchi et al., 2003; Kempf et al., 2005).

However, sensitive detection of pathogenic bacteria is still a challenge for FCM. This is hindered by the small size and consequently the low contents of specific cellular constituents of microbial cells (Vives-Rego et al., 2000). Development of brighter fluorescent labels is needed to improve the sensitivity. Recently, various luminescent nanoparticles, among which are quantum dots, fluorescent latex particles and dye-doped silica nanoparticles, have been successfully applied for biological labeling as well as for detection of pathogenic bacteria because of their superiority over conventional fluorophores in terms of fluorescence intensity and photostability (Su and Li, 2004; Zhao et al., 2004; Edgar et al., 2006; Qin et al., 2007). It seems that these novel luminescent

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nanoparticles might open the opportunity to improve the sensitivity of pathogenic bacteria detection by FCM. Such attempt was reported recently by Ferrari et al. to use quantum dots as single-label for FCM detection of *Cryptosporidium* (Ferrari and Bergquist, 2007). However, beyond expectation, their results showed that the quantum dots were even inferior to organic fluorophores because of the significantly higher false positives. These false positives arose from: (1) high level of background noise caused by aggregates of nanoparticle-conjugates, and (2) high level of nonspecific binding caused by nanoparticle-conjugates to detrital particles in the samples. Therefore, methods developing to overcome the above false positives would offer the possibility to improve the sensitivity of pathogenic bacteria detection with FCM using luminescent nanoparticles as biological labels.

In this paper, we develop an improved two-color flow cytometry by a combination of bioconjugated RuBpy-doped fluorescent silica nanoparticles and SYBR Green I for detection of pathogenic *M. tuberculosis* avoiding false positives. The highly luminescent bioconjugated RuBpy-doped fluorescent silica nanoparticles were used as a specific and bright label for *M. tuberculosis*, and the nucleic acid dye SYBR Green I was used as another monitor to eliminate false positives from aggregates of nanoparticle-bioconjugates and nonspecific binding of nanoparticle-bioconjugates to background debris. This method was used to detect *M. tuberculosis* in buffer solution and spiked urine. The sensitivity of the method was investigated and compared with fluorescent dye FITC-based conventional FCM method.

2. Materials and methods

2.1. Materials

Tris(2,2-bipyridyl)dichlororuthenium(II) hexahydrate (RuBpy), Protein A from Staphylococcus aureus were supplied by Sigma–Aldrich (St. Louis, MO, USA). SYBR Green I (SYBR-I) was obtained from Molecular Probes Inc. (Carlsbad, CA, USA). Bovine serum albumin (BSA) was purchased from Beijing Ding-Guo Biotech. Co. Ltd. (Beijing, China). Cyanogen bromide (CNBr) was received from Acros Organics Inc. (Geel, Belgium). Sodium carbonate, acetonitrile, sodium azide (NaN_3) and Tween 80 were obtained from China National Medicines Group Shanghai Chemical Reagents Company (Shanghai, China). Purified rabbit anti-*M. tuberculosis* IgG and FITC-conjugated rabbit anti-*M. tuberculosis* IgG were purchased from Biodesign International (Saco, ME, USA).

2.2. Bacteria

The H37Ra strain of *M. tuberculosis* was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). *M. tuberculosis* was cultured by Dr. Songlin Yi (Hunan Tuberculosis Hospital, Changsha, China) on modified Lowenstein-Jenson medium at 37 °C for 3–4 weeks to obtain pure bacterial culture for use in establishing detection method. *M. tuberculosis* was harvested in buffer (pH 7.4, 0.01 M PBS, containing 0.05% Tween 80) to form predominantly single-cell suspension using previously described method (Schlesinger et al., 1990). The bacterial suspensions were counted using a Petroff-Hausser counting chamber, and the concentrations of bacteria were adjusted for use in experiments.

2.3. Preparation of antibody-conjugated FSiNPs

The antibody-conjugated FSiNPs were prepared by oriented immobilization of the anti-*M. tuberculosis* antibody via Protein A onto RuBpy-doped FSiNPs. RuBpy-doped FSiNPs with particle diameter of 60 ± 4 nm were synthesized using the water-in-oil (W/O) microemulsion method as described previously (He et al., 2001). Prior to bio-modification, the surface of the FSiNPs was first activated with CNBr. The FSiNPs (30 mg) were suspended in 2 ml of 2 M sodium carbonate by ultrasonication. A solution containing 0.64 g of CNBr in 2 ml of acetonitrile was then added dropwise to the particle suspension under stirring at room temperature for 5 min. After the activation reaction, the particles were washed with ice-cold water and PBS, respectively. To immobilize Protein A onto FSiNPs surface, 100 μl of 1 mg ml^{-1} Protein A was added to 1 ml of 10 mg ml^{-1} freshly activated nanoparticles, and stirring was continued for 24 h at 4 °C. The obtained Protein A-conjugated FSiNPs were then blocked with 1 ml of 1% BSA in PBS at 4 °C for 16 h. The conjugates were washed and resuspended in 1 ml of PBS. To couple the anti-*M. tuberculosis* antibody, the above Protein A-conjugated FSiNPs were incubated with 50 μl of rabbit anti-*M. tuberculosis* antibody at 37 °C for 2 h. The final product was washed, resuspended in PBS containing 1% BSA, 0.05% Tween 80, 0.02% NaN_3, and stored at 4 °C for future usage.

2.4. Procedure for detection of M. tuberculosis

Antibody-conjugated FSiNPs were added to the bacterial suspension in PBS containing 1% BSA, 0.05% Tween 80 at a final concentration of 0.1 mg ml^{-1}. After 1 h of incubation at 37 °C, the mixture was stained with SYBR-I at a final concentration of 1/10,000 dilution of the commercial stock solution at room temperature for 15 min. The sample was then ready for run on FCM as described below without the need for removal of excess nanoparticle-bioconjugates. For one-color FCM with bioconjugated FSiNPs, the procedures were essentially the same as above except that sample was not stained with SYBR-I. For detection of *M. tuberculosis* with FITC-based conventional FCM, FITC-conjugated rabbit anti-*M. tuberculosis* antibody was added to the bacterial suspension in PBS containing 1% BSA, 0.05% Tween 80 at a final concentration of 5 μg/ml. The mixture was incubated at 37 °C for 1 h and analyzed by FCM.

For detection of *M. tuberculosis* in spiked urine samples, samples were collected and pretreated as follows: First, midstream urine of 1 ml was collected from each volunteer without suspicion of TB. Artificial urine samples were prepared by spiking the freshly collected urine samples with different concentrations of *M. tuberculosis*. The spiked samples were pelleted by centrifugation at 12,000 rpm for 5 min, washed again with 1 ml of PBS and resuspended in 200 μl of PBS containing 1% BSA, 0.05% Tween 80. The suspensions were detected with the FSiNP@SG-FCM method or FITC-based conventional FCM. Unspiked urine samples were used as negative controls and processed in an identical manner.

Flow cytometric analyses were performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), equipped with a 15 mW argon ion laser emitting at 488 nm for excitation. Green fluorescence (FITC or SYBR-I) was detected using FL1 detector, 530 ± 15 nm band-pass filter. Red fluorescence (RuBpy-doped FSiNPs) was detected using FL3 detector, 610 nm long-pass filter. All parameters were collected as logarithmic signals. Data analyses were carried out with CellQuest software obtained from BD Biosciences (San Jose, CA, USA). A total of 10,000 cells were analyzed unless mentioned specifically in the sensitivity assay. For quantification of bacterial concentrations, each sample with a known volume was put into a plastic tube and weighed. Then sample run and acquisition were started simultaneously and stopped when the acquisition was lasted for 4 min. The sample was weighed again and subtracted from the initial weight to calculate the analyzed volume.