Highly sensitive detection of *Shigella flexneri* using fluorescent silica nanoparticles

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Robust signal amplification tactic is a good solution for improving detection sensitivity. Unprocessed fluorophores have many limitations. Here we describe an effective strategy for highly sensitive detection of bacteria by using fluorescent conjugated nanoparticles. The nanoparticles were synthesized using silica as matrix. Fluorescein isothiocyanate distributed in the matrix. *S. flexneri* specific antibody was coated on the surface of the nanoparticles. Compared to the traditional fluorescent antibody, these antibody-coated fluorescent conjugated nanoparticles were resistant to photobleaching and could ensure prolonged microscope checking for a small number of target bacteria. These nanoparticles could be used in routine bacterial detection for different species.

KEY WORDS: Nanoparticles, *Shigella flexneri*, Bacteriological techniques

INTRODUCTION

Infectious diseases have been one of the major causes of death throughout the history of human beings. Correct and timely identification of the causative agents can guide treatment options and help to determine appropriate and successful therapy. Traditionally, routine microbiological testing involves acquiring pure cultures, followed by Gram staining, as well as additional biochemical tests for identification. Sometimes, a diagnostic delay of 8.2 days was encountered for intestinal infection (Probert *et al.*, 2004).

The development of highly sensitive, selective and rapid methods to detect target pathogens has long been the concern of both microbiologists and analysts. In order to circumvent the time-consuming culture procedure, many rapid diagnostic methods have been developed, *e.g.* polymerase chain reaction (PCR) (Aranda *et al.* 2004), Fourier transform infrared spectroscopy (Al-Qadiri *et al.*,

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2006) and so on. Although these techniques are promising and have individual strengths, their higher cost, inconvenient maintenance or strict operator requirements compromise their application for common purposes. A simpler and cost-effective approach is warranted especially for large-scale screening, urgent medical diagnosis, and the current fight against bioterrorism.

Fluorescent detection, as an efficient signal amplification method, has been introduced into aiding bacterial detection for many years (Goldman *et al.*, 1956). Because of its high sensitivity compared to traditional Gram stain and the specificity when combined with specific antibodies, this strategy is amenable to many different laboratories. The unprocessed fluorescein is sensitive to photobleaching, so this kind of detection requires fast checking and cannot endure prolonged microscopic analysis.

Shigella is a Gram-negative enteroinvasive genus containing four different species, and known as the causative agents of bacillary dysentery. Shigellosis is an endemic disease throughout the world. Most of the million yearly deaths occur in developing countries, and 70% of the casualties are children between the ages of 1 and 5 years (Sansonetti *et al.*, 1986). As a golden standard, culture-based serological tests still dominate the

diagnostic protocol in clinics. Taking *Shigella flexneri* (*S. flexneri*) as an example, we report a rapid and sensitive bacterium detection method based on fluorescent silica nanoparticles coupled with Concanavalin A coated smear samples. This tactic could endure prolonged microscopic checking without losing sensitivity severely, and could be used to detect a small number of target bacteria.

MATERIALS AND METHODS

Chemicals and instruments

3-aminopropyltrimethoxysilane (APS), fluorescein isothiocyanate (FITC), cyclohexan, 1- hexanol, Concanavalin A (Con A) and glutaraldehyde were purchased from Aldrich Chemie GmbH (Steinheim, Germany). Tetraethoxysilane (TEOS) was the product of Alfa Aesar (MA, USA). Triton X-100 was provided by Solarbio (Beijing, China). Acetone was the HPLC purity product from Tedia (OH, USA).

Ammonia solution and perchlormethane (analytical grade) were purchased from Shenyang Lianbang Regent Factory (Shengyang, China). Water used in the experiments was purified with Milli-Q system (Bedford, MA). Antibody specific to the genus of *Shigella* was the product of Lanzhou Bio-Products Institute (Lanzhou, China) (labeled titer ≥1:3200). *S. flexneri* was provided by Dalian Center of Clinical Laboratory (Dalian, China). *Escherichia. coli* (*E. coli*, 25922) was the strain of American Type Culture Collection (Manassas, VA). FEI (Oregon, USA) Tecnai G2 Spirit transmission electron microscope (TEM) and Quanta 200F scanning electron microscope (SEM) were utilized to acquire photomicrographs when needed. Bacterial turbidity was determined using DENSIMAT transmissometer (bioMerieux, France).

Synthesis of FITC conjugated silica nanoparticles

A water/oil reverse micelle was prepared with a nonionic surfactant using the procedures modified from the literature (Hai *et al.*, 2004). Briefly, 0.05 g FITC was sonicated to dissolve in 0.6 ml deionized water and then 200 µl of TEOS was added to the solution under gentle agitation. Premixed oil phase containing 17 ml cyclohexa-

ne, 4.5 ml 1-hexanol and 4.5 ml Triton X-100 were added and stirred at ambient temperature for 30 min to obtain a clear microemulsion solution. Subsequently, 4 µl of APS were added and stirred for another 30 min. Finally, 200 µl ammonia solution was added dropwise and stirred overnight. All the reactions were under nitrogen flow protection.

The materials generated in the reverse microemulsion were precipitated by acetone, followed by centrifugation, washing with ethanol and deionized water several times to remove any residual molecules. The particles were then dried by nitrogen flow at room temperature. Their sizes were determined by TEM.

Immobilization of the antibody onto the surfaces of the nanoparticles

Antibody against the genus of *Shigella* was immobilized onto the surfaces of FITC conjugated silica particles with well-established glutaraldehyde spacer method (Kasprzyk *et al.,* 1988). 10 mg FITC conjugated particles were dispersed into phosphate-buffered saline (PBS, Na_2HPO_4 8.1 mmol/l, NaH_2PO_4 1.9 mmol/l, NaCl 1.4 mmol/l, Tween-20 0.05%, pH 7.4) containing 5% glutaraldehyde and incubated for about 3 h at room temperature.

These FITC conjugated particles were collected by centrifugation, washing and then redispersed in 1 ml PBS. The suspension was rotation-incubated with 500 µl multiple covalent antibody specific to the genus of *Shigella* at 4°C for 12 h. These antibody-coated FITC conjugated nanoparticles were washed with PBS in triplicate and kept at 4°C in appropriate PBS (final concentration was about 1 mg/ml). Brief sonication was employed prior to each use.

Preparation of Con A modified slides

Glass slides (76.0 mm × 26.6 mm × 1.5 mm) were soaked in 2 M NaOH solution containing 70% ethanol (v/v) for 2 h. After rinsed with deionized water, the slides were dried at 110°C for 30 min and then kept in water- free dryer at room temperature. A 5 Å molecular sieve was dried at 300°C for 5 h and then cooled to room temperature in a water-free dryer.

An appropriate amount of CCl_4 was mixed with the dried molecular sieve over night to remove potential dissolved water. 5% APS solution (v/v)

was freshly prepared using the purified CCl_4, and the slides were immerged in it and incubated for 12 h at 70°C under refluxing. After washing with CCl_4 and ethanol several times, the slides were dried at 110°C for 30 min. Con A was conjugated onto the surfaces of the slides according to the procedures as immobilization of the antibody.

Sample preparation for electronic microscope analysis

Nanoparticle samples for TEM imaging were prepared on 100-mesh Formvar-coated copper grids by pipetting several drops of solution onto the grids directly. For SEM imaging, bacterial solution with nanoparticles was transferred into sterile microcentrifuge tubes.

The pellets were collected by centrifugation at 3000 g for 5 min and then fixed using 2% glutaraldehyde in PBS at 4°C for 4 h. After centrifugation, the pellets were sequentially dehydrated using 30%, 50%, 70%, 85%, 95% and 100% trimethyl carbinol in distilled water for 15 min for each. Finally the pellets were dehydrated utilizing pure trimethyl carbinol for 15 min in trip-

licate. The last dehydration was performed at 4°C, and then the crystal of trimethyl carbinol was lyophilized at -50°C by Freeze dry system/FREE-ZONE 4.5 (Kansas, Mi) as soon as possible. The dried samples were then coated with sputtering gold.

Sandwich immunoassay to detect S. *flexneri* in feces samples

This method combined the bacterium enrichment, purification and fixation procedures, which could facilitate the highly efficient fluorescent detection (Fig. 1). Feces samples were dropped onto the surfaces of Con A modified slides and then incubated at 35°C for 5-10 min under gentle agitation. The slides were then rinsed with PBS in triplicate and covered completely with antibody modified nanoparticle solution. After incubation at 35°C for 5-10 min (under gentle agitation), the slides were rinsed with PBS in triplicate and dried at room temperature. The target bacteria were checked using an OLYMPUS BH-Z fluorescence microscope (Tokyo, Japan) with 10× eye lens and 40× objective lens.
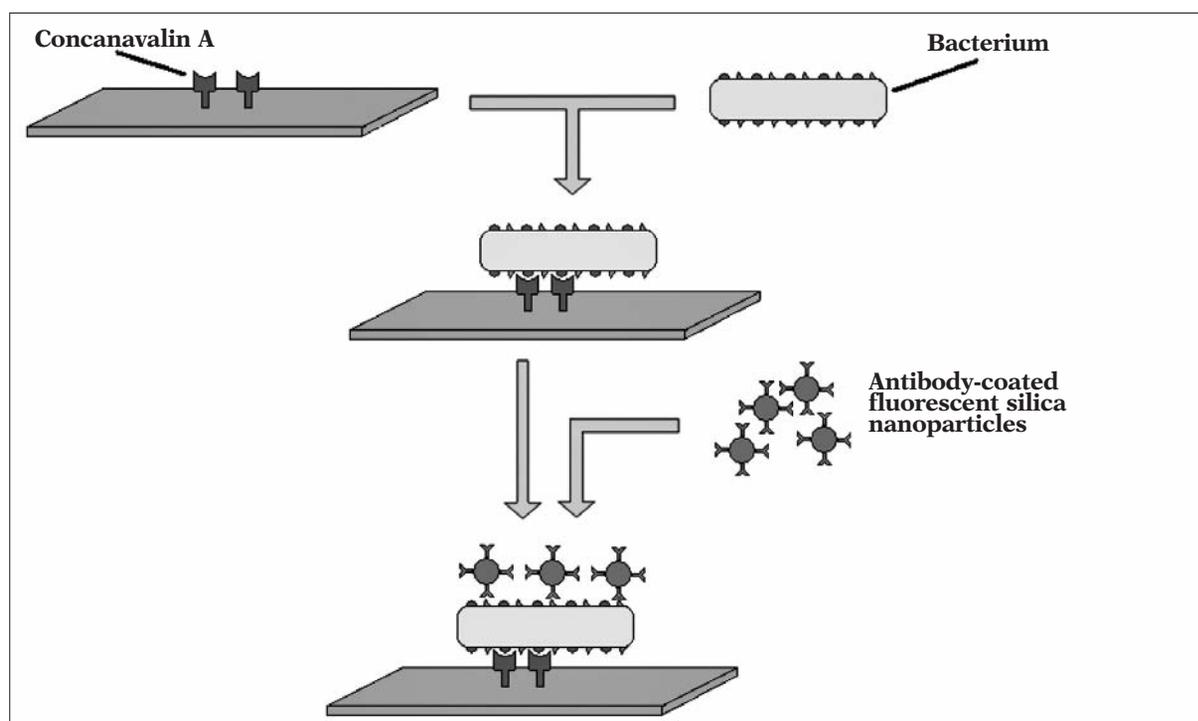

FIGURE 1 - *Schematic representation of the principle to probe the target bacteria by a sandwich method. Con A was immobilized onto the surface of the slide. The bacteria were captured and enriched on the surface. Antibody-coated nanoparticles were used to react with the specific bacteria and then to indicate the existence of target microbes.*

RESULTS

Sizes of the nanoparticles

Figure 2A shows the TEM image of the synthesized fluorescent nanoparticles. Random counting of 100 particles indicated that the average diameter was 50.6±3.3 nm (Fig. 2B).

Specificity of the antibody-coated nanoparticles

E. coli and *S. flexneri* were diluted using PBS (with the final concentration of 1.5×10^8 colony forming unit/ml (CFU/ml) for each) and then dropped individually onto the surfaces of the Con A modified slides. After incubated at 35°C for 5 min, the slides were rinsed with PBS in triplicate. Antibody-coated nanoparticle solution was pipet-ted onto the surfaces of Con A modified slides and incubated in a wet box at 35°C for 10 min with gentle agitation. After rinsing, fixation and dehydrating, the slides were broken into small pieces using diamond knife and then treated with sputtering gold. The SEM images were showed in Figure 3. Clearly, the *S. flexneri* cell was covered with the nanoparticles and the *E. coli* cell was not, demonstrating the selectivity of the bioconjugated particles to *S. flexneri*.

Sandwich assay appraisal

A fecal sample coming from a diagnosed *S. flexneri*-infected patient was collected. About 200 µl of the bloody purulent stool was pipetted onto the surface of a Con A modified slide, and the sandwich immunoassay was performed. The flu-
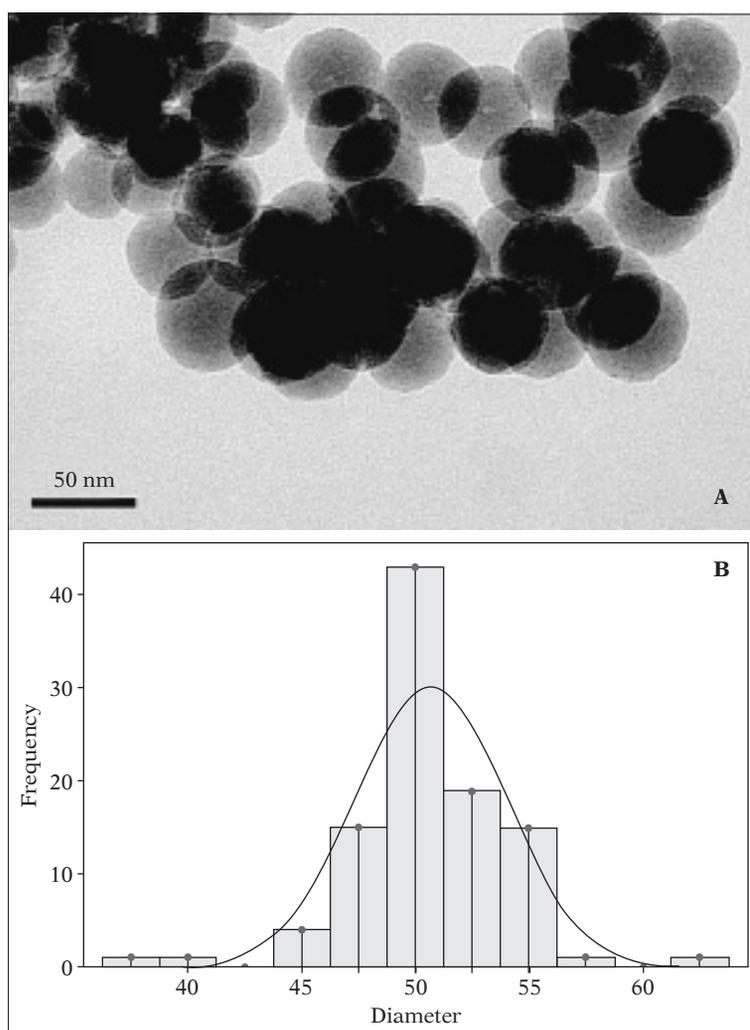
FIGURE 2 - *TEM micrograph of synthesized nanoparticles (265 kV) before bioconjugation (A), and histogram of particle diameter distribution from 100 randomly selected nanoparticles (B).*

FIGURE 3 - *SEM images (50,000×) of bacteria captured by Con A-coated slides. The center of the figure shows a free E. coli with no nanoparticle adhered. The small figure at the right bottom is S. flexneri bound with specific antibody-coated nanoparticles.*
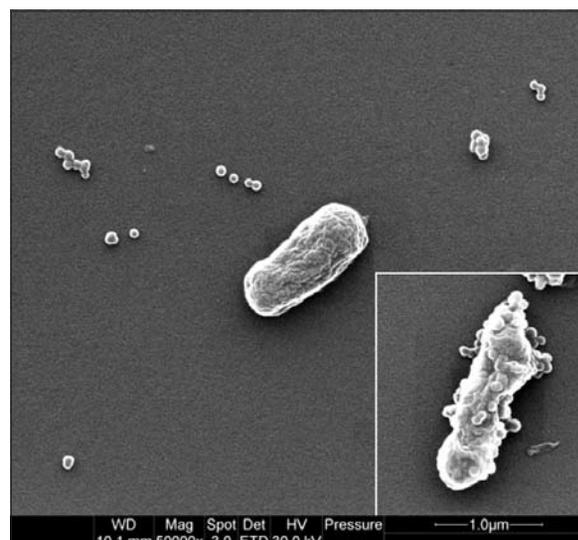

orescence image of the slide was given in Fig. 4A. The target bacteria were very bright in the fields. This proved that the synthesized nanoparticles were perfectly compatible with immunoassay.

DISCUSSION

Nanotechnology has gained broad applications in many fields, because many unexpected new

FIGURE 4 - *(A) Fluorescent image (400×) of single S. flexneri captured by Con A-coated slide and traced by the bioconjugated nanoparticles. The optical image of S. flexneri looks larger than its actual size because of the optical effect. Extremely bright bacteria could be discerned easily in the field, enabling single bacterial detection. Bar indicated 2 μm. (B) Photobleaching comparison between commercial FITC labeled antibody and antibody-coated fluorescent conjugated nanoparticles after exposure to UV light (200W).*
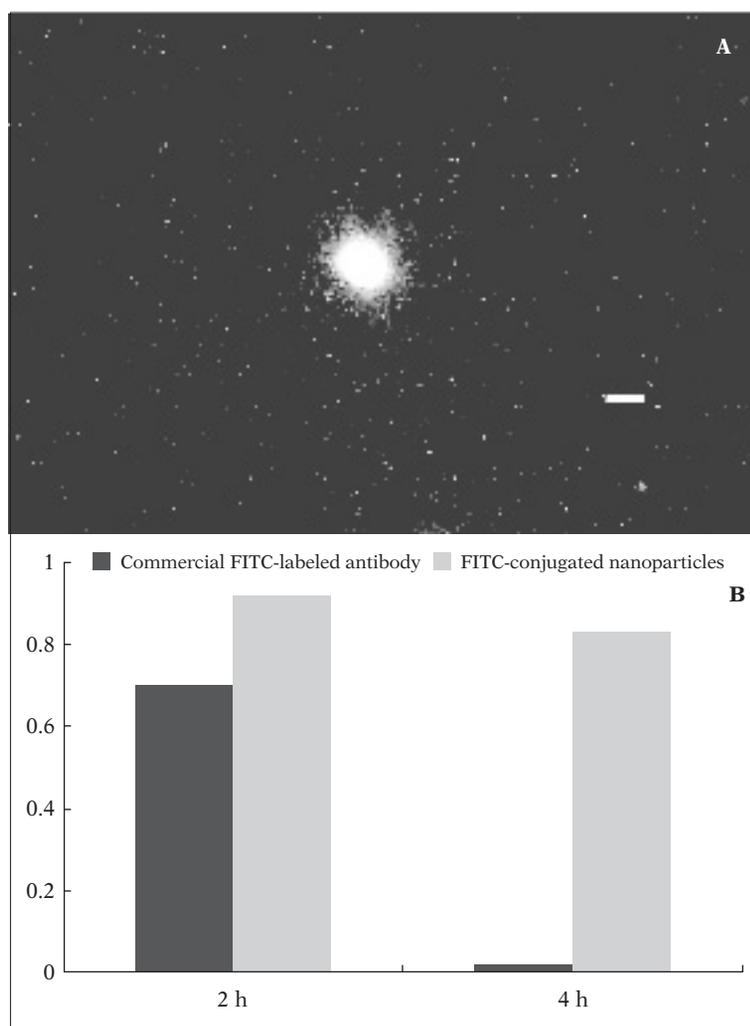
features may arise from the materials in size of nano-scale (Wang *et al.,* 2007; Kim and Fisher 2007; Banta *et al.,* 2007; Navalakhe and Nandedkar 2007). Compared to traditional immunoassays, where only one or a few dye molecules were linked to the antibodies or ligands, our synthesized nanoparticles ensured a highly amplified and reproducible signal for fluorescence-based bioanalysis. From Figure 2, the size distribution range of the nanoparticles was about 50 nm. We also proved that the sizes of the nanoparticles could be manipulated by changing the molar ratio of water to oil phase if needed. The larger the oil phase percentage is, the smaller the diameters of the nanoparticles. This could facilitate the detection of different microorganisms with varied cell sizes.

To evaluate the functionality of the antibody-coated nanoparticles, we adopted the traditional sandwich immunoassay strategy as shown in Figure 1. Using the fecal sample from a diagnosed *S. flexneri* infection patient, we proved that the bioconjugated nanoparticles could bind to the target bacteria definitely as depicted in Figures 3, 4A. Con A is a kind of lectin, which is a carbohydrate-binding protein or glycoprotein of nonimmune origin which agglutinates cells or precipitates glycoconjugates or both (Goldstein and Hayes 1978). Various bacterial polysaccharides are precipitated by Con A, which interacts specifically with bacterial cell walls containing glycosidic residues associated with tecichoic acid. Pathogenic or nonpathogenic microorganisms from various genera, e.g., *Lactobacilli*, *Staphylococus*, *Neisseria*, *Enterobacteriaceae*, could be agglutinated by Con A (Slifkin and Doyle, 1990). In order to confirm the nanoparticle-adhered bacteria were *S. flexneri* instead of others microorganisms, pure cultures of *S. flexneri* and *E. coli* were selected to be separately probed by the nanoparticles. From Figure 3 it is obvious that the bioconjugated nanoparticles were selective to *S. flexneri*. Cells of *S. flexneri* were all coated with nanoparticles in the SEM images, but we seldom found the *E. coli* cells covered with nanoparticles. Definitely, the modified nanoparticles were properly endued with the specificity to *S. flexneri*.

That Con A was selected as the capture component immobilized onto the surfaces of the slides was based on the consideration that this strategy, compared to using specific antibodies as the capture media, could be more convenient to be adapted to detecting other bacteria without the need to prepare different slides. What should be more valuable was the fact that sample slides prepared by Con A could give less dirty backgrounds. Fecal samples contain large amount of debris which usually contaminates the backgrounds of sample smears.

Most of the retained components on the Con A modified slides were bacteria, so dirty raw samples could be checked directly without further purification. Additionally, microorganism enrichment and fixation could be accomplished simultaneously. Other materials such as *S. flexneri* contaminated food or vomit materials from latent patients were all suitable samples for direct check. Because of the protection effects from the silica matrix, the fluorescence became less sensitive to photobleaching. 4-hour exposure to UV light only resulted in 17% lose in fluorescent intensity, whereas, commercial FITC labeled antibody nearly totally photobleached (Fig. 4B). Real smear results showed that target bacteria adhered by specific antibody-coated nanoparticles were still very bright in the high power fields even after exposed to daylight for a week (pictures not shown).

This trait could ensure the availability of checking more fields without severely losing fluorescent intensity for diagnosis purpose utilizing smear samples. In fact, every slide could be checked under the microscope for hours, which is unimaginable for traditional labeling tactics. It was more valuable when the target microbes were in small numbers in the smears and needed to be checked for a long time.

In this study, each synthesized nanoparticle emitted an extremely strong signal, which may make it much easier for us to achieve the goal of considerable signal amplification for ultrasensitive target bacterial detection and to trace rare events which would not be easily monitored by existing labeling techniques. In this research *S. flexneri* was only employed as an example for clinical purposes. The other more dangerous pathogens e.g., *Vibrio cholerae*, can also be detected by this strategy in theory. If fluorophores with different emission wave lengths and multiple antibodies are available, this tactic may also be used to differentiate various serotypes within a certain species.

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