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Fluorescence bio-barcode DNA assay based on gold and magnetic nanoparticles for detection of Exotoxin A gene sequence

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ABSTRACT

Bio-barcode DNA based on gold nanoparticle (bDNA-GNPs) as a new generation of biosensor based detection tools, holds promise for biological science studies. They are of enormous importance in the emergence of rapid and sensitive procedures for detecting toxins of microorganisms. Exotoxin A (ETA) is the most toxic virulence factor of *Pseudomonas aeruginosa*. ETA has ADP-ribosylation activity and decisively affects the protein synthesis of the host cells. In the present study, we developed a fluorescence bio-barcode technology to trace *P. aeruginosa* ETA. The GNPs were coated with the first target-specific DNA probe 1 (1pDNA) and bio-barcode DNA, which acted as a signal reporter. The magnetic nanoparticles (MNPs) were coated with the second target-specific DNA probe 2 (2pDNA) that was able to recognize the other end of the target DNA. After binding the nanoparticles with the target DNA, the following sandwich structure was formed: MNP 2pDNA/tDNA/1pDNA-GNP-bDNA. After isolating the sandwiches by a magnetic field, the DNAs of the probes which have been hybridized to their complementary DNA, GNPs and MNPs, *via* the hydrogen, electrostatic and covalently bonds, were released from the sandwiches after dissolving in dithiothreitol solution (DTT 0.8 M). This bio-barcode DNA with known DNA sequence was then detected by fluorescence spectrophotometry. The findings showed that the new method has the advantages of fast, high sensitivity (the detection limit was 1.2 ng/ml), good selectivity, and wide linear range of 5–200 ng/ml. The regression analysis also showed that there was a good linear relationship ($\Delta F=0.57$ [target DNA]+21.31, $R^2=0.9984$) between the fluorescent intensity and the target DNA concentration in the samples.

1. Introduction

Pseudomonas aeruginosa, a gram-negative obligate aerobe, is found in many natural and manmade environments. It has been isolated from plants, soils, water (Hanaki et al., 2010) and warm, moist environments containing very low levels of organic material. This organism is an opportunistic pathogen that infects humans and animals. In humans, *P. aeruginosa* is a common cause of nosocomial infections in burn and other immunocompromised patients including transplant, cancer and acquired immune deficiency syndrome patients (Hanaki et al., 2010). The numerous virulence factors of *P. aeruginosa* reflect its multifactorial pathogenicity and contribute to several infection stages. Surface factors including pili, lipopolysaccharides, and polysaccharide slime contribute to bacterial adherence and colonization. In contrast, different secreted proteins play a decisive role in dissemination and tissue damage. *Pseudomonas* exotoxin A (ETA) is the most toxic substance in *P. aeruginosa*. Exotoxin A, produced by the most clinical strains of *P. aeruginosa*, is highly toxic for animals and tissue cultures. It belongs to a family of enzymes termed mono-ADP-ribosyltransferases, and inhibits protein synthesis *in vitro* and *in vivo* by enzymatic addition of adenosine 5̄-diphosphate ribose to elongation factor 2 (EF-2). Intake of trace amounts of ETA results in diarrhea, vomiting, septic shock and even death (Wolf and Beile, 2009).

Conventional analytical methods including radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), fluorescence-based fiber optic immunoassay, aptamer microarrays, mass sensitive biosensor, microelectrochemical biosensors, electrochemiluminescent (ECL) (Zhang et al., 2012) quantitative polymerase chain reaction (PCR) assay (Melchior et al., 2010), amperometric immunosensor (Suresh et al., 2010), silica coating magnetic nanoparticle-based silver en-

Abbreviations: SMCC, sulfosuccinimidyl 4-*N*maleimidomethyl cyclohexane-1-carboxylate; APTES, 3-aminopropyltriethoxysilane; MNPs, magnetic nanoparticles; GNPs, gold nanoparticle; TEOS, tetraethoxysilane; DTT, dithiothreitol; ETA, exotoxin A; LB-broth, Luria Bertani broth

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hancement immunoassay are effective for ETA detection (Zhang et al., 2010). Although these analytical methods are effective for ETA detection, but they are time consuming, difficult and expensive. Various PCR methods have been developed for nucleic acid detection in the past decade. Some common fluorescence probes such as fluorescent (Bi et al., 2006) metal ions (Zhang et al., 2013), metal complexes (Krishnamoorthy et al., 2011), nanoparticles (Zanoli et al., 2012), and bio-barcode DNA are the only bio-detection methods that have the PCR-like sensitivity for both protein and nucleic acid targets without any need for enzymatic amplification (Chang et al., 2007). Therefore, searching for new generations of fluorescent probes with especial properties to develop the DNA biosensors with lower detection limits, higher selectivity and very low background is of increasing interest. In this connection, the widespread use of nanomaterials has opened a new era in advancement of bio-analytical technology. Thus, the nanoparticles of metals and semiconductors with unique optical, electronic, magnetic and catalytic properties were introduced as potential candidates for use in optoelectronic nano devices, catalysts and nanobiotransducers (Li et al., 2013). In the past decade, gold nanoparticles (GNPs) have attracted an increasing attention in DNA sensors due to their easy preparation, good biocompatibility and unique optical properties (Saha et al., 2012). The easy modification of gold surface by thiol ended molecules makes it suitable for preparation of different biological assemblies, for using in chemosensing, bio sensing and immunosensing systems (Daniel et al., 2004). Compared to common genomic detection systems, the use of GNPs in DNA detection has increased the sensitivity up to ten-fold in some cases and improved the response characteristics (Kewal et al., 2005).

In the present project, two nanoparticles were utilized in the probes and barcode DNA design: amine-coated magnetic nanoparticles (probe 2-MNP) to separate the target DNA from the sample mixture, and probe1-capped GNPs (probe 1-GNP-barcode DNA) to label the separated target DNA by forming a sandwich structure (GNP-target DNA-MNP) and generating the signal. After conjugation of probes with nanoparticles and hybridization with the target DNA, a magnetic field was used to separate the sandwich complex consisting of GNP-target DNA-MNP. GNPs were used to increase the surface area for more binding amount of probes to target DNA. So in order to enhance the selective separation of target DNA (consequently enhancing the sensitivity), we performed the synthesis and use of amine functionalized iron oxide nanoparticles as highly sensitive affinity probes for detection of target DNA for the first time.

2. Experimental

2.1. Materials

Hydrogen tetrochloroaurate (III) trihydrate ($HAuCl_4{\cdot}3H_2O$) and sodium citrate dehydrate were purchased from Sigma (St. Louis, MO, USA). Dithiothreitol (DTT), Ethyl acetate, NaCl, Luria broth (LB-broth), Tetraethoxysilane (TEOS) and 3-aminopropyltriethoxysilane (APTES) were purchased from Merck, Germany. All oligonucleotides (probes and barcode DNA) were synthesized by Bioneer, Korea. Sulfosuccinimidyl 4-*N*maleimidomethyl cyclohexane-1-carboxylate (SMCC) and Sulfo-NHS acetate were purchased from Thermo (Pierce, Milwaukee, WI, USA). All solutions were prepared in distilled water. *Pseudomonas aeruginosa* ATCC27853.

2.2. Bacterial species

P. aeruginosa ATCC27853 (as a reference species) was purchased from Pasteur Institute, Tehran, Iran. Other species including *Staphylococcus aureus, Escherichia coli, Shigella dysentery* and *Vibrio cholera bacteria* (as the negative controls) were isolated from clinical samples in Mousavi Hospital, Zanjan, Iran.

2.3. Bacterial DNA preparation

P. aeruginosa was grown on LB-broth for 12 h. Subsequently, 5 ml of the bacteria culture was centrifuged at 1400*g* for 5 min. Then, the chromosomal DNA was extracted using DNA purification kit (Roche Diagnostics GmbH, Mannheim, Germany High Pure PCR Template Preparation Kit). Genomic DNA was electrophoresed on 0.8% agarose gel, in order to check the quality of the purified product. The target DNA concentration was determined using a UV–vis spectrophotometer (Cary 4000, U.S) at 260 nm and it was stored at −20 °C.

2.4. Probes and barcode DNA designing

The sequences of the thiol-capped oligonucleotides (Probe1 and prob2) and the barcode DNA were 5′-SH-CAACGACGCACTCAAGCTG-3′, 5′-TTGGTCGCTGAACTGGCTG-SH-3′ and 5′-TEX-TTATTCGTAGCTAAAAAAAAAA-SH-3′ (Deng et al., 2010) respectively. In a BLAST search of Gen Bank DNA sequences (http://www.ncbi.nlm.nih.gov/Genbank/index.html), we found no homology with any known *P. aeruginosa* bacteria genes. The probe1, prob2 and the barcode DNA were synthesized by Bioneer Company, Korea.

2.5. Synthesis of GNPs

Colloidal GNPs of ~15 nm in diameter were synthesized by the citrate reduction method (Yangbao et al., 2015). Typically, 100 ml of $HAuCl_4$ (1 mM) solution was heated to reflux with vigorous stirring, then 10 ml of 38.8 mM sodium citrate was added to the mixture. The solution color changed from pale yellow to wine red within 1 min. The solution was heated under reflux for another 20 min. Then, the heating source was removed and the solution was continuously stirred until it cooled to room temperature (25 ± 2 °C). The resultant GNPs colloids were stored in 4 °C.

2.6. Synthesis of MNPs

Amino group modified silica coating of MNPs was performed using an improved Strober method which produces a relatively thick silica shell, as described by Zhang et al. (2010). 1.6 gr Fe_3O_4 and 0.8 gr Fe_2O_3 nanoparticles were mixed with 2.0 ml 2-propanol and 4.0 ml ethanol. 5.0 ml deionized water and 15 ml 25% (v/v) ammonia solution were added consecutively to the reaction mixture for 2 h at 80 °C. Under continuous mechanical stirring (rpm: 700), 10 ml 3-aminopropyltriethoxysilane (APTES) and 40 ml tetraethoxysilane (TEOS) were added to the reaction solution for 3 h at 80 °C. The hydrolysis and co-condensation of TEOS and APTES was initiated by adding of ammonia solution (25%) to the reaction mixture. The suspension of hybrid particles was separated by a magnetic field to remove all unreacted materials. The obtained pellet was redispersed in 10 ml water under sonication for 10 min. This purification step was repeated three times and the final suspensions were stored at 4 °C.

2.7. Mechanism of bio-barcode fluorescence

In the present study, a novel system based on bio-barcode fluorescence was developed for the detection of ETA gene. The sensing mechanism of the proposed MNP-target DNA-GNP bio-barcode for *P. aeruginosa* gene detection was shown in Scheme 1. We used MNPs for conjugation of probe 2 as a capture probe and GNPs for conjugation of the probe 1 and barcode DNA as the capture and reporter probes (Scheme 1A and B). Otherwise we had false positive response due to the release of barcode DNA from both free MNPs and those connected to the target DNA. This is because we have the probes and barcode DNA on the surface of all the MNPs and not just on the surface of MNPs connected to target DNA. The added target DNA of *P. aeruginosa* was co-hybridized with capture probes on GNPs and