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DNA probe functionalized QCM biosensor based on gold nanoparticle amplification for *Bacillus anthracis* detection

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ABSTRACT

The rapid detection of *Bacillus anthracis*, the causative agent of anthrax disease, has gained much attention since the anthrax spore bioterrorism attacks in the United States in 2001. In this work, a DNA probe functionalized quartz crystal microbalance (QCM) biosensor was developed to detect *B. anthracis* based on the recognition of its specific DNA sequences, i.e., the 168 bp fragment of the Ba813 gene in chromosomes and the 340 bp fragment of the *pag* gene in plasmid pXO1. A thiol DNA probe was immobilized onto the QCM gold surface through self-assembly via Au–S bond formation to hybridize with the target ss-DNA sequence obtained by asymmetric PCR. Hybridization between the target DNA and the DNA probe resulted in an increase in mass and a decrease in the resonance frequency of the QCM biosensor. Moreover, to amplify the signal, a thiol-DNA fragment complementary to the other end of the target DNA was functionalized with gold nanoparticles. The results indicate that the DNA probe functionalized QCM biosensor could specifically recognize the target DNA fragment of *B. anthracis* from that of its closest species, such as *Bacillus thuringiensis*, and that the limit of detection (LOD) reached 3.5×10^2 CFU/ml of *B. anthracis* vegetative cells just after asymmetric PCR amplification, but without culture enrichment. The DNA probe functionalized QCM biosensor demonstrated stable, pollution-free, real-time sensing, and could find application in the rapid detection of *B. anthracis*.

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1. Introduction

Bacillus anthracis is the cause of anthrax, a serious and often fatal infection among livestock and human beings (Edwards et al., 2006). *B. anthracis* can form dormant spores that are able to survive in harsh conditions, such as high temperatures, ultraviolet radiation, and high pressures (Mizak, 2004), and can persist for centuries (Brossier and Mock, 2001; Mock and Fouet, 2001). To date, there have been several reports of anthrax infections in humans as a result of occupational exposure, including such occupations where contact with infected animal hides, fur, wool or leather is common (Edwards et al., 2006). Of greater concern, concentrated anthrax spores were used in acts of bioterrorism in the United States in 2001, in which letters containing the spores were mailed to a number of targets. Such acts have highlighted the dangers of anthrax and its impact on our society and normal life. To prevent and control *B. anthracis*, the detection of this bacterium is the first and significantly most important process (Edwards et al., 2006).

Conventional detection methods for *B. anthracis*, based on bacteriology, serology–immunology and molecular biology, are usually insensitive, cross-reactive, and labor- or cost-intensive, and therefore, many new assays are required. Accordingly, biosensors have been developed to meet this challenge in terms of sensitivity, specificity, time- and cost-efficiency (Lim et al., 2005). Typically, the fabrication of biosensors involves the immobilization of various sensitive materials including for instance antibodies and DNA probes onto the transducer surface of the biosensor to capture different analytes (Choi et al., 2005; Grubor et al., 2004). Analyte capture is usually recognized through the signals generated from enzymes or fluorescently labeled antibodies or DNA probes (Grabarek et al., 2002; Xie et al., 2004). However, background fluorescence from the organism analyte, as noise, inevitably interrupts

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the signal to decrease the resolving power, increase detection cost, or prolong the assay time. Therefore, label-free bio-sensing technology, such as surface plasma resonance (SPR) (Wang et al., 2009) and piezoelectric sensors (Campbell and Mutharasan, 2006) have distinct advantages in detection. Among these label-free assay techniques, the quartz crystal microbalance (QCM) piezoelectric sensor relies on a bulk acoustic wave frequency shift due to the corresponding mass change after capturing analytes by the functionalized QCM transducer surface, and has proved to be a useful platform in the efficient detection of pathogens including *B. anthracis*. Based on this platform, we have developed a monoclonal antibody functionalized QCM biosensor to realize the simultaneous detection of *B. anthracis* spores and its vegetative form (Hao et al., 2009).

At present, along with the development and application of gene recombinant technology, it is now possible to artificially exchange the toxin genes among *B. anthracis*, *Bacillus cereus*, *B. thuringiensis*, *Bacillus subtilis*, or other pathogenic bacteria or viruses, to produce new pathogens. Traditional methods such as utilizing antibody-based assays, are challenged in identifying the *B. anthracis* pathogen (Koehler, 2002; Rasko et al., 2005), so the development of a DNA probe-based biosensor assay is necessary to detect this pathogen at the gene level. The method for selecting conserved and specific gene sequences is of primary importance in identifying *B. anthracis*. It was reported that the Ba813 chromosomal sequence has been found in 28 strains of *B. anthracis* but not in the other 33 strains closest to *B. anthracis* (Patra et al., 1996). Ba813 has been regarded as a specific sequence of the *B. anthracis* chromosome; however, false positive results identifying *B. anthracis* using this method were also reported (Patra et al., 1998). Therefore, more accurate identification should be confirmed through combination of this sequence with other specific sequences of *B. anthracis*. The *B. anthracis* toxin is known to be composed of the cell-binding protein, protective antigen (PA), and two enzyme components, called the edema factor (EF) and the lethal factor (LF) which act together to impart their physiological effect. The causal component of this pathogen is encoded by the three genes on plasmid pXO1, but neither the LF nor the EF can separately present a toxic effect on the organism, because each of them should be combined with the third toxin protein, the PA, to form effective virulence (Abrami et al., 2005). In fact, PA plays an important role in the *B. anthracis* toxic effect through binding of the receptor of the organism to induce LF or EF to display their respective function (Moayeri and Leppla, 2004), so the PA encoding gene, *pag*, is another important DNA sequence to identify *B. anthracis*. Conventional assays for *B. anthracis* specific DNA sequences are normally based on gel electrophoresis after PCR, which is labor-intensive and insensitive with pollution risk of ethidium bromide (Fasanella et al., 2001). As mentioned above, QCM has many advantages in the assay of DNA sequences; however, DNA analytes, which are usually lighter than bacteria, spores, or proteins, produce weaker signals on the QCM biosensor, so signal amplification is needed to enhance their sensitivity (Mao et al., 2006; Patolsky et al., 2000). An amplified microgravimetric gene sensing system was developed by Liu et al. (2002) and in this system gold nanoparticles were used to modify the QCM surface to enhance the detection sensitivity. Mao et al. (2006) applied QCM DNA sensor in the detection of bacterial gene, i.e., *Escherichia coli* O157:H7 *eaeA* gene, and biotin labeled primers were used to amplify the target gene sequences through asymmetric PCR, and then streptavidin conjugated Fe_3O_4 nanoparticles were used to enhance the sensitivity.

Here, we report the fabrication of a DNA probe functionalized QCM biosensor for the detection of *B. anthracis* at the gene level, towards its specific sequences on two sites, i.e., the 168 bp fragments on the Ba813 chromosome and the 340 bp fragments on the *pag* gene of the PXO1 plasmid. Gold nanoparticle functionalized DNA probe, complementary to the target DNA, was used to amplify the detection signal. To our knowledge, this is the first application of a QCM biosensor for the detection of *B. anthracis* DNA.

2. Materials and methods

2.1. Materials and apparatus

DL15000 DNA Marker, dNTP and rTaq DNA were purchased from Takara-Bio, Inc. (Shiga, Japan); ethidium bromide (EB) as a fluorescent dye was purchased from Sigma–Aldrich (USA). Gold nanoparticles (GNPs) of size 30 nm for signal amplification were supplied by Ted Pella (USA); Nap-5 column for purifying the thiol DNA probe used in functionalizing the GNPs was purchased from GE Healthcare (UK); other materials, in or above analytical grade, were supplied by the Country Medicine Group (Chengdu, China). The buffer for the modification of the DNA probe or hybridization with target sequences, namely the reaction buffer, was prepared as follows: 0.3 M NaCl, 10 mM phosphate buffer with pH 7.4.

B. anthracis isolates were kindly provided by Professor Ruifu Yang. Other strains, such as *E. coli*, *B. thuringiensis*, *B. subtilis*, and *B. cereus* as control, were obtained from the Wuhan Institution of Virology, Chinese Academy of Sciences (Wuhan, China). All bacterial strains were grown as previously described (Wang et al., 2004). The 168 bp fragments on the Ba813 chromosome and 340 bp fragments on the *pag* gene of the PXO1 plasmid were chosen as specific target gene sequences to identify *B. anthracis*, and the target sequences, i.e., TA-Ba813 and TA-pag, are listed in Table s1 (see Supplementary data). This table also presents the primers used to amplify the target sequences, artificial targets or control sequences, and the thiol DNA probe. The capture probes used in this study, i.e., P-BA1-SH (SH-$(CH_2)_6$-TTTTTTTTTTCATTTAGCGAAGATCCAGT) and P-PAG1-SH (SH-$(CH_2)_6$-TTTTTTTTTTACGGCTCCAATCTACAAC), were complementary in sequence to their target, and 10 T bases and 1 thiol group were added at the 5′ end of the probes to facilitate their self-assembling immobilization on the gold surface. The signal amplifying probe was complementary to the target sequences at the other end, and modified with thiol groups at the 3′ end to link with gold nanoparticles through self-assembly. All the artificial sequences mentioned above were synthesized by the Sangon Biotech Company (Shanghai, China).

The QCM detection system, as shown in Fig. 1, consisted of a sensitive element (QCM wafer), an inlet subsystem, and a frequency acquisition unit. The 6 MHz AT-cut quartz crystals, purchased from Factory 707 (Beijing, China) were first coated with a chromium layer followed by coating with gold to yield a gold electrode surface on both sides, where the thickness of the quartz, chromium, and gold layers was 0.3, 20 and 50 nm, and their diameters were 12.5, 6 and 6 mm, respectively. The inlet subsystem used in the injection and washing of the analytes was composed of a detection cell, a peristaltic pump, and pipes connecting the detection cell and the peristaltic pump. The detection cell was modified from a CHI127 electrolytic cell obtained from the CHI Company (USA), and comprised a 270 μl chamber with a shorter inlet and a longer outlet to adapt the flow-through detection procedure. The peristaltic pump was the Minipuls 3 (Gilson, France). The frequency acquisition unit contained an oscillator and frequency counter: the oscillator was used to supply energy to oscillate the QCM wafer under 5 V of direct current; the frequency counter, 53131A obtained from Agilent, was used to monitor the resonant frequency of the QCM and transmit the frequency to a computer. The whole QCM detection system was run in a condition of constant-temperature and magnetic screening, which was provided by an environment controlling chamber. In addition, an LS55 Ultraviolet-Visible spectrophotometer from PerkinElmer (USA), and a PCR instrument and gel imaging system

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