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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*.

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

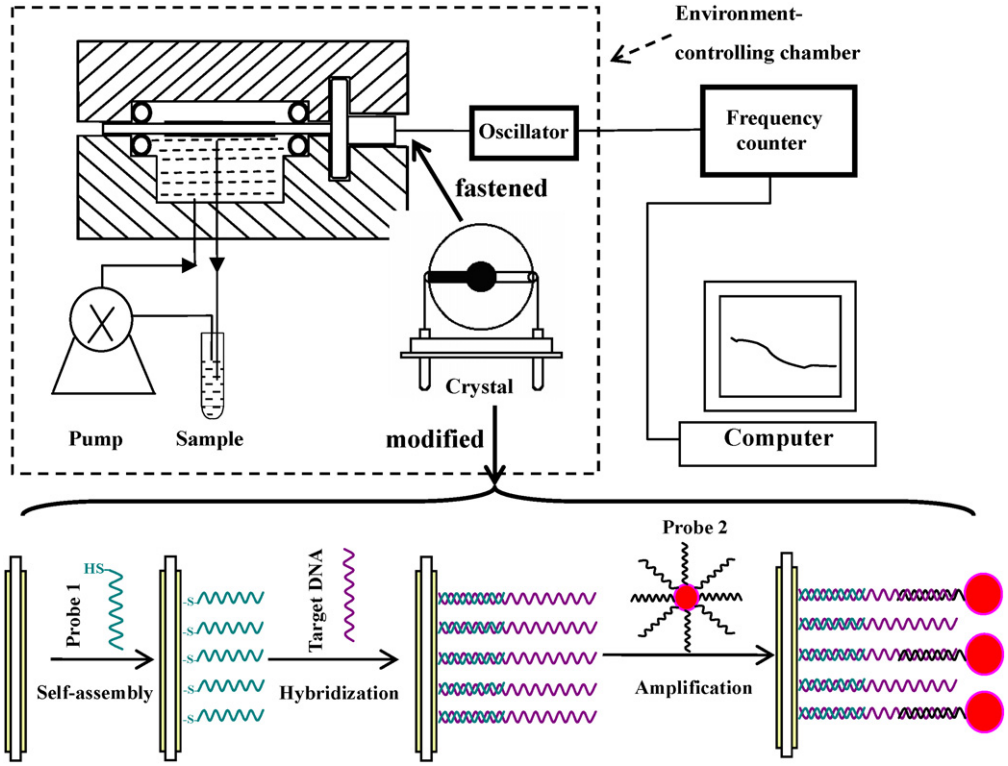

Fig. 1. Schematic illustration of the QCM detection system and the immobilization of DNA probe 1 on the QCM surface, followed by hybridization with a target DNA sequence, and subsequent signal amplification by DNA probe 2 functionalized with gold nanoparticles (GNPs). (Not drawn to scale.)

from Biometra (Germany) and Alpha Innotech (USA), respectively, were used in this study.

2.2. Methods

2.2.1. The modification and immobilization of DNA probe

DNA probes were modified with a thiol group at its 3′ or 5′ end to facilitate their immobilization on the gold surface of the QCM or gold nanoparticles (GNPs), and the probe for signal amplification was prepared through conjugating the thiol DNA probe and GNPs via a self-assembling method (Hill and Mirkin, 2006). Before the conjugation, the thiol DNA probe was first reduced by dithiothreitol (DTT) to increase the efficiency of conjugation with the GNPs. The main modification procedures (Hill and Mirkin, 2006) were as follows: first, mix 1 OD of the thiol DNA probe with 200 μl of 0.1 M DTT in the absence of light for about 2.5 h to reduce the thiol DNA probe; second, the above mixing solution was passed through a Nap-5 column to purify the reduced thiol DNA probe; third, the thiol DNA probe was mixed with 1 ml of GNP solution and the resulting mixture kept in the dark overnight; fourth, phosphate buffer was added to the above mixture to 9 mM phosphate as final concentration, then sodium dodecyl sulfate (SDS) was injected into the mixture to a final concentration of 0.1% (w/v) and the mixture stirred for 30 min; fifth, sodium chloride (NaCl) solution was then added to the above solution for 6 times over 2 days until 0.3 M as final concentration; last, the solution was centrifuged at 11,000 rpm for 25 min and re-suspended with 200 μl of 100 mM phosphate buffer to a pH 7.0. Thus, the DNA probe for signal amplification was prepared.

The immobilization of the thiol DNA probe on the QCM gold surface was also carried out through self-assembly via Au-S bond formation. The DNA probe with the final concentration of 0.25, 0.5, or 1 μM was added into 100 μl of reaction buffer and the buffered DNA probe was then incubated for 2 h. After washing, 100 μl of 1 mM 6-mercaptohexan-1-ol (6-MHO) in ethanol was then introduced for 2 h to block the active sites on the QCM surface and to decrease the non-specific sorption during the detection procedures. All the procedures were performed at 30 °C, and the frequency changes were recorded.

2.2.2. The preparation of single-stranded target sequences

The target sequences should be single-stranded so as to hybridize with the DNA probes immobilized on the QCM surface. In this study, artificial single-stranded target sequences, T-BA-58 and T-PAG-36 as shown in Table s1 (see Supplementary data), were used to optimize the detection condition. Then, the 168 bp fragment of Ba813 on the *B. anthracis* chromosome and the 340 bp fragment of the *pag* gene on the *B. anthracis* plasmid PXO1 were amplified as single-stranded target sequences through the asymmetric PCR method; the main procedures were as follows: first, a certain concentration of *B. anthracis* or control bacteria were boiled for 20 min, and the centrifuged at 4000 × *g* for 3 min, and the supernatant was kept as a PCR template; constitutions for PCR were as follows: 5 μl of 10× Taq PCR buffer, 4 μl of 2.5 mM dNTP, 1 μl (for normal PCR) or 0 μl (for asymmetric PCR) of 20 μM upstream primer (BA1 or PAG1 in Table s1 of Supplementary data), 1 μl (for normal PCR) or 2 μl (for asymmetric PCR) of 20 μM downstream primer (BA2 or PAG2 in Table s1 of Supplementary data), 3 μl of 0.1% BSA, 5 μl of template, 0.3 μl of 5 U/μl Taq DNA polymerase, and a certain volume of water with the whole final volume of 50 μl. The PCR reaction was performed on a thermal cycler PE2400 with the following condition: 94 °C for 5 min with 1 cycle; 94 °C for 1 min, 45 °C for 45 s, 72 °C for 45 s, with 5 cycles; 94 °C for 45 s, 45 °C for 40 s, 72 °C for 40 s, with 40 cycles; 72 °C for 5 min with 1 cycle. The PCR product was detected through electrophoresis in 1.2% agarose gel, and the asymmetric PCR product, as single-stranded DNA sequences, could be used in the detection with a QCM biosensor.

2.2.3. The detection of target sequences

As shown in Fig. 1, the detection of target sequences was accomplished by the DNA probe functionalized QCM biosensor through

two main procedures: the direct hybridization detection for single-stranded target sequences and the consequent signal amplification with a gold nanoparticle functionalized DNA probe. The main detection procedures were performed in the flow-through model as follows: first, reaction buffer (in 200 μl/min) was flowed through the detection cell comprising the DNA probe functionalized QCM wafer fastened to the cell, and then a certain quantity of target DNA solution was injected into the sample bottle when the QCM frequency steadied; second, the real time frequency change was recorded over a period of 30 min, and then neat buffer was flowed into the detection cell to wash the unbound target sequences from the QCM wafer surface; third, when the QCM frequency steadied, 20 μl of gold nanoparticle functionalized DNA probe solution was injected into the sample bottle and flowed into the detection cell for signal amplification, and the detection cell was then washed. The frequency changes of the QCM biosensor were recorded in real-time during the whole detection procedure, and the QCM system was maintained at 30 °C during the whole procedures.

2.2.4. Characterization of gold nanoparticles and the QCM surface

The gold nanoparticles (GNPs) used for signal amplification in this study, were scanned using UV–visible absorption spectrometry to characterize their homogeneity in diameter and change of property before and after modification by the DNA probes. During the procedures of modification and detection, the QCM gold surface was characterized using electrochemical methods such as cyclic voltammetry (CV) or electrochemical impedance spectroscopy (EIS), and the experimental methods were given in Fig. s9 (see Supplementary data).

3. Results and discussion

3.1. The immobilization of DNA probes on QCM surface

The immobilization of DNA probes on the QCM biosensor surface is of great importance in the fabrication of DNA biosensors. In this study, a concentration of DNA probe, 0.5 μM, was optimally chosen, in order that there was enough space left around the DNA probe on the QCM biosensor surface to hybridize with the target DNA sequence. In addition, 6-MCHO was demonstrated to be able to block the non-specific sites of the QCM gold surface, after the immobilization of the thiol-DNA probe on the QCM gold surface. The particular experimental data and discussions could be found in Figs. s1 and s2 (see Supplementary data).

3.2. Response of QCM sensor to artificial oligonucleotides

After modifying the QCM surface with DNA probes, the specificity of the QCM biosensor was studied by testing its response to artificial oligonucleotides as targets or control sequences, and to GNP functionalized oligonucleotides as amplifying probes. The result is shown in Fig. 2, where the response curve (e) and (f) present the response of the positive probe (P-PAG1-SH) functionalized QCM biosensor to the positive target sequence (T-PAG-36), −12 Hz, and the subsequent signal amplified by the GNP functionalized probe, −37 Hz; and the response curve (a) and (d) show the response of the same QCM sensor to the negative target sequence (T-con-36), +0.3 Hz, and its amplifying signal to the GNP functionalized probe, −3 Hz; and the response curve (b) and (c) give the response of the negative probe (P-BA1-SH) functionalized QCM biosensor to the positive target sequence (T-PAG-36), 0 Hz, and its amplifying signal, −2 Hz. These experimental results show that this gene probe functionalized QCM biosensor could yield strong response signals to its target oligonucleotide and a stronger amplifying signal to the GNP functionalized probe, but a very weak response signal to the negative target sequence and even to the GNP functionalized probe.

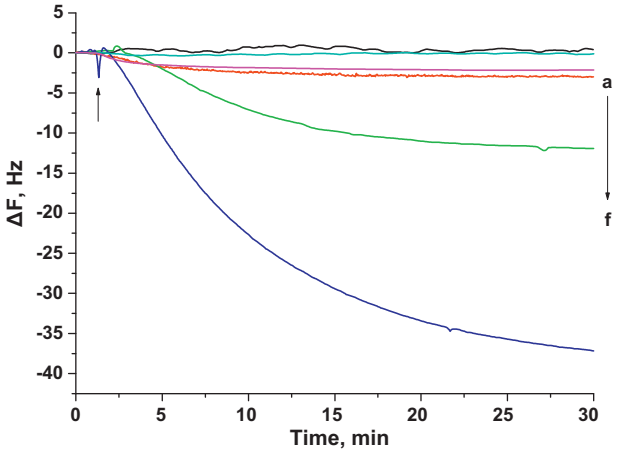

Fig. 2. Response of DNA probe functionalized QCM to a target nucleotide and sequential signal amplification with DNA probe functionalized gold nanoparticles (GNPs). (Target detection: response of P-PAG1-SH functionalized QCM to 0.5 μM T-PAG-36 (e) and signal amplification with P-PAG2-SH functionalized GNPs (f); Negative control for target: response of P-PAG1-SH functionalized QCM to 0.5 μM T-con-36 (a) and signal amplification with P-PAG2-SH functionalized GNPs (d); Negative control for capture DNA probe: response of P-BA1-SH functionalized QCM to 0.5 μM T-PAG-36 (b) and signal amplification with P-PAG2-SH functionalized GNPs (c)).

In a word, the specificity of the QCM biosensor was demonstrated by this positive result and these two negative results as probe or target control.

3.3. Response to B. anthracis specific gene sequences

3.3.1. Real-time response to target sequences

The results of gel electrophoresis for the fragments of 168 bp in the Ba813 chromosome and 340 bp in the *pag* gene of PXO1 plasmid of *B. anthracis*, as shown in Fig. s3 (see Supplementary data), demonstrated that the primers designed in this study could correctly amplify their target DNA sequences from *B. anthracis* vegetative cells. After the amplification of the *B. anthracis* specific DNA sequences through asymmetric PCR, the single-stranded target sequences obtained could be detected by the DNA probe functionalized QCM biosensor. And the real-time response of this QCM biosensor to the asymmetric PCR products for the 340 bp fragments of the *B. anthracis pag* gene and the amplifying signal by the GNP functionalized probe is shown in Fig. 3. In this figure, the whole detection process and signal response are also presented. When only buffer was cycling and contacting the QCM biosensor, the frequency baseline was less than 0.4 Hz. After the addition of the single-stranded target DNA sequences, the frequency decreased by 5 Hz within 10 min, while washing with neat buffer increased the frequency to 2 Hz within 2 min. Based on these results, the direct signal produced by the hybridization of the target sequences with the DNA probe was regarded as 3 Hz. Consequently, the GNP functionalized probe solution was flowed into the detection chamber once a steady state frequency was obtained, and the frequency presented a rapid decrease, and the total change of frequency even after the washing could reach 43 Hz. In a word, the direct signal produced by the target sequences was 3 Hz (as signal 1) which could be amplified 14 fold (as signal 2) to 43 Hz, by GNPs.

During the whole detection process, although the small perturbation to the frequency existed at the moment of injection of the target sequences, amplifying probe, and buffer, it could immediately return to its baseline. A little increase of frequency after the wash was probably due to the removal of some substances non-specifically absorbed on the QCM surface. As shown in Fig. 3, this method for the detection of target sequences could pro-

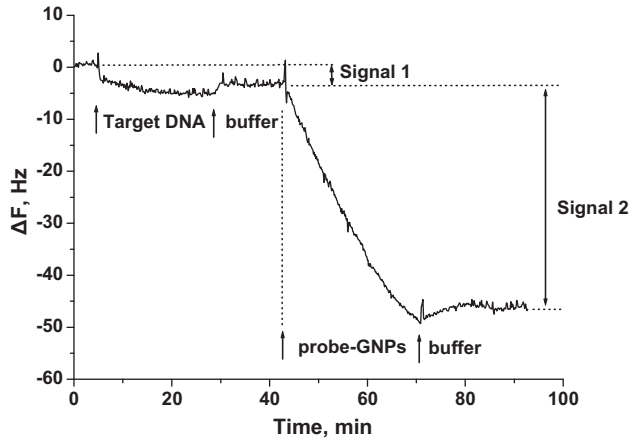

Fig. 3. Real-time response of the DNA probe (P-PAG1-SH) functionalized QCM biosensor to target DNA (T-PAG-340 with 0.1 μM of final concentration) and sequential signal amplification with gold nanoparticle immobilized DNA probe (P-PAG2-SH). (Conditions: 30 °C, 200 μl/min of buffer, upward arrow represents injection of sample.)

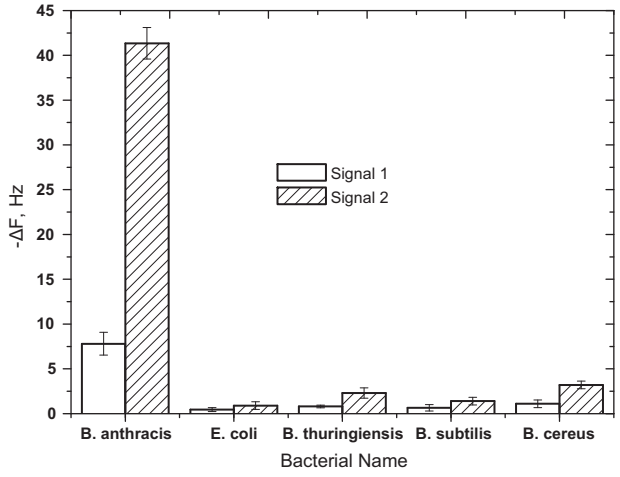

Fig. 4. Frequency shift of the DNA probe (P-PAG1-SH) functionalized QCM biosensor to the target DNA sequence amplified by asymmetric PCR to 3.5×10^7 CFU/ml of *B. anthracis*, *E. coli*, *B. thuringiensis*, *B. subtilis*, and *B. cereus*.

vide a continuous frequency shift signal, suggesting the real-time hybridization between the probe and its target.

3.3.2. Specificity of the QCM biosensor to B. anthracis

In this study, the 340 bp fragment of the *pag* gene on the PXO1 plasmid of *B. anthracis* was amplified by asymmetric PCR, and detected as a target sequence on the DNA probe functionalized QCM biosensor. To test the specificity of this biosensor to *B. anthracis* gene sequences, several species of bacteria including its closely relative species, such as *B. thuringiensis, B. subtilis,* and *B. cereus,* as well as *E. coli*, a representative gram negative bacteria, were chosen to perform the same procedures as that for *B. anthracis*, and the results are presented in Fig. 4. As shown in this figure, not only the direct response signal by the target sequences but also the amplifying signal by the GNP functionalized probes was far higher than that for the negative control bacteria, demonstrating that this biosensor could specifically recognize *B. anthracis* from its closest species through the specific gene sequences.

3.3.3. Response to different concentrations of B. anthracis

As mentioned above, the detection of *B. anthracis* was not used to directly test the response of *B. anthracis* bacteria on the QCM biosensor, but it was used to amplify its specific gene fragments through asymmetric PCR from the bacteria, to apply the target sequences on the DNA probe functionalized QCM biosensor, and to enhance the frequency signal through the use of GNP modified DNA probes. All the detection procedures were strictly performed in parallel.

3.3.4. Detection of 340 bp fragments from the pag gene on PXO1 plasmid

Fig. 5A presents the amplifying signal of the DNA probe (P-PAG1-SH) functionalized QCM biosensor taken at 30 min by the GNP functionalized probe to the 340 bp fragments of the *pag* gene on PXO1 obtained by the asymmetric PCR method from different concentrations of *B. anthracis* bacteria. The results show that the frequency shift signal was distinguishable and enhanced along with the increase of *B. anthracis* concentration from 3.5×10^1 to 3.5×10^8 CFU/ml. But the signal for 3.5×10^5 CFU/ml of *B. thuringiensis* bacteria was closely equal to the baseline and far lower than that for *B. anthracis* at the same condition. The whole response procedures could be accomplished within 30 min; moreover, the small deviation of the parallel experiment results ($n = 3$) demonstrates their good accordance.

3.3.5. Detection of 168 bp fragments from B. anthracis Ba813 chromosome

The detection results for the 168 bp Ba813 chromosomal fragments amplified by asymmetric PCR from *B. anthracis* bacteria are shown in Fig. s4 (see Supplementary data), where the biosensor was functionalized by the DNA probe (P-BA1-SH). Similar to that for the *pag* gene in Fig. 5A, this biosensor also presented a strong signal, increasing with *B. anthracis* concentration, but a weak signal to the blank or negative control target, which proved its specificity and quality determining capability in the detection for its target.

3.3.6. Limit of detection (LOD)

The limit of detection (LOD), the lowest concentration of analytes distinguishable from the background signal, is generally equal to the concentration of analytes with the corresponding signal exceeding the noise by 3 times the standard deviation (Meyers, 2000). Fig. 5B gives the response signal caused by the 168 bp fragment from the Ba813 chromosome and the 340 bp fragment from the *pag* gene of the PXO1 plasmid at different concentrations of *B. anthracis* on the QCM biosensor. For Ba813, the background signal was 2 Hz, and its standard deviation was 0.4 Hz, thus the distinguishable signal should be above 3.2 Hz; the signal from 3.5×10^2 CFU/ml of *B. anthracis* was 5.2 Hz, larger than 3.2 Hz, so the LOD of this biosensor when applied to the Ba813 chromosome of *B. anthracis* as a target should be below 3.5×10^2 CFU/ml. For the *pag* gene, the background signal and its standard deviation were 3.7 Hz and 0.6 Hz, respectively, therefore the distinguishable signal was 5.5 Hz; the signal for 3.5×10^2 CFU/ml of *B. anthracis* was 6.4 Hz, so the LOD for the *pag* gene would also be under 3.5×10^2 CFU/ml of *B. anthracis*. The linear correlation between the frequency shift of the biosensor and logarithmic number of *B. anthracis* cell concentration was demonstrated from 3.5×10^2 to 3.5×10^7 CFU/ml of cells, for both types of gene fragments of *pag* and Ba813.

To compare with the detection method of DNA fragments using agarose gel electrophoresis, we carried out the assay for the 168 bp fragments of the Ba813 chromosome and the 340 bp fragments of the *pag* gene through normal PCR towards different concentrations of *B. anthracis*, and the results are shown in Fig. s5 (see Supplementary data). The results indicate that the LOD in the agarose gel electrophoresis assay for *B. anthracis* was 3.5×10^3 CFU/ml, which was one order of magnitude larger than that in the QCM biosensor developed in this study.

Except for the advantage that the DNA probe functionalized QCM sensor based detection method had higher sensitivity than

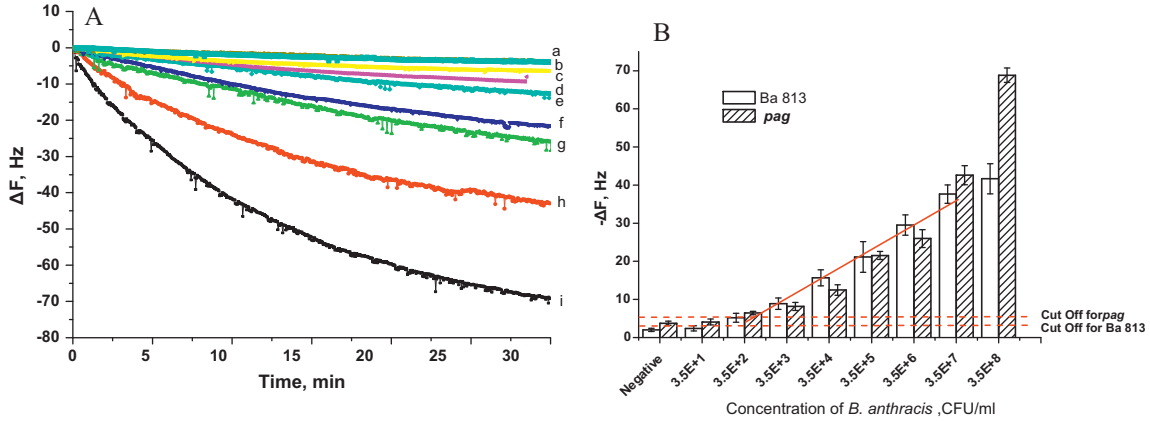

Fig. 5. Response of the DNA probe 1 (P-PAG1-SH in A) functionalized QCM biosensor with time (A), and the response value at 30 min (B) to asymmetric PCR samples (T-PAG-340 and T-BA-168) of different concentrations of *B. anthracis* vegetative cells after signal amplification with the GNP-functionalized DNA probe 2 (P-PAG2-SH and P-BA2-SH). (a), negative control for that of 3.5×10^5 CFU/ml *B. thuringiensis* vegetative cells; from (b) to (i), 3.5×10^1 to 3.5×10^8 CFU/ml *B. anthracis* vegetative cells, respectively. The linear range of the biosensor was from 3.5×10^2 to 3.5×10^7 CFU/ml of cells, for both types of gene fragments of *pag* and Ba813.

the agarose gel electrophoresis assay, this QCM biosensor based detection method was a clean assay for DNA sequences, with the absence of fluorochrome pollution, such as ethidium bromide (EB), commonly required in agarose gel electrophoresis assays. We also investigated the storage stability and the regeneration of this DNA probe functionalized QCM biosensor, and the results indicated that, after storage for 2 months at −20 °C, the response signal from this biosensor to its targets could approach the signal from the new one at the same detection condition. Moreover, after being treated by 0.1 M HCl for 2 min, the QCM biosensor could be regenerated owing to the release of the target DNA sequences hybridized with the DNA probe, such that the remaining DNA probe could be used to detect another target sequence (see Figs. s6 and s7 in Supplementary data).

However, the detection of pathogens using a QCM biosensor is still partially dependent on polymerase chain reaction (PCR) technology to amplify the limited DNA sequence of the target. Although PCR-free piezoelectric sensor detection for DNA has been reported, it has so far only been available for analytes containing lots of repeat sequences, such as specific DNA fragments of transgenic products (Minunni et al., 2005). We think the limit of PCR-dependent QCM detection may be improved in two ways: concentration of analyte DNA from the sample such as through immuno-magnetic particles, and perfect signal amplifying techniques through more efficient mass enhancement.

3.4. Characterization of gold nanoparticles and QCM surface

The UV–visible spectrum of the GNPs showed in Fig. s8 (see Supplementary data) that 3.6 nm red shift of peak absorbance appeared after the modification with the thiol-DNA probe, which was probably due to the increase of the diameter of the GNPs. The result was consistent with the report that conjugation of streptavidin with nanoparticles caused a red shift of their peak absorbance (Wang et al., 2008). Moreover, the results of the CV as well as the EIS demonstrated that the modification of the DNA probe and hybridization of target sequences increased impedance of the QCM surface, and hybridization of the GNPs decreased its impedance, which was shown in Fig. s9 (see Supplementary data).

4. Conclusion

Several thiol-DNA probes for the 168 bp of the Ba813 gene in the chromosome or the 340 bp fragment of the *pag* gene in the pXO1 plasmid of *B. anthracis* were designed and immobilized on the QCM gold surface through self-assembly to fabricate the DNA QCM biosensor. It was demonstrated that this DNA probe functionalized QCM biosensor could specifically recognize the target single-stranded DNA sequences from *B. anthracis* within 30 min, 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. Besides its rapidness and specificity, this DNA probe functionalized QCM biosensor also demonstrated pollution free biosensing avoiding the use of ethidium bromide for the *B. anthracis* DNA assay.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.01.010.

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