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A reusable quartz crystal microbalance biosensor for highly specific detection of single-base DNA mutation

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ABSTRACT

A reusable quartz crystal microbalance (QCM) biosensor based on the toehold-mediated strand displacement reaction (SDR) is first developed for highly specific detection of single-base DNA mutation. N^5, N^{10}-methylenetetrahydrofolate reductase (MTHFR) gene C677T mutation is chosen as a model to investigate the performance of the constructed biosensor. The capture DNA immobilized on the gold electrode surface of QCM hybridizes with the conjugate of biotinylated reporter DNA and streptavidin (abbreviated to reporter probe) to form the recognition layer. A toehold domain in the reporter DNA is specifically designed to bind the complementary mutant DNA and trigger the SDR, eventually resulting in the release of the complex of reporter probe and mutant DNA from the gold surface, and then the recognition layer is mildly regenerated by subsequently injecting the reporter probe again. The fabricated biosensor shows good repeatability for the mutant DNA, as more than 87% signal intensity of the sensor remained after six repetitive measurements. A linear range of 1–75 nM is achieved with a detection limit of 0.8 nM at room temperature. This biosensor also affords remarkable specificity to the mutant DNA against its wild-type DNA with a discrimination factor of 33. The potential of applying this biosensor in screening real samples is confirmed by spiked HeLa cells lysate with a recovery of 108%. The proposed sensing strategy provides a simple and universal solution for repetitive and highly specific detection of single-base DNA mutation.

1. Introduction

Biosensors that convert the DNA hybridization into measurable optical (Dolatabadi et al., 2011), electrochemical (Dolatabadi et al., 2011; Ronkainen et al., 2010), or mechanical (Arlett et al., 2011) signals have been developed to accomplish sensitive and sequence-specific DNA detection, which shows growing significance in many fields, including clinical diagnosis, genetic analysis, and forensic application (Sassolas et al., 2008). As a mass-sensitive device, quartz crystal microbalance (QCM) is a prominent technology to construct real-time, label-free, and sensitive biosensors for nucleic acids (Speight and Cooper, 2012). However, the application of QCM biosensors is limited for rapid and high-throughput detection of DNA samples, because on-line regeneration strategies are lacked to reduce the loss of crystal chip and shorten the detecting period. Acidic or alkaline solutions with high salt concentration were used to dissociate the conjugated antibody–antigen (Reddy et al., 2012) or protein–aptamer (Chen et al., 2010; Liu et al., 2010; Xia et al., 2013) in QCM or other surface sensing platforms, while such methods are invalid in dissociating hybridized duplex. Despite the hybridized strands could be separated by water rinsing (Lubin et al., 2006; Yang and Lai, 2012; Yu and Lai, 2012) and thermal denaturation (Flechsig and Reske, 2007), the strategies are also inadequate for real-time monitoring and rapid detection since an extra washing step was essential to recover the drastically changed baseline. Therefore, mild and wash-free regeneration methods that selectively release the target DNA from the recognition layer are expected to construct rapid and reusable QCM biosensors.

Strand displacement reaction (SDR) is the process through which one single strand displaces another from pre-hybridized strands, initiated at a complementary single-stranded overhang region (known as a toehold) (Zhang and Seelig, 2011). The toehold-mediated SDR is reversibly fueled by single DNA trigger at mild conditions (Zhang and Winfree, 2009), so it has been applied in constructing autonomous DNA nanomachines which can open and close repeatedly by alternately adding the fuel sequence and its complementary sequence (Alberti and Mergny, 2003; Yurke et al., 2000). The reported works provide the feasibility to construct reusable biosensing systems based on the SDR for nucleic acids. Additionally, utilizing a hybridized duplex containing a toehold domain as the recognition probe, the SDR

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shows improved specificity compared with traditional molecular beacons (Kolpashchikov, 2010; Wang et al., 2012). Overall, the toehold-mediated SDR is an ideal candidate to fabricate reusable and specific QCM biosensors for nucleic acids, which is coupled with flow injection analysis to ensure the stable density of recognition layer.

N^5, N^{10}-methylenetetrahydrofolate reductase (MTHFR) gene C677T mutation is widely considered as the most relative genetic candidate underlying folate-responsive neural tube defects (NTDs) (Beaudin and Stover, 2009) and also a risk factor of coronary heart disease (CHD) (Klerk et al., 2002). Specific detection of this mutant gene sequence containing the mutation hotspot (abbreviated to mutant DNA) is useful in predicting relative diseases. Recent works mainly focus on optical and electrochemical methods based on the DNA hybridization or ligation reaction (Chen et al., 2011; Nakamura et al., 2007), which are hard to achieve real-time and repetitive detection. Herein, we aimed to develop an on-line and mild regeneration strategy by combining the toehold-mediated SDR with the QCM technique for specific detection of the mutant DNA without enzyme at room temperature.

In this work, the toehold-mediated SDR was first utilized to fabricate a reusable QCM biosensor for specific and sensitive detection of the mutant DNA. The capture DNA was thiol-modified to self-assemble on the gold-coated chip surface of QCM, then hybridized with the conjugate of biotinylated reporter DNA and streptavidin (named as reporter probe) to form a recognition layer and produce a sharp frequency decrease. Then the mutant DNA hybridized with the reporter probe and displaced it from the chip surface, making the self-assembled layer of the capture DNA and the frequency response recover simultaneously. The recognition layer was easily regenerated by subsequently injecting the reporter probe again. Using this new strategy, a cost-effective and reusable QCM biosensor was constructed for specific detection of the mutant DNA.

2. Material and methods

2.1. Materials

All synthesized and HPLC-purified DNA sequences as shown in Table 1 and Table S1, and tris(2-carboxyethyl)phosphine (TCEP) were ordered from Sangon Biotech Co., Ltd. (Shanghai, China). Streptavidin and 6-mercaptohexanol (MCH) were from Sigma-Aldrich (St. Louis, MO, USA). (3-Aminopropyl)trimethoxysilane (APTMS) was obtained from Acros Organics (New Jersey, USA). Glutaraldehyde (50%) was of analytical reagent grade from Beijing Yili Fine Chemical Co., Ltd. (Beijing, China). Tris(hydroxymethyl) aminomethane (Tris) was purchased from Novon (USA). HeLa cells lysates were prepared according to the reported procedure (Wang et al., 2012). All DNA samples, streptavidin, and MCH were prepared by dissolving in Tris–HCl buffer (20 mM Tris, pH 7.4, containing 137 nM Na^+, 5 mM K^+, and 5 mM Mg^{2+}) and filtered by a 0.45 μm filter membrane prior to use. Deionized water was used in all experiments.

Table 1
Sequences of DNA mainly used in this study.

Name	Sequences (5′–3′)
Capture DNA	$HS(CH_2)_6$-TTTTTGTCTGCGGGAG
Reporter DNA[a]	Biotin-*TGATG***AAATCGA**CTCCCGCAGACA
Mutant DNA	TGTCTGCGGGAGTCGATTT
Wild-type DNA	TGTCTGCGGGAGCCGATTT

[a] The spacer domain is italicized, the toehold domain is bolded, and the hybridization domain is underlined.

2.2. QCM biosensing procedure

All on-line detections were implemented on a Q-Sense E4 QCM-D instrument (Q-Sense AB, Västra Frölunda, Sweden), and the frequency shift of the normalized ninth overtone which has the lowest noise was used to quantify. The real-time dissipation responses of the QCM biosensor and the corresponding discussion are in Figs. S1 and S2, and Supplementary materials. Prior to modification, the gold-coated crystal chips (5 MHz, AT-cut) (Dongwei Co. Ltd., Hangzhou, China) were immersed in a boiling mixture of 30% H_2O_2, 28% ammonia, and deionized water with a volume ratio of 1:1:5 for 10 min, then rinsed with deionized water and dried by nitrogen gas. In a typical experiment with precise temperature controlled at 25 °C, the mixture of the capture DNA (200 nM) and TCEP (400 μM) was injected for 15 min. The chip surface was then blocked by MCH (4 μM) for 15 min to form a self-assembled layer. The 1:1 mixed streptavidin and reporter DNA (100 nM) were injected for 15 min to hybridize with the capture DNA and produce remarkable frequency decrease. The frequency was recovered as the response signal by injecting the mutant DNA for 30 min. The flow rate was 30 μL/min set by Ismatec IPC tubing pump (Glattbrugg, Switzerland).

2.3. Surface regeneration and successive detection

In the sensing procedure, the reporter probe was released from the gold surface by hybridizing with the mutant DNA, resulting in a recovered self-assembled layer of the capture DNA. After rinsing with Tris–HCl buffer, the recognition layer was directly rebuilt with subsequently injecting the reporter probe again.

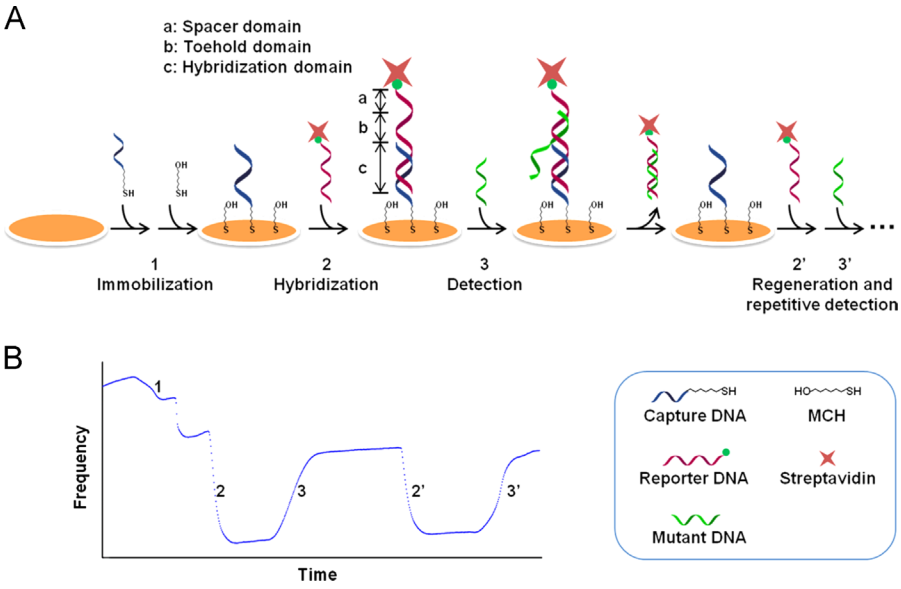
2.4. AFM characterization

Freshly cleaved micas were immersed in ethanol containing 1% APTMS for 5 min, then washed with ethanol and dried in air. The mica surface was modified with 5% glutaraldehyde for 30 min to immobilize 1 μM amino-modified capture DNA (5′-$NH_2(CH_2)_6$-TTTTTGTCTGCGGGAG-3′) through an amino-coupling reaction overnight, followed with 2.6% sodium borohydride to close down uncombined aldehyde group. 1 μM reporter probe was added to the dried surface and incubated for 1 h, followed with addition of 1 μM mutant DNA, 1 μM wild-type DNA, or Tris–HCl buffer as a control for 1 h. Finally the micas were rinsed with deionized water and dried in air for atomic force microscopy (AFM) characterization. The surface morphology of the prepared micas was observed and recorded using a SPI3800/SPA400 AFM (Seiko Inc., Tokyo, Japan) in contact mode with a Si cantilever (SI-AF01-A, Seiko Inc., Tokyo, Japan).

3. Results and discussion

3.1. Recognition and regeneration rationale of the QCM biosensor

The design rationale of the QCM biosensor based on the toehold-mediated SDR is shown in Scheme 1A. The reporter DNA is composed of hybridization, toehold, and spacer domains from the 3′ to 5′ end, in which the hybridization domain is complementary to the capture DNA, both the toehold and hybridization domains are complementary with the mutant DNA, and the spacer domain is used to reduce the steric hindrance. In a typical procedure, the capture DNA is immobilized on a chip surface to form a self-assembled monolayer within the assist of blocking reagent MCH (step 1). Then the reporter probe hybridizes with the capture DNA to produce a sharp frequency decrease due to the mass contribution of streptavidin (step 2). The toehold domain in



Scheme 1. (A) Schematic representation of the designed QCM biosensor for on-line detection and regeneration processes based on the toehold-mediated SDR. (B) Typical real-time QCM frequency response for the above five steps.

the reporter DNA hybridizes with the mutant DNA to mediate the SDR, forming a more stable duplex, so the reporter probe is removed from the chip surface and the frequency signal is simultaneously recovered (step 3). The sensing surface is easily regenerated by subsequently injecting the reporter probe again (step 2′) for next detection (step 3′). The designed detection and regeneration procedures are confirmed by the real-time frequency responses of QCM biosensor (Scheme 1B). For the wild-type DNA, the SDR is interrupted at the 3′ end of the toehold domain, so the corresponding frequency change is negligible and the response is close to that of the control sample, as shown in Fig. 1A.

3.2. Verification of the discrimination capability by AFM

The discrimination capability of the QCM biosensor based on the toehold-mediated SDR was further conformed by AFM, as shown in Fig. 1. From the topographic images, many "islands" represent streptavidin molecules on the mica surface in the control sample (Fig. 1B). For the mutant DNA sample, the mica surface is relatively smooth because the reporter probe is released (Fig. 1C). The mica surface in wild-type DNA sample is similar to that of the control sample, showing that the wild-type DNA cannot remove the reporter probe from the recognition layer (Fig. 1D). The significant difference in the surface morphology indicates that the SDR selectively occurred under the addition of mutant DNA.

3.3. Optimization of the sensing system

Considering that the steric hindrance of streptavidin may affect the efficiency of the SDR, the reporter DNA and the mutant DNA with different lengths were compared together (the sequences are shown in Table S1). From the results shown in Fig. S3, with the increasing spacer length of the reporter DNA, the frequency shift first increases and then decreases for each mutant DNA, so the spacer length should be suitable, being neither too long nor too short. To obtain the highest frequency shift, Rep5 and Mut7 were selected to fabricate the QCM biosensor in the following experiments.

3.4. Sensing performances of the QCM biosensor

3.4.1. Surface regeneration and repetitive detection

After detecting the mutant DNA, the recognition layer was regenerated by subsequently injecting the reporter probe again to hybridize with the capture DNA. To quantitatively research the repetitive performance of the QCM biosensor and shorten the detecting period, 100 nM reporter probe and 200 nM mutant DNA were flowed through the chip surface alternately, and the durations were 20 and 30 min, respectively. One typical real-time frequency response containing six cycles was recorded as shown in Fig. 2A. More than 87% signal intensity of the sensor remained after six cycles of detection and regeneration, indicating that this SDR based sensing strategy has good repeatability. Moreover, we investigated the successive detecting ability of QCM biosensor for the mutant DNA of low concentration (10 nM). Our preliminary result indicates that the fabricated biosensor has the potential for successive detection using the same sensing chip without the regeneration step (Fig. 2B).

3.4.2. Sensitivity

The proposed QCM biosensor shows different frequency responses to the mutant DNA of different concentrations (Fig. 3A). The calibration plot displays a good linear relationship between the frequency shift and the mutant DNA concentration in the range of 1–75 nM ($r=0.9982$) (Fig. 3B) with the detection limit of 0.8 nM calculated by the triple signal-to-noise method, indicating that this sensing system is sensitive.

3.4.3. Specificity

To verify the specificity of the QCM biosensor, the mixtures of mutant DNA and wild-type DNA at various ratios with a total concentration of 50 nM flowed through the sensing surface for 30 min. As shown in Fig. 4, the frequency shift of QCM biosensor increases with the increasing mutant DNA percentage in the sample solution. The frequency shifts of the mixtures are in accordance with that of the mutant DNA samples. To quantitatively characterize the specificity of this biosensor, the discrimination factor (D.F.) is defined as the ratio of the net frequency shift obtained with the mutant DNA to that with the wild-type DNA. The D.F. of single nucleotide difference between 50 nM mutant

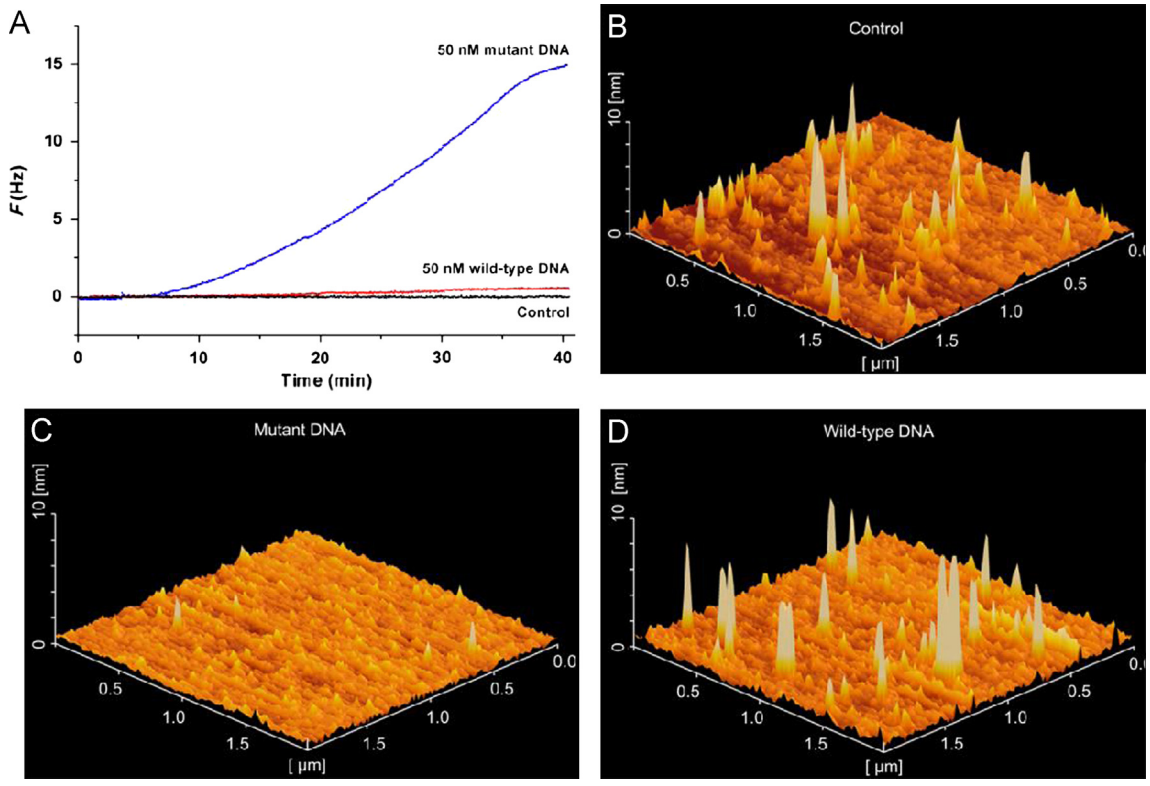


Fig. 1. (A) Frequency responses of the QCM biosensor to mutant DNA, wild-type DNA, and control sample. AFM topographic images of streptavidin-loaded mica surface after been incubated with (B) control sample, (C) mutant DNA, and (D) wild-type DNA.

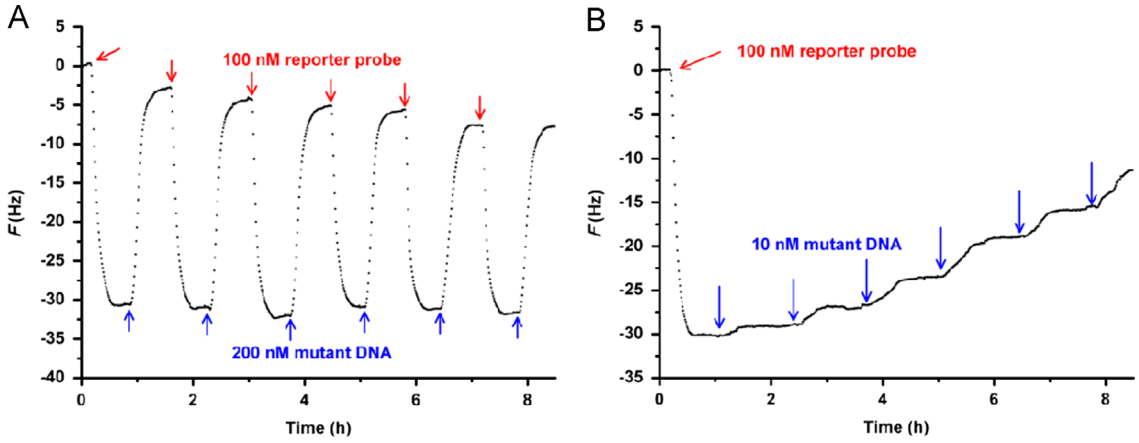


Fig. 2. Real-time frequency responses of the QCM biosensor to (A) repetitive and (B) successive detections.

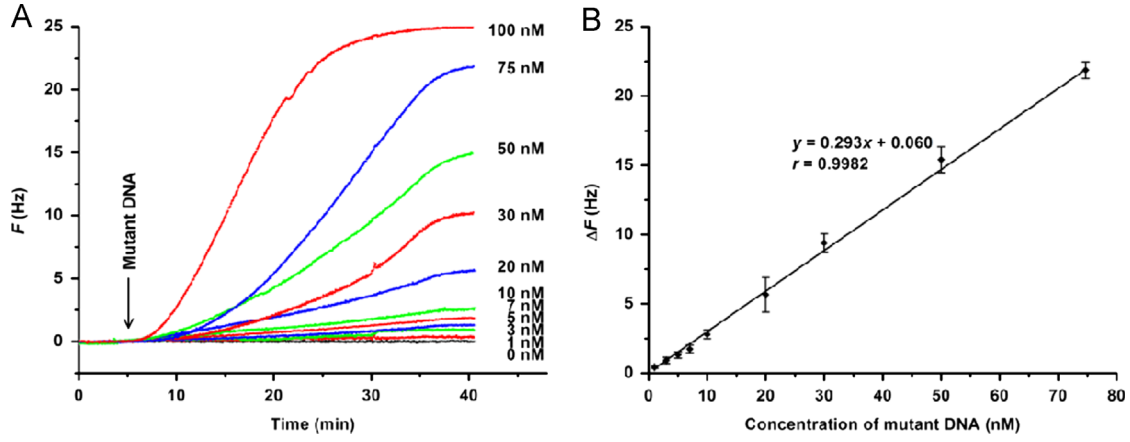


Fig. 3. (A) Real-time frequency responses of the QCM biosensor to the mutant DNA of different concentrations. (B) Linear relationship between the frequency shifts and the mutant DNA concentrations. The error bars are standard deviations of four repetitive measurements.

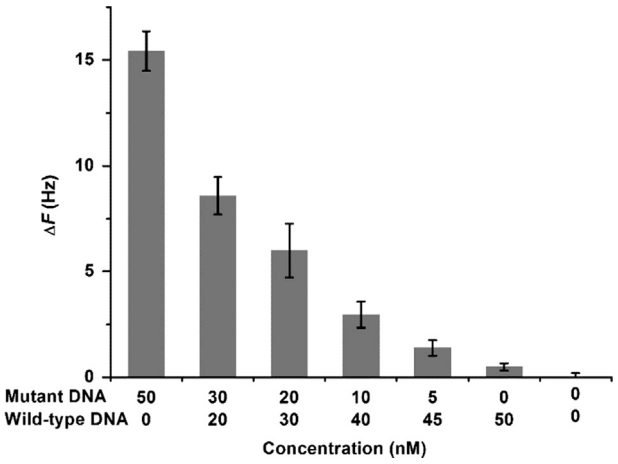


Fig. 4. Frequency responses of the QCM biosensor to the mixtures of mutant DNA and wild-type DNA. The error bars are standard deviations of four repetitive measurements.

DNA and 50 nM wild-type DNA is 33, demonstrating that the designed biosensor has high specificity to the mutant MTHFR gene fragment.

3.5. *Mutant DNA detection of spiked sample*

To evaluate the feasibility of applying the developed QCM biosensor for real samples, a 10% HeLa cells lysate was spiked with 50 nM mutant DNA. For three repetitive measurements, the found mutant DNA concentrations in the spiked samples were 47.5, 55.3, and 58.6 nM, using the unspiked 10% lysate as a control. Obtained recovery of mutant DNA was 108% (RSD = 10.6%, $n = 3$). Our preliminary result indicates that combining with pre-amplification technologies such as polymerase chain reaction (PCR), this QCM-based sensing strategy has the potential for biological sample application.

4. Conclusions

In this work, we have demonstrated that the toehold-mediated SDR can be utilized to develop a reusable QCM biosensor for specific detection of single-base DNA mutation at room temperature. The recognition layer on the chip surface was easily regenerated without obvious degradation of response signal, so the fabricated biosensor was performed for the repetitive DNA detections to shorten the detecting period and reduce the sensing cost. Considering the high specificity and repeatability, the mild and wash-free regeneration strategy developed here could possibly be used widely in other surface sensing platforms for cost-effective and specific detection of nucleotide sequences.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.04.035.

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