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Highly Selective Detection of Single-Nucleotide Polymorphisms Using a Quartz Crystal Microbalance Biosensor Based on the Toehold-Mediated Strand Displacement Reaction

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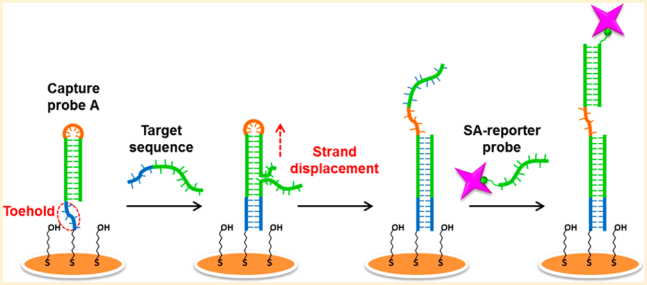
ABSTRACT: Toehold-mediated strand displacement reaction (SDR) is first introduced to develop a simple quartz crystal microbalance (QCM) biosensor without an enzyme or label at normal temperature for highly selective and sensitive detection of single-nucleotide polymorphism (SNP) in the p53 tumor suppressor gene. A hairpin capture probe with an external toehold is designed and immobilized on the gold electrode surface of QCM. A successive SDR is initiated by the target sequence hybridization with the toehold domain and ends with the unfolding of the capture probe. Finally, the open-loop capture probe hybridizes with the streptavidin-coupled reporter probe as an efficient mass amplifier to enhance the QCM signal. The proposed biosensor displays remarkable specificity to target the p53 gene fragment against single-base mutant sequences (e.g., the largest discrimination factor is 63 to C−C mismatch) and high sensitivity with the detection limit of 0.3 nM at 20 °C. As the crucial component of the fabricated biosensor for providing the high discrimination capability, the design rationale of the capture probe is further verified by fluorescence sensing and atomic force microscopy imaging. Additionally, a recovery of 84.1% is obtained when detecting the target sequence in spiked HeLa cells lysate, demonstrating the feasibility of employing this biosensor in detecting SNPs in biological samples.



Single-nucleotide polymorphisms (SNPs) are the most frequent forms of gene variant. As many SNPs are important tumor and genetic biomarkers, highly selective and sensitive detection of SNPs is a requisite for heredity-related risk assessment, disease diagnostics, drug development, and so on.[1] Existing methods can be mainly categorized into enzyme-assisted and hybridization-based ones. The enzyme-assisted approach is typically composed of a key single-nucleotide-specific enzymatic reaction, such as primer extension,[2,3] invasive cleavage,[4,5] and ligation,[6−9] with following detection of reaction products. However, most of these methods suffer from tedious procedures, labile enzyme, and costly labeling reagents. In hybridization-based approaches, DNA probes are designed to report the hybridization difference between perfectly matched and single-nucleotide mismatched targets.[10] Among these strategies, molecular beacon (MB) technology, known for its prominent features of easy designability and good performances at room temperature,[11] is widely applied in solution-phase sensing systems[12,13] and surface sensing platforms such as electrochemical biosensors[14−16] and fluorescence imaging.[17,18] The limited discrimination capability born from the simple stem-loop structure could be improved with more complex probe designs such as the triple-stem probe,[19,20] which effectively resolves the small difference in thermodynamic stability between the target and mutant sequences. However, it remains necessary and meaningful to develop a simple and highly specific sensing strategy without a label or enzyme for SNPs detection at normal temperature.

The strand displacement reaction (SDR) can be initiated by a fuel strand hybridization with the complementary single-stranded overhang domains (known as toeholds) of two or more prehybridized strands and progresses through a branch migration process.[21] Toehold-mediated SDR can take place without an enzyme at room temperature, and the kinetic rate can be controlled by adjusting the length and sequence composition of the toehold.[22−24] So it is an ideal design ingredient in DNA nanotechnology, such as logic gates and a network,[25] a noncovalent DNA catalytic amplifier,[26] an autonomous DNA nanomachine,[27] and a hybridization chain reaction.[28] With these prominent advantages, it is also a promising technology in analyzing nucleic acid sequences. However, toehold-mediated SDR was used in only a few works for detecting single strand DNA (ssDNA) by fluorescence microscopy[29] or atomic force microscope (AFM) imaging,[30] in which the substructures for immobilizing the capture probe were complex and costly. Some articles reported the sensing strategy combining toehold-mediated SDR with enzyme, which effectively increased the sensitivity of detecting ssDNA but

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Table 1. Sequences of Oligonucleotides Used in This Study[a]

Name	Sequences (5' to 3')
Capture probe A	$HS(CH_2)_6$-AAACACGCACCTCAAAGC*CATGCT*GCTTTGAGGTGC
Capture probe A*	FAM-AAACACGCACCTCAAAGC*CATGCT*GCTTTGAGGTGC-DABCYL
Capture probe B	$HS(CH_2)_6$-TCACAAACACGCACCTCAAAGC*CATGCT*GCTTTGAGGTGC
Capture probe B*	$NH_2(CH_2)_6$-TCACAAACACGCACCTCAAAGC*CATGCT*GCTTTGAGGTGC
Reporter probe	Biotin-GCACCTCAAAGC
$Target_{18}$	GCTTTGAGGTGCGTGTTT
$Target_{19}$	GCTTTGAGGTGCGTGTTTG
$Target_{17}$	GCTTTGAGGTGCGTGTT
$Target_{16}$	GCTTTGAGGTGCGTGT
Mutant A	GCTTTGAGGTGCATGTTT
Mutant T	GCTTTGAGGTGCTTGTTT
Mutant C	GCTTTGAGGTGCCTGTTT
Mutant D[b]	GCTTTGAGGTGCTGTTT
Random	TGTCTGCGGGAGCCGATT

[a]In the capture probes, the toeholds are underlined and the loops are italicized. In the target and mutant sequences, the bases at the mutational position are highlighted in the box. [b]The mutant type is the deletion of the relative base.

suffered from very complicated and time-consuming procedures.[31,32] Therefore, it is a great challenge to develop a simple and effective sensing platform for SNPs detection based on toehold-mediated SDR.

The quartz crystal microbalance (QCM) biosensor as a real-time and label-free sensing platform has been widely applied in biochemical analysis.[33,34] Nevertheless, present strategies for SNPs detection on a QCM platform are mainly based on the ligase reaction, which are not satisfying due to their tedious and nonisothermal procedures.[35,36] As a mass-sensitive platform, the QCM biosensor shows low sensitivity to directly detect ssDNA by hybridization without further signal amplification, not to mention discrimination of SNPs. In the current efforts, we aimed to introduce toehold-mediated SDR into the QCM sensing platform utilizing a streptavidin-coupled reporter probe (SA-reporter probe) as an efficient mass amplifier and develop a new strategy for simple, selective, and sensitive detection of SNPs.

In this work, we develop a simple and sensitive QCM biosensor to detect a target sequence specifically which combines the enzyme-free and normal-temperature features of toehold-mediated SDR with a real-time and label-free QCM technique for the first time. A 18-nucleotide (nt) sequence in the p53 gene including the mutation hotspot R273H as the target (named as $target_{18}$) was chosen.[37] A hairpin capture probe with an external toehold was designed to anchor on the chip surface by the Au−S bond. Once hybridizing with the external toehold, $target_{18}$ could initiate the unfolding of the capture probe through a strand displacement process. Subsequently, the open-loop capture probe hybridized with the SA-reporter probe for signal amplification. Otherwise, single-base mutant sequences with the mutant types at codon 273 could hardly unfold the capture probe. Specificity of this sensing system was also verified by fluorescent sensing and AFM imaging. This work provides a simple and universal strategy for SNPs detection using toehold-mediated SDR by a QCM biosensor.

EXPERIMENTAL SECTION

Materials. All synthesized and HPLC-purified sequences of oligonucleotides as depicted in Table 1 and tris(2-carboxyethyl)phosphine (TCEP) were ordered from Sangon Biotech Co., Ltd. (Shanghai, China). Streptavidin (SA) and 6-mercaptohexanol (MCH) were purchased from Sigma-Aldrich (St. Louis, MO). RIPA cell lysis solution was from Yuanpinghao Bio (Beijing, China). Tris(hydroxymethyl)-aminomethane (Tris) was obtained from Novon. (3-Aminopropyl)triethoxysilane (APTES) was from Acros Organics (New Jersey). Concentrated sulfuric acid, hydrogen peroxide (30%), and ammonia−water (28%) were analytical reagent grade from Beijing Chemical Works (Beijing, China). Glutaraldehyde (50%) was also analytical reagent grade from Beijing Yili Fine Chemical Co., Ltd. (Beijing, China). All DNA samples and SA were prepared by dissolving in Tris−HCl buffer (20 mM Tris, pH 7.4, containing 5 mM $MgCl_2$, 137 nM NaCl, and 5 mM KCl) and filtered by 0.45 μm filter membrane prior to use. Deionized water was used in all experiments.

QCM Measured Procedure. All online experiments were executed on a Q-Sense E4 QCM-D instrument (Q-Sense AB, Västra Frölunda, Sweden), which has four channels and can

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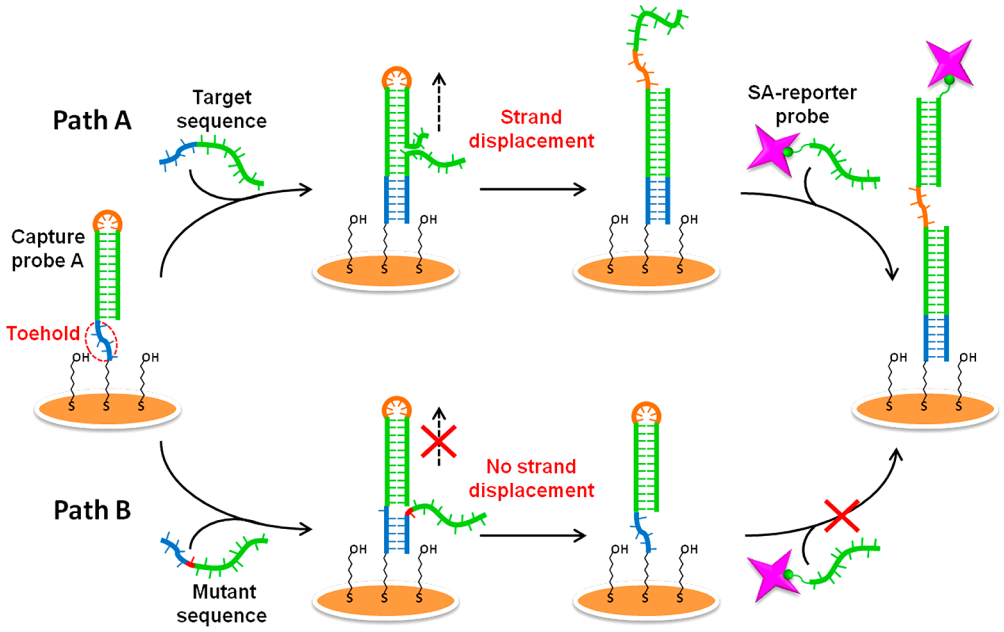
Scheme 1. Schematic Representation of the Sensing Processes of Amplified QCM Biosensor Based on Toehold-Mediated SDR[a]



[a]Paths A and B are for detecting the target sequence or mutant sequences, respectively.

provide real-time responses of multiovertone frequencies. Prior to use, the gold-coated crystal chips (5 MHz, AT-cut) (Hrbio Co. Ltd., Beijing, China) were immersed in newly prepared piranha solution, a 3:1 mixture of concentrated sulfuric acid and 30% hydrogen peroxide (CAUTION: piranha solution is extremely corrosive, reactive, and potentially explosive; please be careful to avoid skin contact), for 5 min followed with thorough rinsing with deionized water; then they were immersed in a boiling mixture of 30% hydrogen peroxide, 28% ammonia−water, and deionized water with a volume ratio of 1:1:5 for 10 min. Before loading to measuring cells, the chips were rinsed with deionized water and dried by nitrogen gas. In a typical experiment, after the mixture of the capture probe (100 nM) and reducer TCEP (10 μM) was injected for 50 min, MCH (1 mM) flowed through the channels for 20 min to block the gold surface and remove weakly bound capture probe. Then target$_{18}$ was injected for 1.5 h to open the hairpin capture probe. The amplified response was obtained from the final injection of the SA-reporter probe (the mixture of 100 nM streptavidin and 100 nM reporter probe) for 40 min. The running rate was 10 μL/min set by a Ismatec IPC tubing pump (Glattbrugg, Switzerland), and all injected samples were preincubated for 1.5 h at room temperature. To reduce the effect of system error, the frequency shift of the normalized ninth overtone which has the smallest noise was used to quantify.

Fluorescence Measurement. The F-7000 fluorescence spectrometry (Hitachi, Japan) was utilized to verify the occurrence of toehold-mediated SDR between capture probe A* and target sequences. Capture probe A* (50 nM) was mixed with 100 nM target$_{18}$ or mutant A at room temperature for 1 h, then the intensity of emitted light at 520 nm was measured.

AFM Characterization. Freshly cleaved micas were immersed in 95% acetone containing 1% APTES for 5 min, then rinsed with acetone and dried in air. Capture probe B* (1 μM) was immobilized on the mica surfaces through an amino-coupling procedure using 5% glutaraldehyde as the linker, then blocked with 2.6% sodium borohydride. Target$_{18}$ or mutant A (1 μM) was dropped to the dried surface and reacted for 4 h, followed with addition of 1 μM SA-reporter probe and reacting for 2 h. Finally the micas were washed with deionized water and dried in air for AFM characterization. The surface morphology of the prepared micas was observed and recorded by using a SPI3800/SPA400 AFM (Seiko Inc., Tokyo, Japan) in tapping mode. A Si cantilever with an oscillation frequency of 125 kHz and a spring constant of 14 N/m (SI-DF20, Seiko Inc., Tokyo, Japan) was used for AFM imaging.

Preparation of HeLa Cells Lysate. Incubated HeLa cells were collected by trypsinization and centrifugation, washed with 0.1 M PBS, and pelleted at 3 000 rpm for 5 min at 4 °C. The cells were resuspended in RIPA cell lysis solution at a concentration of 5.0 × 10^6 cells/mL, incubated for 30 min at −20 °C, and then centrifuged at 12 000 rpm for 30 min at 4 °C. The supernatant was collected and filtered by a 0.45 μm filter membrane prior to storing at −20 °C.

RESULTS AND DISCUSSION

Discrimination Rationale of the QCM Biosensor. The rationale of the QCM biosensor based on toehold-mediated SDR for specific discrimination SNPs is shown in Scheme 1. Considering loop opening via an external toehold is 10−100 times faster than via an internal toehold,[24] the hairpin capture probe A consists of a 6-nt loop, 12-basepair (bp) stem, and 6-nt external toehold at its 5′ end, in which the sequence of the external toehold and linked stem strand is exactly complementary to target$_{18}$, the stem sequence at the 3′ end is exactly complementary to the reporter probe, and the loop sequence is not complementary to any sequence. A sensing strategy separating target-initiated strand displacement and signal amplification is employed to accomplish the selective and sensitive detection. In a typical sensing process as path A, the capture probe A and MCH are immobilized on the chip surface by a Au−S bond in sequence. Then strand displacement is

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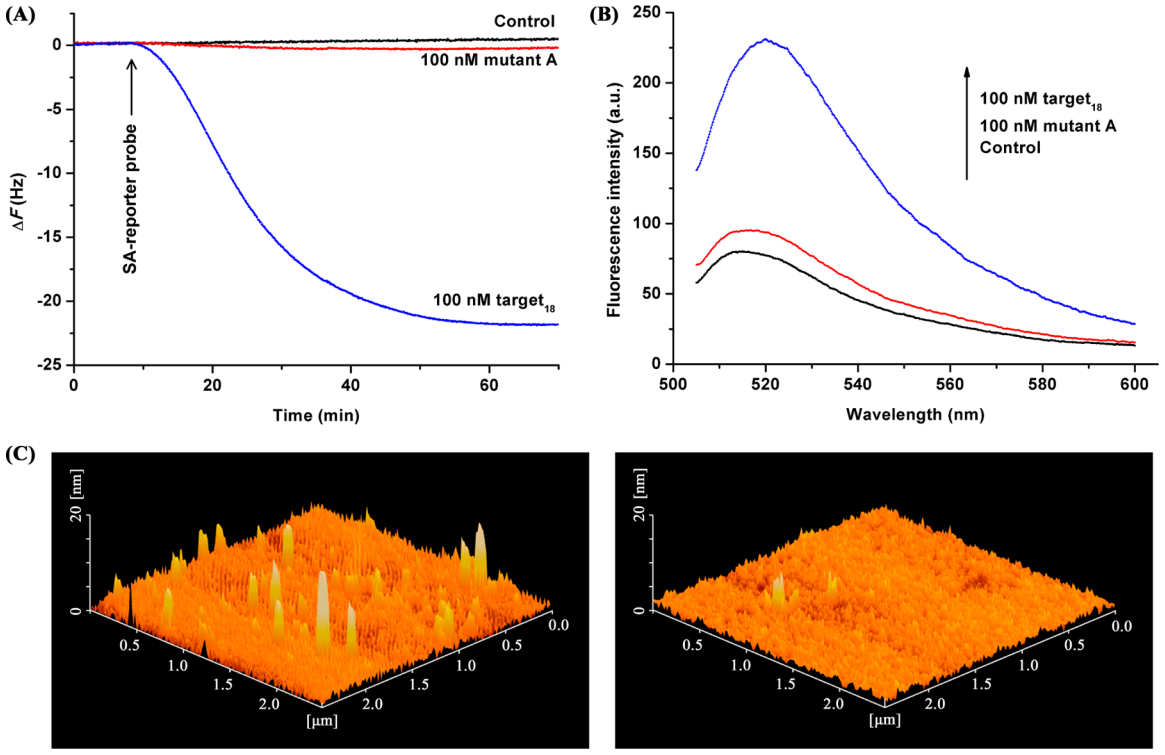

Figure 1. (A) Real-time frequency shifts of amplified QCM biosensor to target$_{18}$, mutant A, or control sample injected for 3 h. The concentration of capture probe A was 100 nM. (B) Fluorescence spectra of capture probe A* (50 nM) with target$_{18}$, mutant A, or control sample. (C) AFM images of SA-loaded mica surfaces. The 1 μM capture probe B* immobilized on the mica surfaces hybridized with 1 μM target$_{18}$ (left) or mutant A (right), followed by the addition of 1 μM SA-reporter probe.

initiated by target hybridization with the toehold domain and accomplished by the migration of the stem strand at the 3′ end. Finally this new emerging single strand hybridizes with the SA-reporter probe, producing an amplified frequency shift of the crystal chip as the output signal. As path B is shown in Scheme 1, the strand displacement process is prevented because the single-base mismatch at the 3′ end of the external toehold greatly suppresses the hybridization between the capture probe A and single-base mutant sequences. So it is very difficult for the SA-reporter probe to load on the surface. Following this design strategy, the target sequence can be specifically discriminated from the single-base mutant sequences.

Verification of the Discrimination Capability. The design rationale of this sensing system was verified by the typical result that a real-time frequency shift of amplified QCM biosensor to target$_{18}$ is much larger than to mutant A and control samples, as shown in Figure 1A.

The capture probe is the crucial component for specific detection of target$_{18}$ in this sensing platform. To verify the highly-selective reaction between the designed capture probe and target$_{18}$, fluorescence sensing with a labeled capture probe A (named as capture probe A*) was used. The fluorophore FAM at the 5′ end of capture probe A* was quenched by DABCYL at the 3′ end when the target sequence was absent. With the addition of target$_{18}$, fluorescence was recovered because the unfolding of capture probe A* increased the spatial distance of FAM and DABCYL; however, fluorescence was recovered much less under single addition of mutant A, showing this hairpin probe was rarely opened (Figure 1B).

The discrimination capability of this surface sensing strategy was further verified by AFM imaging. The capture probe B* was immobilized on the mica surfaces with an amino coupling procedure. Through an open-loop process initiated by target$_{18}$, the SA-reporter probe can load on the surface, shown as numerous "islands" (Figure 1C, left). On the other hand, the smooth surface (Figure 1C, right) illustrates that mutant A fails to open the capture probe B*. The agreement of these data clearly indicates the specificity of the designed capture probe.

Influence of Temperature on Toehold-Mediated SDR. For investigating the influence of temperature on toehold-mediated SDR, online sensing experiments were executed under fixed chamber temperatures of 15, 20, 25, or 30 °C, respectively. The QCM responses to the capture probe A and SA-reporter probe were shown in Figure 2. The impact of temperature to frequency change of quartz crystal can be ignored from the nearly invariable responses to the capture probe A. So the different QCM responses to the SA-reporter probe indicate that toehold-mediated SDR is impacted by temperature. The effect of temperature on QCM response to the SA-reporter probe is in accordance with the simulated results from NUPACK algorithms (www.nupack.org): providing the concentrations of both capture probe A and target$_{18}$ were 100 nM, in the presence of 137 mM Na^+ and 5 mM Mg^{2+}, the concentrations of produced complex are 95.6, 90.5, 81.1, and 65.7 nM at 15, 20, 25, and 30 °C, respectively. According to both the experimental and simulated results, temperature mainly impacts toehold-mediated SDR through thermodynamic factors. For satisfying the demand of good sensitivity, 20 °C was chosen for later experiments. More significantly, it is demonstrated that the toehold-mediated SDR based QCM biosensor is capable of detecting ssDNA at normal temperatures.

Concentration Optimization of Reporter Probe. It is well-known that one SA molecule has four binding sites for

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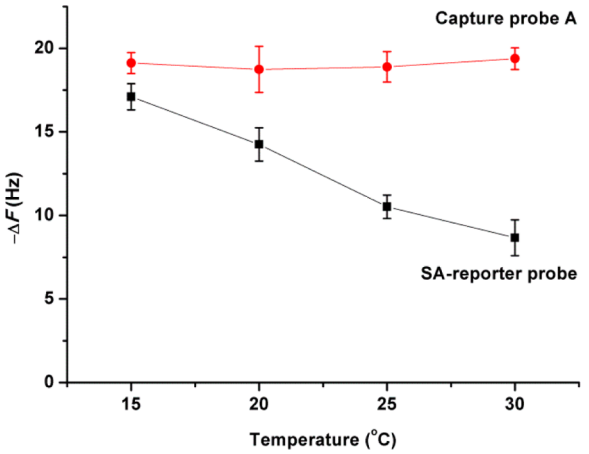

Figure 2. Effect of temperature on frequency response of the QCM biosensor. Capture probe A (100 nM), SA-reporter probe (mixture of 100 nM SA and 300 nM reporter probe), and target$_{18}$ (100 nM, injected for 2 h). The error bars are standard deviations of three repetitive measurements.

coupling with biotin, so the concentration of the reporter probe may affect the responses of this sensing system when the concentration of SA is fixed. Taking this into account, the reporter probe of various concentrations (0−400 nM) was incubated with 100 nM SA before injection, respectively. As shown in Figure 3, when only SA was injected, the frequency

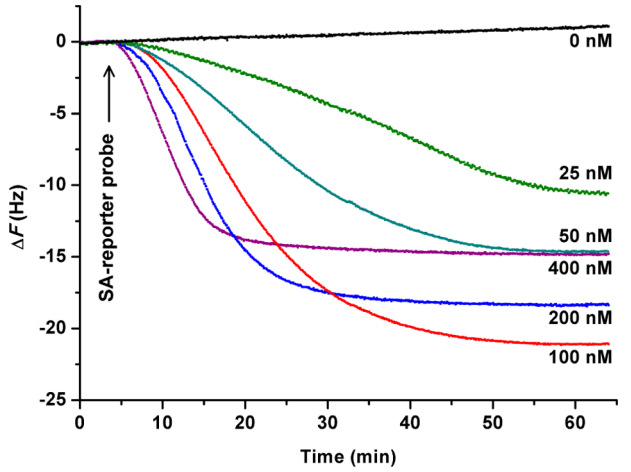

Figure 3. Effect of reporter probe concentration (100 nM SA) on frequency response of amplified QCM biosensor to 100 nM target$_{18}$.

did not change, ruling out the possibility of the adsorption of SA on the chip surface. With the concentration of reporter probe increasing from 25 to 400 nM, the equilibrium time was shortened as expected. The stoichiometric ratio of the reporter probe and SA is critical in obtaining high-frequency responses. With a ratio less than 1, the SA may not be very well coupled by the reporter probe, thus the lower frequency response was observed. At a ratio higher than 1, one SA-reporter probe may bind to more than one opened capture probes. As a result, the amount of SA anchored on the chip surface was reduced, and thus the frequency response was also decreased. The maximum frequency response was obtained with a stoichiometric ratio of 1. Thus, 100 nM reporter probe was used in the later sensing process.

Kinetics of Toehold-Mediated SDR on the Chip Surface. As the SDR was initiated near the solid−liquid interface in this sensing system, the capture probe B with an extra 4-nt spacer at the 5′ end was compared with the capture probe A to investigate the influence of steric hindrance on the kinetics of toehold-mediated SDR. The capture probe B displayed a much shorter equilibrium time (about 60 min in Figure 4B) than the capture probe A (about 150 min in Figure

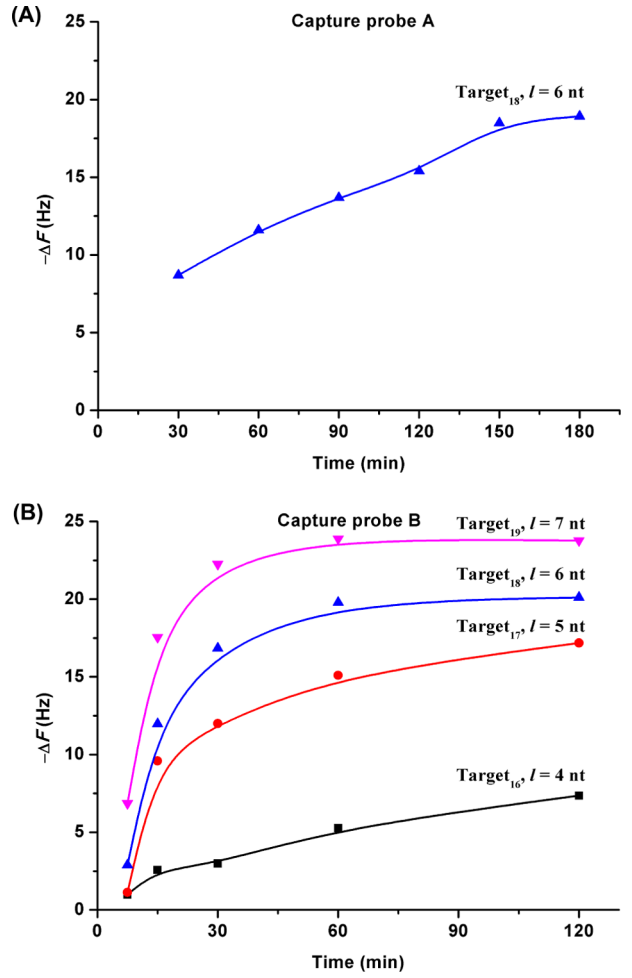

Figure 4. Frequency responses of QCM biosensor based on toehold-mediated SDR to targets with different toehold length (l). Each concentration of capture probes and targets is 100 nM.

4A) to target$_{18}$, indicating that the capture probe with a longer spacer results in smaller steric hindrance for target$_{18}$ hybridization.

The kinetic rate of toehold-mediated mainly depends on the length of the toehold. A related article reported that the toehold length of 6 nt was the smallest for initiating SDR at the maximum rate in solution.[22] However, the influence of toehold length on the kinetics of surface SDR has not been reported yet. We changed the toehold length through altering the length of the target p53 gene fragment, just like target$_{19}$ to target$_{16}$ made the effective toehold length varying from 7 nt to 4 nt, respectively. As shown in Figure 4B, the equilibrium time was shortened with the increased toehold length. Target$_{18}$ had an equilibrium time of about 60 min, similar to target$_{19}$ but much shorter than target$_{17}$ and target$_{16}$, illustrating that a toehold length no less than 6 nt generates the maximum SDR kinetic

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rate on the chip surface, which is in accordance with solution-phase SDR.[22]

Sensing Performances of the QCM Biosensor. To investigate the analytical performances of this sensing platform, experiments were carried out under fixed conditions of capture probe B and SA-reporter probe (100 nM for each) at 20 °C, and the injection time of target$_{18}$ or mutant sequences was 1.5 h.

Selectivity. The selectivity of the biosensor is a critical indicator for SNPs detection. To quantitatively characterize the specificity of this sensing system, the discrimination factor (DF) is defined as the ratio of the net signal gain obtained with target$_{18}$ to that obtained with mutant sequences under the same conditions. According to the results in Figure 5, the present

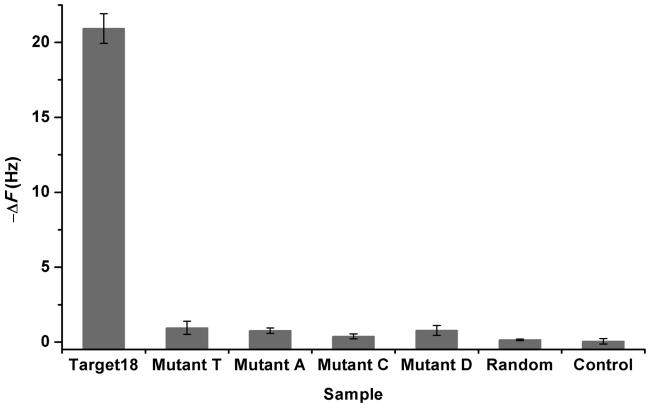

Figure 5. Frequency responses of amplified QCM biosensor to target$_{18}$, single-base mutant sequences, random sequence, or control sample based on toehold-mediated SDR. Each concentration of p53 samples is 100 nM. The error bars are standard deviations of three repetitive measurements.

biosensor showed perfect selectivity to target$_{18}$, with remarkable DF values to mutant T, mutant A, mutant C, and mutant D that are 23, 29, 63, and 28, respectively, much higher than the reported data from some enzyme-free technologies such as molecular beacon[12−18] and triple-stem probe.[19,20] The highest DF value was achieved against mutant C, showing the C−C mismatch has weaker thermostability than C−A and C−T mismatches, which is in accordance with the reported results.[19] The high DF values may be attributed to the following factors: the first, from a dynamic point of view, toehold-mediated SDR processes step by step, so the mismatch between the mutant sequence and the capture probe at the 3′ end of the external toehold greatly prevents the impending strand displacement; the second, the hairpin capture probe B including a 12-bp stem duplex has good thermal stability unless hybridizing with a longer complementary ssDNA such as target$_{18}$ to form a more stable duplex, so mutant sequences are insufficient to unfold the capture probe; finally, in this surface sensing platform, the toehold domain is located near the gold membrane−solution interface and isolated from the mobile phase by the loop, which enhances the signal-to-noise ratio.

Sensitivity. This sensing system is a sandwich-like strategy. Without amplification of the SA-reporter probe, the QCM biosensor could only detect target$_{18}$ over 10 nM directly. After injection of the SA-reporter probe, the QCM biosensor showed different amplified responses to target$_{18}$ of various concentrations (Figure 6A). This QCM biosensor showed the linear relationship in the range of 0.5−20 nM target$_{18}$ (Figure 6B)

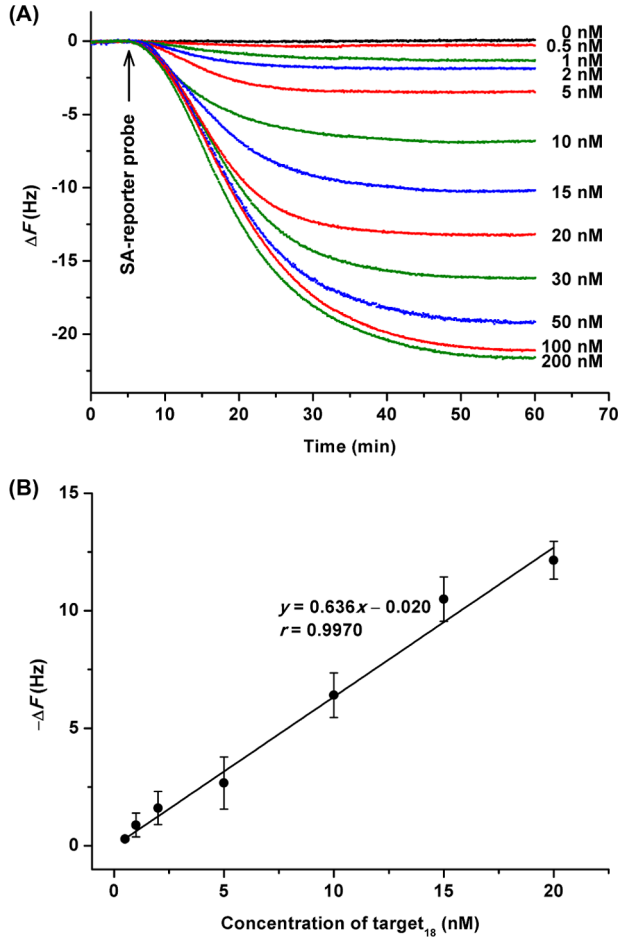

Figure 6. (A) Real-time frequency responses of amplified QCM biosensor to target$_{18}$ of different concentrations. (B) Linear relationship between the frequency shifts and the target$_{18}$ concentrations. The error bars are standard deviations of four repetitive measurements.

with the detection limit of 0.3 nM calculated by the triple signal-to-noise method, showing an obvious improvement in sensitivity.

Target$_{18}$ Detection in Spiked Sample. To evaluate the feasibility of applying this sensing platform for real samples, a 10% HeLa cells lysate was spiked with 20 nM target$_{18}$ and analyzed using the developed QCM biosensor. Obtained recovery of target$_{18}$ was 84.1% (RSD = 6.7%, n = 4). Meanwhile, no evident frequency shift was observed using unspiked lysate sample. Our preliminary results indicated the possibility of applying this sensing strategy for SNPs detection in biological samples.

■ CONCLUSIONS

In this work, toehold-mediated SDR was first applied in developing a simple, selective, and sensitive QCM biosensing strategy without a label or enzyme for SNPs detection at normal temperature. The proposed QCM biosensor displayed significant discrimination capability to target p53 gene fragments against single-base mutant sequences. Further research is focused on increasing the performances of detecting the target sequence in biological samples. The proposed sensing strategy not only extends the application of the toehold-mediated SDR and QCM technique but also provides a simple and universal strategy for SNPs detection through easily

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altering the sequences of probes according to the sequences around target SNPs.

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Notes

The authors declare no competing financial interest.

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