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ORIGINAL PAPER

Streptavidin-enhanced assay for sensitive and specific detection of single nucleotide polymorphism in TP53

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Abstract This paper reports an approach to detection of single nucleotide polymorphism based on special amplification assay and surface plasmon resonance biosensor technology. In this assay, a part of the target DNA is recognized by a probe (probe A) coupled with streptavidin–oligonucleotide (SON) complexes ex situ, and when the mixture is injected in the sensor, another part of the target DNA is recognized by a DNA probe (probe B) immobilized on the sensor surface. To achieve high sensitivity and specificity, the assay is optimized in terms of composition of SON complexes, probe design, and assay temperature. It is demonstrated that this approach provides high specificity (no response to targets containing single-mismatched bases) and sensitivity (improves sensor response to perfectly matched oligonucleotides by one order of magnitude compared to the direct detection method). The assay is applied to detection of a short synthetic analogue of TP53 containing a "hot spot"—single nucleotide mismatch frequently mutated in germ line cancer—at levels down to 40 pM.

Keywords Surface plasmon resonance · TP53 · Single nucleotide polymorphism · Point mutation · Amplification

Introduction

The largest degree of sequence variation in human genomes can be attributed to the differences due to single base substitution polymorphism, commonly referred to as a single nucleotide polymorphism (SNP). Although the majority of these genetic alterations are not harmful, some of them may disrupt or alter the coding of amino acids resulting in protein dysfunction. Recent advances in genomic techniques [1] have led to identification of dozens of such SNPs and related them to genetic and hereditary diseases [2]. TP53 gene plays a key role in regulation of cell cycle and is therefore the most frequently mutated gene in human cancers [3, 4]. The majority of those mutations are missense substitutions affecting the DNA-binding core of the protein [5, 6] and influencing patient prognosis and therapy response. Detection of those specific mutations is therefore of fundamental importance for the early diagnosis and monitoring of development and treatment of numerous serious diseases.

The number of SNP genotyping methods aimed at detection of known mutations has soared in recent years (for review, see [7]), and many robust techniques are currently available, such as denaturing high-performance liquid chromatography [8, 9], single-strand conformation polymorphism [10, 11], and denaturing gradient gel electrophoresis [12]. Several recently developed technologies based on microarrays, microelectrophoresis, and beads have made steps towards miniaturization and high throughput [13, 14]. However, the search for rapid, high-throughput, high-sensitivity, and cost-effective methods still continues.

Biosensors, such as those based on surface plasmon resonance (SPR), present a promising alternative to conventional techniques and allow for rapid, sensitive, and on-site analysis [15]. In recent years, SPR biosensor technology has made vast advances both in terms of technology and applications. SPR biosensors provide a sensitive platform for detection of various types of molecules [16] (proteins, peptides, nucleic acids, etc.) and

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species (bacteria and viruses) and make it possible to detect nucleic acids at subnanomolar levels [17]. SPR biosensors have also made substantial advances towards high-throughput screening [18]. However, providing both high sensitivity and specificity towards target analyte remains one of the main challenges for SPR biosensors [19-21].

Various approaches have been proposed to improve sensitivity of SPR DNA biosensors. These include nanoparticle-enhanced surface plasmon spectroscopy [22] or employment of enzymes providing femtomolar detection limits [23, 24]. Another way of SPR signal amplification is the introduction of agents with high molecular mass. Typical amplification strategies employ sandwich assay format and introduce hydrogel nanospheres [25] or gold nanoparticles to achieve picomolar detection limits [26, 27]. Corn et al. developed an assay with avidin-DNA multilayers which enhanced the biosensor signal by a factor of ~4 [28]. This approach has been further elaborated by Uludag et al. combined it with a quartz crystal microbalance (QCM) and achieved the limit of detection of ~50 pM [29]. However, the majority of the above-mentioned assays have been optimized to achieve high sensitivity, while paying no attention to specificity. Sensitivity and specificity have been addressed, for instance, in the work by Okumara et al., who performed detection of point mutation in K-ras by employing hydrogel nanospheres with the lowest measured concentration of ~60 nM [25].

In this paper, we report on a novel assay for detection of SNP using SPR methodology. The assay employs streptavidin–oligonucleotide (SON) complexes, which are attached to the target and then injected in the sensor in a single step. The effect of composition of SON complexes, temperature, and probe design is investigated. We demonstrate that the optimized assay (a) significantly increases specificity of short oligonucleotide detection and (b) enhances sensor response by a factor of up to 15. The assay is demonstrated for detection of short synthetic analogue of TP53 containing a "hot spot"—single nucleotide mismatch in codon 273 frequently mutated in germ line cancer.

Materials and methods

Amplification assay

The assay reported herein is based on biomolecular amplification of the mass-sensitive (e.g. SPR, QCM, and interferometric) sensor signal with SON complexes. Concept of the assay is illustrated in Fig. 1. Target oligonucleotides contain two specific regions (part A and part B). Part A is complementary to probe A (P_A) in SON complex, and part B is specifically recognized by probe B (P_B), which is immobilized on the sensor surface. Creation of SON

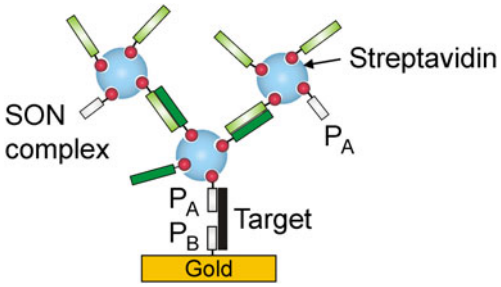

Fig. 1 Assay based on streptavidin–oligonucleotide (SON) complexes: capture of SON–target complex by the DNA probes immobilized on the sensor surface

complexes is performed ex situ by linking together streptavidin, biotinylated oligonucleotides (poly(dA) and poly(dT)), and a 9-mer probe (P_A). The solution of SON complexes is mixed with the target and then injected in the SPR sensor, where the binding of the target–SON complexes to the P_B is monitored.

In this work, we shall compare performance of this novel assay with the conventional direct detection of oligonucleotides. These experiments will be performed using the same SPR sensor and immobilization method.

Materials

Oligonucleotides (Table 1) were purchased from Integrated DNA Technologies, USA. For the SPR-chip functionalization and immobilization of oligonucleotides, the following reagents were used: alkanethiols HS-C_{11}-$(EG)_4$-OH were obtained from Prochimia, Poland and sodium acetate, KH_2PO_4, Na_2HPO_4, KCl, NaCl, $MgCl_2$, NaOH, formic acid (98%), and Tris ($C_4H_{11}NO_3$) were purchased from Sigma Aldrich, USA, in mol. biol. grade or higher. Streptavidin from *Streptomyces avidinii* was also obtained from Sigma Aldrich, USA. SuperBlock® T20 (PBS) blocking buffer was obtained from Thermo Scientific, USA. All buffers were prepared using high purity water (18 MΩ/cm resistance, Direct-Q from Millipore).

SPR sensor

In this work, a four-channel SPR biosensor developed at the Institute of Photonics and Electronics, Prague, was used. The sensor is based on the attenuated total reflection method and wavelength spectroscopy of surface plasmons [30]. In this sensor, white light is used to excite surface plasmons on a thin gold film in four areas, which are interfaced with four separate channels of a flow-cell. Reflected light is monitored by a four-channel spectrograph. The samples are delivered by peristaltic pump to the detection chambers allowing for independent functionalization and detection at four areas on SPR chip.

Table 1 The sequences of the used oligonucleotides

TP53	5′-AAA CAC GCA *C*CT CAA AGC TG-3′
MismB	5′-AAA CA**T** GCA *C*CT CAA AGC TG-3′
MismA	5′-AAA CAC GCA *C*CT CA**G** AGC TG-3′
MismAB	5′-AAA CA**T** GCA *C*CT CA**G** AGC TG-3′
MismB2	5′-AA**G** CA**T** GCA *C*CT CAA AGC TG-3′
sTP53	5′-AAA CAC GCA CTC AAA GCT G-3′
sMismB	5′-AAA CA**T** GCA CTC AAA GCT G-3′
sMismA	5′-AAA CAC GCA CTC A**G**A GCT G-3′
sMismAB	5′-AAA CA**T** GCA CTC A**G**A GCT G-3′
sMismB2	5′-AA**G** CA**T** GCA CTC AAA GCT G-3′
P1	HS-5′-CAG CTT TGA GGT GCG TGT TTG-3′
P_B	5′-TGC GTG TTT GAT TAT T-3′-HS
P_A	Biotin-$(TEG)_2$-5′-CAG CTT TGA G-3′
BdA_{23}	Biotin-$(TEG)_2$-5′-$(dT)_{23}$-3′
BdT_{23}	Biotin-$(TEG)_2$-5′-$(dA)_{23}$-3′

The letters in bold represent mismatches in the sequence of CTP53. Central nucleotide of the targets, which does not hybridize with neither of probe P_A nor P_B, is rendered in italic. The biotinylated probes contain two triethylene glycol (TEG) spacers between the biotin and DNA sequence

Immobilization of oligonucleotides on SPR chip

A method employed herein for the functionalization of the SPR sensor chips is based on the attachment of thiol-derivatized DNA probes to the gold layer on the sensor surface. The clean sensor chip was rinsed with ethanol and Milli-Q water and dried with stream of nitrogen. The chip was then mounted to the SPR sensor, and the 1-μM solution of thiolated probes in PBS buffer (137 mM NaCl, 1.4 mM KH_2PO_4, 8 mM $Na_2HPO_4{\cdot}12H_2O$, 2.7 mM KCl, pH 7.4 at 25 °C) was injected in the flow-cell and flowed through at a constant flow rate of 5 μL/min for 1 h. For the direct detection, the sensor chip was coated with 23-mer probe complementary to the whole target (P1), whereas for the amplified detection, shorter 9-mer probes P_B were used. The sensor surface was then incubated in 1 μM blocking alkanethiol (HS-C_{11}-$(EG)_4$-OH) solution in PBS for 1 h. The flow rate was then increased to 30 μL/min, and the PBS buffer was injected for a few minutes. Then, the $Tris_{Mg}$ buffer (10 mM Tris–HCl buffer, 30 mM $MgCl_2$, pH 7.4 at 25 °C) was injected. To remove all non-covalently bound alkanethiols, the surface was rinsed with 5 min injection of PBNa buffer (10 mM phosphate buffer, 0.75 M NaCl, pH 7.6 at 25 °C) and then flushed with $Tris_{Mg}$ buffer. Prior to the detection, a regeneration solution (2 mM NaOH) was injected for 5 min, followed with $Tris_{Mg}$ buffer and 5 min injection of SuperBlock® T20 (PBS) blocking buffer and returned to the $Tris_{Mg}$ buffer.

DNA detection

Sensor chip functionalized with thiolated DNA probes was used for detection of the target. After sensor response had been stabilized in $Tris_{Mg}$ buffer, sensor chip was incubated for 10 min with sample with or without the addition of SON amplifier (amplified or direct detection, respectively). The sample solution was then changed for $Tris_{Mg}$. Bound target could be then stripped away by 5 min injection of 3% formic acid (direct detection) or 2 mM NaOH (amplified detection). The surface was then washed with $Tris_{Mg}$. To keep the nonspecific binding of the SON complexes to the sensing surfaces low, the 5-min incubation with SuperBlock® T20 (PBS) blocking buffer was performed after each regeneration of the sensor surface. Finally, $Tris_{Mg}$ buffer was injected in the flow-cell.

Preparation of streptavidin–oligonucleotide complexes

Preparation of SON complexes was performed ex situ, and its scheme is described in Fig. 2. Of streptavidin, 4.72 μM solution was spiked with 562 nM of P_A solution to the final molar ratio of 1 molecule of P_A per 3.2, 1.7, or 1.13 streptavidin molecules. The solution was shaken for a few seconds, let to rest for 5 min, and then mixed with concentrated 600 nM solution of BdA_{23} or BdT_{23} to produce mixtures A or T, respectively. Concentrations of BdA_{23} and BdT_{23} in respective mixtures are detailed in Table 2. The mixtures A and T were then shaken for a few seconds, let to rest for 5 min, and then mixed to a final ratio of BdA_{23} and BdT_{23}.

Results

To compare detection of short oligonucleotides using the SON assay and direct detection, detection of CTP53 was performed under the same conditions using different areas of a single SPR chip. Initially, 5-nM CTP53 was injected in the sensing channel with DNA probes P1 (direct detection), while 5-nM CTP53 mixed with SON complexes was injected in the sensing channel with DNA probes P_B (amplified detection). Respective sensor responses are shown in Fig. 3. Upon injection of CTP53, its hybridization with the immobilized probes is observed as an increase in the sensor response. As can be deduced from Fig. 3, the amplified detection yields about 14 times higher sensor response than direct detection. This enhancement is mainly due to the difference in mass between surface-bound P_B–CTP53–SON complexes (SON assay) and with P1-CTP53 duplexes (direct detection). To test the nonspecific binding of SON complexes to the sensor surface, SON complexes without CTP53 were injected over the P_B-coated sensor

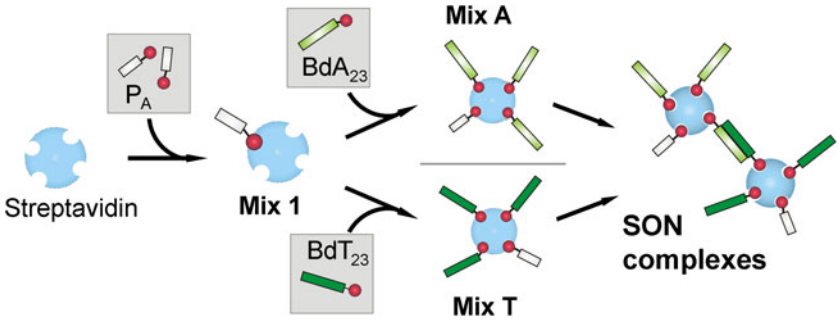

Fig. 2 Formation of streptavidin–oligonucleotide complexes: biotinylated probe P_A is attached to streptavidin via streptavidin–biotin bond to form mix 1. BdA_{23} or BdT_{23} are then added to mix 1 to form mixtures A and T (mix A or mix T), respectively. Upon mixing mix A and mix T, streptavidin molecules connect via BdA_{23} and BdT_{23} hybridization and give rise to streptavidin–oligonucleotide complexes

surface (see inset in Fig. 3). No significant binding was observed suggesting that SON complexes bind to the sensor surface specifically via CTP53.

Optimization of sensitivity of the assay

To optimize the composition of SON complexes, we investigated the influence of concentrations and mutual ratios of SON complex components, i.e., streptavidin, P_A, and connection oligonucleotides (biotinylated poly(dA) and poly(dT) strands) on assay sensitivity.

First, the effect of the ratio of streptavidin and biotinylated oligonucleotides (ONs) was examined. Initial concentration of streptavidin was set to 85 nM, while the concentration of P_B was set to 50 nM and the total concentration of BdA_{23} and BdT_{23} was varied (while maintaining their ratio at 1:1). The sensor response to target oligonucleotide at a concentration of 5 nM is shown in Fig. 4a. The sensor response increases with the total concentration of ONs until ~375 nM, driven by the binding of biotinylated ONs to the four binding sites of streptavidin. The biotinylated oligonucleotide attached to streptavidin via high affinity bond (1) increases the mass of the complex and (2) mediates the association of streptavidin molecules. The decrease in the sensor response observed for biotinylated ON concentrations higher than ~375 nM is believed to be associated with a gradually decreasing size of the SON complexes. When the concentration of biotinylated ONs exceeds the number of binding sites on streptavidin molecules, substantial amount of free ONs remains in solution. The competition of free BdA_{23} and BdT_{23} and those on streptavidin for binding with their counterparts on other streptavidin molecules gives rise to a reduced size of the SON complexes. The maximum sensor response is observed at the concentration of biotinylated ONs, which is about 10% higher than the concentration of binding sites on streptavidin molecules (375 nM). This suggests that small excess of free connection oligonucleotides (BdA_{23} and BdT_{23}) over streptavidin does not significantly affect the size of the SON complexes, but may slightly increase the SON mass by attachment to the SON complexes via hybridization.

Subsequently, concentration of P_A was varied, while the overall concentration of biotinylated ON was kept at

Table 2 Concentrations of oligonucleotides used for preparation of streptavidin–oligonucleotide complexes in respective mixtures

Final composition of SON solution			Mixture A		Mixture T	
Total concentration of ONs [nM]	P_A [nM]	Ratio BdA_{23}:BdT_{23}	P_A [nM]	BdA_{23} [nM]	P_A [nM]	BdT_{23} [nM]
200	50	1:1	75	133.4	75	133.4
250	50	1:1	109.1	100	109.1	100
350	50	1:1	80.0	150.0	80.0	150.0
375	50	1:1	75.0	162.5	75.0	162.5
425	50	1:1	66.7	187.5	66.7	187.5
375	25	1:1	35.3	175	35.3	175
375	75	1:1	120	150	120	150
375	50	2:1	59.0	216.7	102.9	108.3
375	50	1:2	102.9	108.3	59.0	216.7
375	50	3:1	53.3	243.8	126.3	81.3

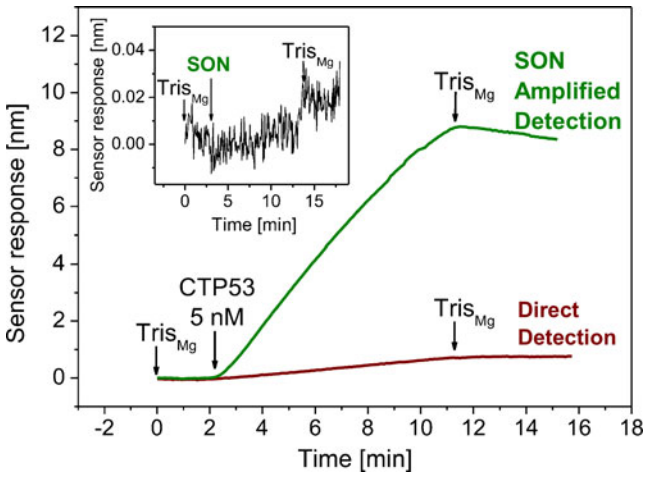

Fig. 3 Sensor response to 5 nM target oligonucleotide using direct detection and streptavidin–oligonucleotide (SON)-amplified detection. Composition of SON: 85 nM streptavidin, 50 nM P_A, 162.5 nM BdA_{23} and BdT_{23}, temperature 25 °C (direct detection) and 30 °C (amplified detection). The *inset* shows sensor response to SON amplification in the absence of the target

375 nM and the ratio of BdA_{23} and BdT_{23} was kept at 1:1. As the attachment of SON complexes to the target oligonucleotide is ensured with the P_A, its low concentration may decrease probability of the target binding. On the other hand, too high concentration may reduce the sensor response due to a lower mass and stability of SON complexes caused by a portion of interconnecting 23-mer ONs being substituted for 9-mers. The concentration of P_A was set to 1 molecule per 3.4, 1.7, and 1.13 streptavidin molecules (25, 50, and 75 nM), and respective solutions were together with 5 nM target injected to the sensor. As follows from Fig. 4b, the sensor response depends on this parameter rather weakly, probably due to concentration of P_A exceeding that of the target. The issues raised above may become more important when concentrations of P_A and target ON are comparable.

In further experiments, the concentration of P_A was fixed at 1 P_A molecule per 1.7 molecules of streptavidin (50 nM concentration). Sensor response to 5 nM CTP53 and SON with different ratios of connection oligonucleotides BdA_{23} and BdT_{23} (1:2, 1:1, 2:1, and 3:1) is shown in Fig. 4c. The smallest sensor response was obtained for amplification with higher concentration of BdA_{23} than BdT_{23}. Clearly,

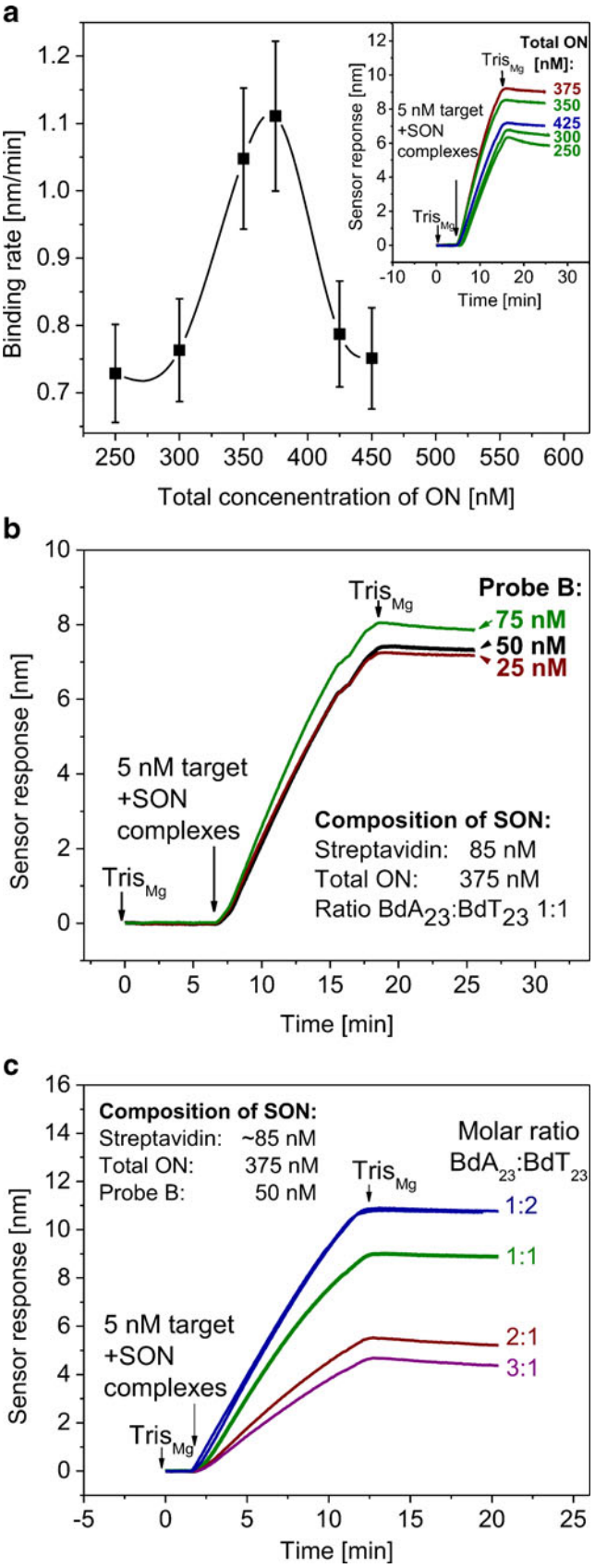

Fig. 4 Sensor response to mixture of 5 nM target amplified with streptavidin–oligonucleotide (SON) complexes of various compositions. Temperature was set to 20 °C. **a** Optimization of total oliognucleotide concentration in final solution of SON. The concentration of probe A was kept constant on 50 nM. Binding rate was determined from initial slope of sensor response (*inset*). **b** Influence of apportionment of probe B in biotinylated ON on the sensor response. **c** Optimization of BdA_{23}:BdT_{23} ratio, when keeping the total concentration of oligonucleotides constant

higher levels of BdT_{23} resulted in a higher sensor response. Moreover, the BdA_{23}–BdT_{23} ratio 1:2 resulted in considerably higher sensor response than 1:1 ratio. This suggests that the ability of interconnecting oligonucleotides to form triplexes may further improve stability of SON complexes and consequently improve sensitivity of the assay. The formation of the BdA_{23}*BdT_{23}·BdT_{23} triplexes may be supported by a high concentration of magnesium ions in the running buffer [31].

The best amplification was obtained when using 85 nM streptavidin and 375 nM biotinylated ONs, 50 nM P_A, and 1:2 ratio of BdA23 and BdT23. This optimized composition of SON complexes containing 85 nM streptavidin and 375 nM biotinylated ON (50 nM P_A; ratio BdT_{23} and BdA_{23}, 2:1) was used in further experiments.

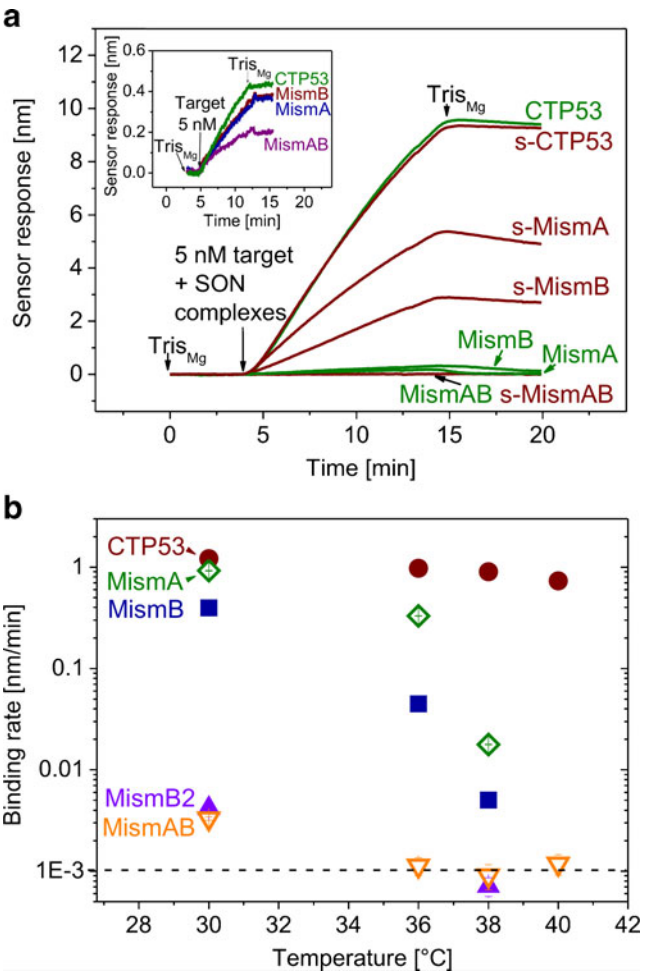

Fig. 5 Optimization of specificity of the amplified detection. Composition of streptavidin–oligonucleotide: 85 nM streptavidin and 375 nM biotinylated ON (50 nM P_A; ratio BdT_{23} and BdA_{23}, 2:1). **a** Sensor response corresponding to the detection of 5 nM target ONs with various mismatches using assay design with one-nucleotide gap between probes A and B (*green line*) and without gap (*red line*). *Arrows* indicate injection of respective solutions. *Inset*: direct detection at 25 °C. **b** Sensor response to amplified detection of matched and mismatched targets at various temperatures. *Dashed line* indicates instrumental resolution of the surface plasmon resonance sensor. Concentration of the target, 5 nM

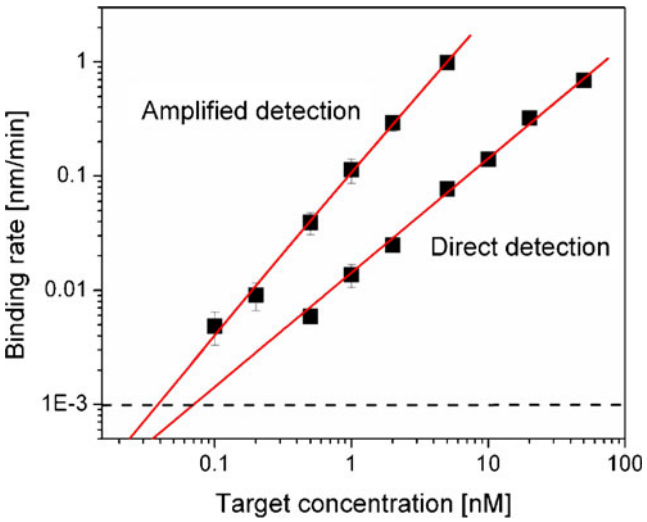

Fig. 6 Calibration curves for the detection of CTP53 using direct detection (at 25 °C) and amplified detection using streptavidin–oligonucleotide (SON) complexes (at 40 °C). Composition of SON: 85 nM streptavidin and 375 nM biotinylated ON (50 nM P_A; ratio BdT_{23} and BdA_{23}, 2:1). *Dashed line* indicates instrumental resolution of the surface plasmon resonance sensor

Optimization of specificity of the assay

In order to improve mismatch specificity, we examined two designs of the assay. Either (a) the two probes P_A and P_B were neighboring when hybridized to the CTP53 target or (b) one-nucleotide "gap" was introduced between the probes P_A and P_B. To allow for comparison of these two assay designs, detection experiments were performed using these two designs and the same probes while the targets were modified. Targets for assay design without "gap" were missing the central non-hybridizing nucleotide (these targets are denoted with s- in Table 1). Several targets were detected: fully matched oligonucleotide corresponding to part of TP53 gene (CTP53), target containing mismatch in codon 273 (MismB, mismatch in P_B*CTP53 duplex), mismatch in another position (MismA, mismatch towards probe A), or two mismatches, one in each of the duplexes with probes (MismAB) and two towards probe B (MismB2). Figure 5a shows sensor responses to the 5-nM concentration of targets at a temperature of 38 °C. The sensor response to fully matched targets is the same in both designs. However, although the assay design with "gap" exhibits almost no response for targets with one mismatch, in the other design, sensor response to one-mismatch target ONs is considerable. It seems that despite the disconnected phosphate backbone, the duplexes of P_A and P_B with mismatched target are mutually stabilized by base stacking

interactions between the duplexes. By insertion of one-nucleotide "gap" between the probes, the stacking interaction is diminished which cause an increase in assay specificity. For comparison, the inset in Fig. 5a demonstrates the specificity of direct detection using 23-mer probes P1. In contrast to the amplified detection, hybridization of fully matched target and the target with one or two mismatches yield almost identical responses and therefore cannot be distinguished.

The "gapped" assay design was further investigated with respect to the influence of temperature. Figure 5b displays temperature dependence of the sensor response to SON-enhanced detection of 5 nM CTP53 and targets with mismatches. It was demonstrated that the specificity of the assay gradually increases with temperature due to decreasing probe-mismatched target stability. The optimal temperature was determined to be 40 °C. At this temperature, the sensor response to targets with one mismatch fell below the instrumental resolution of the sensor. When increasing temperature up to 40 °C, the sensor response to CTP53 was decreasing only moderately, ensuring high sensitivity of the assay.

Detection of CTP53

To compare the sensitivity of the optimized assay with direct detection of short synthetic analogue to the TP53, the calibration was performed using both methodologies. Figure 6 displays the respective calibration curves, in which each point was measured in at least three replicas on different chips. It can be seen that the amplification results in more than tenfold improvement of the detection of higher concentrations and about fivefold amplification of SPR signal at low concentrations. This difference is believed to be due to the fact that the assay is performed at the verge of stability P_A–CTP53–SON complexes, which is required to obtain high specificity. The surface attachment of CTP53–SON complexes is probably stabilized by the surrounding complexes, and at lower surface concentrations, the role of this stabilization is diminished resulting in a lower amplification factor. The limit of detection (LOD)—i.e., the concentration of analyte that results in the sensor response equal to three standard deviations of the baseline noise—was determined for CTP53 (molecular weight, 6 kDa) to be 40 pM—two times lower than the LOD determined for direct detection.

Discussion

The reported assay presents a simple platform for improving both sensitivity and specificity of detection of short oligonucleotides. Although the assay requires additional reagents (streptavidin and biotinylated oligonucleotides), only limited amounts (3 μg of streptavidin and 0.2 nmol of oligonucleotides) need to be added in a single step which does not increase the cost and complexity of the detection process. Clearly, the assay recognizes one-nucleotide mismatch within the 23-mer sequence. Detection of other SNPs in the same or a different sequence can be easily implemented (and done in parallel using a high-throughput sensor platform) by varying probe P_B (while keeping the P_A probe the same) or by varying both probes. In principle, it is also possible to detect presence of two and three mutations by decreasing the measurement temperature. It should be, however, noted that applicability of the assay may be more limited when (1) detecting SNP in areas of genome containing abundance of various one-point mutations as many different combinations of P_A and P_B would be required or (2) other types of mutation (e.g., insertions or deletions) are present.

Conclusions

In this paper, we present a novel assay for sensitive and specific detection of oligonucleotides and combine it with SPR method making it possible to detect SNP. In this assay, SON complexes are employed for amplifying SPR sensor signal generated by the hybridization of target ONs with the probes immobilized on the sensor surface. The influence of SON composition on assay performance is discussed. The probe design and temperature are demonstrated to have significant effect on specificity of the assay. It is shown that the assay can detect short oligonucleotides at concentrations as low as 40 pM. Moreover, the assay makes it possible to discriminate fully matched targets from those containing one mismatch. The assay can be easily adapted to other transduction schemes including fluorescence-based sensors, QCM, etc. and is compatible with high-throughput biosensor technologies such as SPR imaging.

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