Discrimination of single base mismatched oligonucleotides related to the *rpoB* gene of *Mycobacterium tuberculosis* using a surface plasmon resonance biosensor

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Abstract

Single base mismatched oligonucleotides related to the *rpoB* gene of *Mycobacterium tuberculosis*, the mutations of which cause drug resistance of the infectious agent, were detected and discriminated using a surface plasmon resonance biosensor system. Thiol-modified oligonucleotides of the selected sequence (the probe) and 1-mercapto-6-hexanol were immobilized on a gold sensor surface. Hybridization between immobilized probe **P2** and perfectly matched target **T2** as well as a single base mismatched target **TN** was investigated in buffer solutions of various stringencies. Discrimination of perfectly matched and single base mismatched targets is achieved due to a difference in the level of their hybridization with the immobilized probe depending on stringency of the buffer solution. In 0.5×SSC buffer solution (7.5 mM sodium citrate, pH 7, containing 75 mM NaCl), sensor response at **T2** injection into the measuring sensor cell was 16 times that at **TN** injection. The experimental results on surface hybridization between the studied oligonucleotides demonstrated a good correlation with theoretical calculations of thermodynamic parameters of these interactions in the solution. The described approach could be proposed as a basis for creating a biosensor for real-time label-free diagnostics of drug-resistant tuberculosis. Volume 00, Number 0, Pages 1–6, 2013

Keywords: *hybridization DNA sensor, mismatch discrimination, rpoB gene, stringency control, surface plasmon resonance, tuberculosis*

1. Introduction

Tuberculosis continues to be a serious threat to people's health and life worldwide. In 2010, there were 8.8 million incident cases of the disease, 1.1 million deaths from tuberculosis among human immunodeficiency virus (HIV)-negative people, and an additional 0.35 million deaths from HIV-associated tuberculosis [1]. One of the most serious global problems in the prevention of tuberculosis transmission is a steady increase in the frequency of *Mycobacterium tuberculosis* strains that are resistant to at least one of the antituberculous agents commonly used in the treatment. Diagnosis and appropriate treatment of multidrug-resistant tuberculosis remain major challenges.

The availability of the complete genome sequence of *M. tuberculosis* and investigations of the molecular genetic basis of resistance to antituberculous agents reveal that virtually all isolates resistant to rifampicin have mutations in the 81-bp rifampicin resistance defining region (RRDR) of the *rpoB* gene (GenBank ID: NC 000962), which alter the sequence of a 27-amino-acid region of the beta subunit of RNA polymerase [2,3]. Conventional methods for the analysis of specific gene sequences are based on direct sequencing or DNA hybridization methods. DNA sequencing methods are generally labor intensive, time and reagent consuming, and expensive. Therefore, the second option is more attractive for diagnostic purposes. Rapid detection of drug resistance not only could

Abbreviations: *PNA, peptide nucleic acid; RIU, refractive index unit; RRDR, rifampicin resistance defining region; SPR, surface plasmon resonance; SSC, saline sodium citrate.*

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optimize treatment and improve outcome for patients with drug-resistant tuberculosis but also is especially important in the prevention of its spread [4].

Currently, for the most commonly employed detection systems, DNA hybridization monitoring relies on the use of fluorescent, enzymatic, or some other molecular labels, and/or electrochemically active indicators. Besides obvious advantages, the use of molecular labels leads to some problems and inconveniences. For example, fluorescence, due to low background noise, has a high sensitivity but requires the use of costly reagents and time-consuming labeling procedures [5]. DNA hybridization biosensors are based on the immobilization of single-stranded oligonucleotide probes onto a transducer surface and the recognition of complementary targets contained in analyzed samples by their hybridization with the surface-bound probes [6–8]. We believe that methods for detecting specific gene sequences via hybridization on the sensor surface in combination with label-free real-time measurements using surface plasmon resonance (SPR) spectrometry are reliable and promising. The principle of the SPR biosensor is based on the change in the refractive index occurring on a thin gold film surface modified with various materials and within few hundred nanometers of the sensor surface [8–12]. Our previous studies describe the development of the method based on homemade SPR setup for the detection of the specific oligonucleotide sequences related to the *rpoB* gene of *M. tuberculosis* [13,14].

A truly high performance DNA biosensor should be able to discriminate as few as a single base pair mismatch between different target DNA strains. Discrimination between matched and mismatched hybrids on the sensor surface can be achieved by employing various factors influencing hybridization efficiency, including target length and concentration, temperature, composition of hybridization buffer, posthybridization stringency wash, and a combination of the factors [15,16]. Although various stringency control strategies are available, including high temperature and adding hybridization suppressors or denaturants into the hybridization buffer, the choice of a proper one would be subjected to the sensing platform used. For SPR spectrometry–based DNA analysis, employment of high temperature is not recommended because the refractive index sensitivity of SPR is influenced by temperature and high temperature will generate considerable complications in SPR responses. Moreover, not all SPR equipments have temperature control functions. Hybridization suppressors like formamide with a high refractive index, which is out (or nearly out) of the detectable range of most SPR equipment, result in a technical challenge for SPR detection of DNA hybridization [15–19].

Therefore, stringency control based on the ionic strength of the hybridization buffer, which strongly influences hybridization of perfect matched and mismatched DNA sequences and does not lead to strong refractive index change, appears quite attractive and could be used for discrimination. The aim of this study is to demonstrate the feasibility of the biosensor method based on SPR for detecting and discriminating oligonucleotides related to the normal and mutated *rpoB* genes of *M. tuberculosis* by using a stringency control. The successful realization of this aim enables creation of the biosensor for detecting *M. tuberculosis* drug-resistant strains, which would be a helpful and important tool in the efforts to prevent the alarming transmission of this dangerous disease.

2. Materials and Methods

2.1. Reagents

The single-stranded oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). Urea and KH_2PO_4 were purchased from Fluka (Buchs, Switzerland). 6-Mercapto-1-hexanol and saline sodium citrate (SSC) buffer 20×concentrate (0.3 M sodium citrate, pH 7, containing 3 M NaCl) were obtained from Sigma (Oakville, Canada). All solutions were made with deionized Milli-Q water.

2.2. Experimental setup

To perform DNA hybridization tests, we specially designed and built a sensitive, cost-efficient SPR instrument [13]. Briefly, it consists of 10 mW He–Ne laser as the light source, a polarizer, a rotating diffusing disk to remove the laser speckles effects and smoothen the laser intensity profile, two lenses, which produce a convergent beam through a glass coupling prism on a sensing surface (a 50-nm gold thin film on a glass plate), and a flow injection measuring cell with 20-μL volume. The SPR effect was accompanied by a drastic decrease in the intensity of p-polarized component at the SPR angle. Primary SPR response values were obtained in arbitrary units (a.u.), where 1 a.u. is equal to the 2.7×10^{-3} degree (2.7 mdeg) of angular shift. The application of a rotating diffuser and imaging two-dimensional charge-coupled device (CCD) camera allows to detect multiple SPR resonance curves with a convergent incident beam and after numerical filtering obtaining a SPR response with sensitivity comparable to the Biacore system (3×10^{-6} RIU). Moreover, this homemade SPR system possesses larger flexibility in biosensor chip choice and offers the possibility to work with various configurations of a measuring cell, and a wide range of scanned angles.

2.3. Probe immobilization

Prior to modification, the gold surface of the glass plate was cleaned with freshly prepared "piranha" solution (3:1 mixture of concentrated H_2SO_4 and 30% H_2O_2) at 23°C for 2 Min, then rinsed thoroughly with Milli-Q water, and dried in the air. Extreme care was taken in handling these chemicals as the "piranha" solution reacts violently with organic compounds.

The cleaned plate was placed on the spectrometer prism using an immersion liquid. At the beginning of probe immobilization, a stable base line was obtained during pumping of the immobilization solution (0.5 M KH_2PO_4, pH 3.8) through the measuring cell at the flow rate of 50 μL Min^{-1} at 23°C; then at 1 μM thiol-modified probe **P2** in the same immobilization solution was pumped for 20 Min. After that, the flow was stopped for 40 Min incubation. Then, the measuring cell was washed

by the immobilization solution until a stable sensor signal was obtained.

For blocking sites on the gold surface, which left free from thiol-modified oligonucleotides, 1 mM aqueous solution of 1-mercapto-6-hexanol was used. It was suggested that the hybridization efficiency could be improved by occupying areas of the surface that are not coated by DNA, thereby preventing interactions of DNA from the analyzed sample with the gold surface [20].

2.4. Target hybridization

On the stage of hybridization, 2×SSC (30 mM sodium citrate, pH 7, containing 300 mM NaCl) was used as a running buffer solution. To reduce the analysis time, a rather short hybridization time was chosen—3 Min of sample injection and 10 Min of incubation at stopped flow. After that, the measuring flow cell was washed by the running buffer solution at a standard flow rate (50 μL Min^{-1}). For regeneration of the bioselective element, 8 M urea was used.

3. Results and Discussion

The selection of the most appropriate oligonucleotide as a probe for a hybridization DNA sensor is one of the most important stages of the investigation. When a probe should be selected from a whole gene or other long DNA sequence, a computational-assisted tool such as OligoWiz [21] could take into account the different parameters involved in optimizing a probe design [22]. In our case, it was necessary to select a probe from a very limited DNA fragment containing the mutated codon 531 TCG→TTG of the *rpoB* gene of *M. tuberculosis*, because it is the most frequent mutation in RRDR of the *rpoB* gene [2, 3].

To estimate the level of intra- and intermolecular interactions of potential probes, their thermodynamic parameters were obtained with the DINAMelt Web server [23–25]. We considered the following criteria:

(1) The length of the probe should be in the range of 18–30 nucleotides (to ensure the uniqueness of the sequence);
(2) the GC content of the probe should be in the range of 40–60% (to provide an optimal level of interaction with complementary DNA sequences);
(3) the level of self-hybridization of the probe should be as low as possible (to prevent strong intramolecular secondary structures, which would be able to deteriorate interactions with target DNA sequences and their detection).

At first, the values of thermodynamic parameters of self-hybridization of various fragments of the gene sequence containing the codon 531 were determined. It was shown that the oligonucleotides containing 18–21 bases could not form strong intramolecular structures (e.g., in 2×SSC at 22°C, $\Delta G = -0.39$ kcal mol^{-1}), whereas the oligonucleotides containing 22–30 bases demonstrated a gradually increased ability for self-hybridization (ΔG was from −1.02 until −8.92 kcal mol^{-1}).

A very high GC content (more than 85%) of the gene sequence adjacent to the codon 531 from the right (from the 3′-end) determined our choice of the 21-mer with the location of the mutation site at its 3'-end. The GC content of the 21-mer is near to a satisfactory level (61.9%). The observation of Hughes et al. [26] showed that for their probes the solution end was more sensitive in detecting mismatches than the surface end, which gave us some additional assurance about the correctness of our choice.

Finally, a comparison of the free energy changes for hybridization of potential probes with a perfectly matched or mismatched oligonucleotide depending on a mismatch location within target oligonucleotides shows that $\Delta\Delta G$ varied around −2.5 kcal mol^{-1} at the mismatch location on 17–20 positions and $\Delta\Delta G$ was −0.6 kcal mol^{-1} only at the mismatch location on position 21. Thus, the oligonucleotide acccacaagcgccgactgttg with a mutated base on position 20 was selected as the probe and was named **P2**. For immobilization on a gold surface of the sensor chip 5′-end of the probe, the oligonucleotide was modified by the SH group via a spacer of six methylene groups. This spacer is necessary to decrease a probability of steric hindrances for hybridization near the sensor surface (Fig. 1). For hybridization selectivity testing, the following target oligonucleotides were used: **T2** (CAACAGTCGGCGCTTGTGGGT) (complementary to the probe

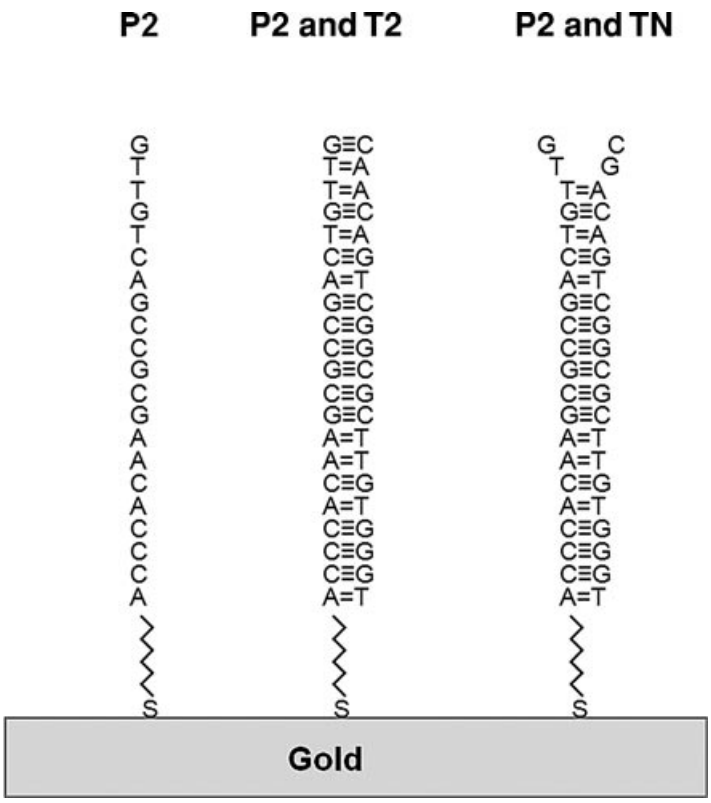

FIG. 1 *Scheme of hybridization between the probe **P2** immobilized on the sensor surface and the targets **T2** (perfect match) and **TN** (single base mismatch).*

P2), **TN** (CGACAGTCGGCGCTTGTGGGT) containing a single-base mismatch (complementary to the sequence containing normal TCG codon 531 of the *rpoB* gene), and noncomplementary oligonucleotide **TC** (GCTATCAGCCACGAACACCCA). The calculated free energy changes of their self-hybridization showed that the formation of the stable internal secondary structure within the mentioned oligonucleotides is hardly probable.

The chosen conditions (0.5 M KH_2PO_4, pH 3.8) have led to an efficient immobilization of the probe **P2** on the gold surface of the SPR biosensor. The changes in the resonance angle at the **P2** immobilization were converted into surface mass uptakes using a factor of 120 mdeg per 100 ng cm^{-2} [27, 28], and they were equal to about 180 ng cm^{-2} (28 pmol cm^{-2} or 1.7×10^{13} molecules cm^{-2}) [13].

The main stage of DNA hybridization sensor performance is the recognition of nucleic acid molecules contained in analyzed samples by surface-bound probes. Selectivity is critical for the evaluation of the DNA hybridization biosensor, especially for detecting mutations, early stage diagnosis of critical illness, and forensic applications. Stringency control based on the ionic strength of the hybridization buffer is effective for discrimination because it strongly influences hybridization of perfectly matched and mismatched DNA sequences. According to calculated thermodynamic parameters of intermolecular interactions in solution under various stringencies (from 0.06×SSC to 6×SSC), hybridization between **P2** and **T2** and **P2** and **TN** should be efficient, whereas the probability of strong hybridization between **P2** and noncomplementary **TC** is very low (Table 1). These values show that an increase in the ionic strength leads to gradual strengthening of intermolecular interactions that can be explained by shielding of negatively charged phosphate residues of oligonucleotides by counterions of the buffer solution and, consequently, creating more favorable conditions for hydrogen bonding between complementary nucleic bases.

Our experimental results showed very low SPR responses observed at **T2** hybridization with the surface-bound probe **P2** in 0.1×SSC (under high stringency conditions). The increase in the buffer concentration to 0.5×SSC and then to 2×SSC led to significant signal growth due to hybridization on the sensor surface (Fig. 2), whereas the increase of the corresponding ΔG values, which were calculated for hybridization in the solution, was not so impressive (since −27.8 to −31.3 and then to −32.7 kcal/mol). An obvious dependence of the biosensor response on the T2 concentration when using 0.5×SSC or 2×SSC as the running buffer solution confirmed the successful **T2** hybridization on the sensor surface.

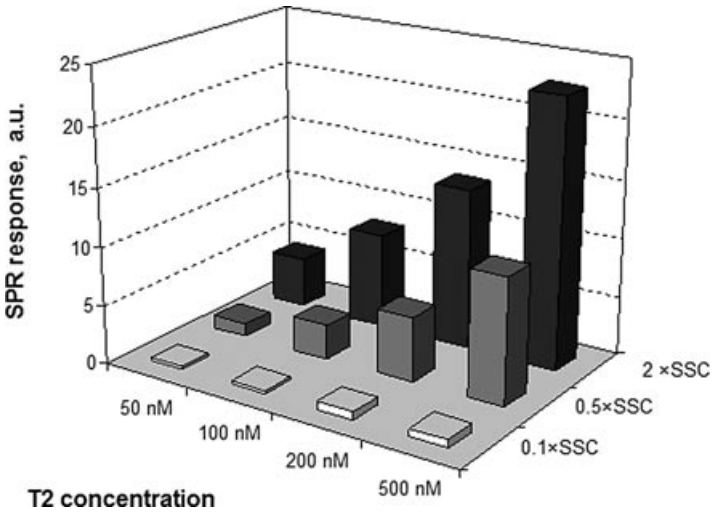

FIG. 2 *SPR response at hybridization of the surface-bound probe **P2** with various concentrations of the perfectly matched target **T2** in 0.1×SSC, 0.5×SSC, and 2×SSC.*

A comparison of the experimental SPR responses caused by hybridization of surface-bound probe **P2** and the perfectly matched target **T2** with that of **P2** and the single-base mismatched target **TN** (Fig. 3) demonstrated that under relatively low stringency conditions (high buffer concentration, in this case, 2×SSC), even mismatched oligonucleotides could hybridize quite efficiently. On the contrary, higher stringency conditions (a lower buffer concentration, in this case, 0.5×SSC) reduced the level of hybridization of both targets but were much stronger for less complementary oligonucleotides. For example, at 100 nM target concentration, in 2×SSC SPR

TABLE 1 ***Thermodynamic parameters of hybridization in the solution between the probe P2 and the targets T2, TN, and TC depending on the buffer stringency***

	Free energy change ΔG (kcal/mol)						
Pairs of oligonucleotides	*0.06×SSC*	*0.1×SSC*	*0.5×SSC*	*1×SSC*	*2×SSC*	*4×SSC*	*6×SSC*
P2 and **T2**	−26.7	−27.8	−31.3	−32.7	−34.2	−35.8	−36.6
P2 and **TN**	−24.8	−25.8	−29.0	−30.4	−31.9	−33.3	−34.1
P2 and **TC**	−3.1	−3.3	−3.8	−4.0	−4.3	−4.9	−5.2

All calculations are performed for 22°C, 0 M Mg^{2+}, and 100 nM concentration of the oligonucleotides.

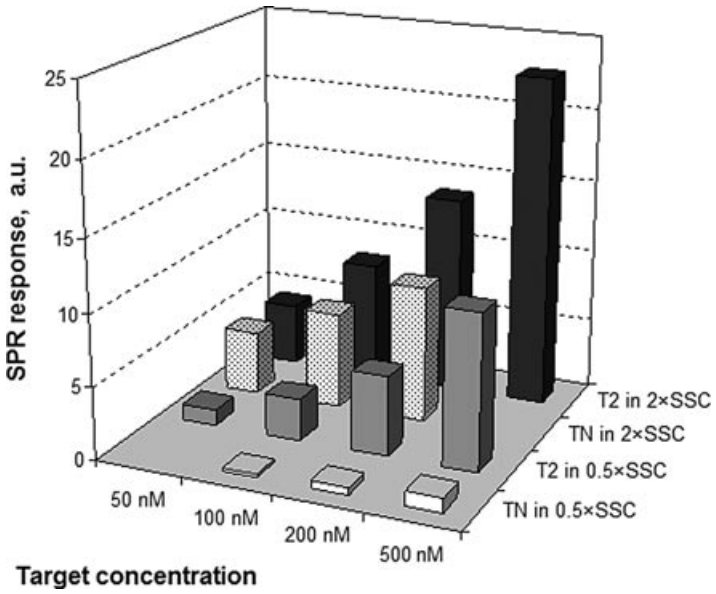

FIG. 3 *SPR response at hybridization with the surface-bound probe **P2** of the perfectly matched target **T2** and the single-base mismatched target **TN** in 0.5×SSC and 2×SSC. In the case of 50 nM **TN** in 0.5×SSC, the obtained SPR response could not be distinguished from the noise.*

response at **TN** hybridization was 1.2 times less than that at **T2** hybridization and in 0.5×SSC this index increased to 16 times.

Lao and coworkers [19] reported that under optimal conditions (using formamide) a 500-nM single base mismatched 22-mer was detected with 1.7 and 2.8 times less hybridization signals compared with the perfectly matched oligonucleotide, for DNA probe and peptide nucleic acid (PNA) probe, respectively. Thus, using 0.5×SSC allowed much more pronounced discrimination between the perfectly matched and the single base mismatched oligonucleotides related to the normal and mutated *rpoB* genes of *M. tuberculosis*.

It is interesting to note that near-minimum SPR response for **P2–TN** hybridization on the sensor surface was obtained in 0.5×SSC. ΔG value calculated for **P2–TN** hybridization in the solution of the same concentration was equal to −29.0 kcal/mol. The near-minimum SPR response for **T2** hybridization with the surface-bound probe **P2** was observed in 0.1×SSC, whereas the corresponding ΔG value, which was calculated for hybridization in the solution of this buffer concentration, was equal to −27.8 kcal/mol. Results suggest that the above-mentioned ΔG values for solution hybridization could serve as a threshold for more or less pronounced hybridization on the surface and, consequently, for the probable SPR sensor response (under specified conditions).

In conclusion, successful detection and discrimination of perfectly matched and single base mismatched oligonucleotides related to the normal and mutated *rpoB* genes of *M. tuberculosis* using the SPR sensor system and stringency control were presented for the first time. A DNA hybridization biosensor of this level of sequence discrimination and the rapid response time reported here would be well suited to the identification of highly virulent microbial organisms. The SPR technology could be employed to elaborate this single sensor into a sensor array that will be useful in the antimicrobial resistance profiling of *M. tuberculosis* and play an important role in the efforts to prevent the alarming spread of this dangerous disease.

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