



Surface plasmon resonance detection of oligonucleotide sequences of the *rpoB* genes of *Mycobacterium tuberculosis*

A. Rachkov^{a,*}, S. Patskovsky^b, A. Soldatkin^a, M. Meunier^b

^a Institute of Molecular Biology and Genetics of National Academy of Sciences of Ukraine, 150 Academician Zabolotny Street, Kyiv 03143, Ukraine

^b Engineering Physics Department, Laser Processing and Plasmonics Laboratories, École Polytechnique de Montréal, C. P. 6079, succ. Centre-Ville, Montréal (Québec), Canada H3C 3A7

ARTICLE INFO

Article history:

Received 30 September 2010

Received in revised form 1 July 2011

Accepted 9 July 2011

Available online 22 July 2011

Keywords:

SPR

Hybridization

DNA sensor

Tuberculosis

rpoB gene

ABSTRACT

Oligonucleotide sequences related to the normal and mutated *rpoB* genes of *Mycobacterium tuberculosis* are detected using a surface plasmon resonance (SPR) biosensor system. A bioselective element was prepared by immobilizing the thiol-modified oligonucleotides of the selected sequence (the capture probe **P2**) that contains the mutated TCG → TTG codon 531 (evoking drug resistance) of the *rpoB* gene of *M. tuberculosis* on a gold sensor surface. Specific hybridization between immobilized probe **P2** and complementary target **T2** gave the highest sensor response, single-base mismatched oligonucleotide **TN** (corresponding to the normal gene sequence) produced somewhat smaller response and no response was observed at injection of noncomplementary oligonucleotide **TC**. The **P2**–**T2** hybridization efficiency is calculated ca. 30% (5×10^{12} molecules cm^{-2}), and the lowest detection limit of **T2** was 10 nM. An extended **T2E** oligonucleotide sequence consisting of **T2** sequence and additional 24 nucleotides was shown to cause more pronounced sensor response (at least 5 nM **T2E** was easily detected). Injection into the sensor cell of the oligonucleotides complementary to the free additional part of **T2E** after **P2**–**T2E** hybridization gave a significant additional SPR response, thus showing that the sandwich hybridization format further improves the sensor sensitivity and decreases the lowest detection limit. The experimental results on surface hybridization between the studied oligonucleotides were in good agreement with thermodynamic parameters of the hybridization calculated for solution conditions. The described approach could be proposed as a basis for creating a biosensor for real-time and label-free diagnostics of drug resistant tuberculosis.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

There is approximately one-third of the world's population that is infected by *Mycobacterium tuberculosis* and about 3 million people globally die of tuberculosis each year, and another 8 million new individuals become infected. Although once thought possible to be virtually eradicated by the end of the twentieth century, tuberculosis has resurged sharply since the mid-1980s [1,2]. Probably, the most serious global problem is a steady increase in the frequency of *M. tuberculosis* strains resistant to at least one of the antituberculous agents commonly used in the treatment. The last report prepared by the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease Global Project based on the data from 93 different countries/geographical settings collected between 2002 and 2007 shows that the proportion of drug resistance ranged up to more than 55%. In addition, the multi-drug resistant (MDR) tuberculosis, which is defined as

tuberculosis with resistance to isoniazid and rifampicin, the two most powerful first line drugs, is reaching now a critical proportion of more than 20% in some countries of the former Soviet Union. China and India carry approximately 50% of the global burden of MDR cases and the Russian Federation an additional 7% [3].

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 rifampin and related rifamycins have a mutation in a discrete region of the *rpoB* gene (rifampicin resistance region) that alters the sequence of a 27-amino-acid region of the RNA polymerase beta subunit [4]. However, when drug susceptibility testing is culture based, detection still takes 2–9 weeks [5]. Recent advances in nucleic acid-based methods to detect drug resistance to *M. tuberculosis* makes it possible to create new rapid diagnostic tests, for example, polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP), heteroduplex analysis and procedure involving nucleic acid hybridization such as dot-blot hybridization [6].

Rapid detection of drug resistance could not only optimize treatment and improve outcome for patients with MDR tubercu-

* Corresponding author. Tel.: +380 44 526 4397; fax: +380 44 526 0759.

E-mail address: oleksandr.rachkov@yahoo.com (A. Rachkov).

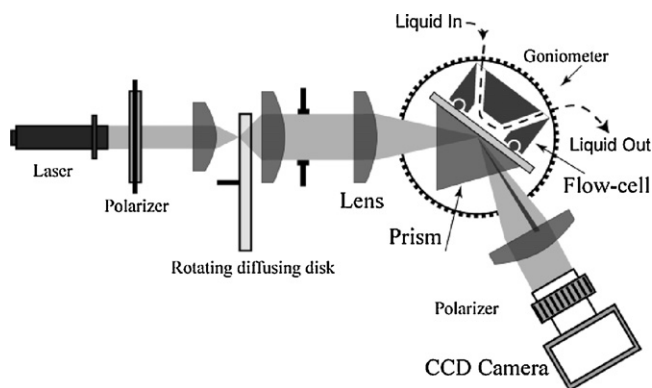


Fig. 1. Experimental CCD camera based SPR set-up.

losis but would also be especially important in the prevention of its transmission and spreading [7]. We believe that methods for detecting specific gene sequences via hybridization with complementary surface-bound oligonucleotide probes would be reliable and promising especially in combination with such a powerful tool for biomolecular interaction analysis like surface plasmon resonance (SPR) sensor. The ideal diagnostic device would need to be a cost-effective, portable, point-of-source or point-of-care detection system that would be highly sensitive, accurate and could differentiate multiple pathogens. Since the conditions of plasmon excitation are extremely sensitive to the refractive index changes occurring within a thin layer of liquid (~ 300 nm) near a metallic (commonly, gold) surface, SPR biosensing is now considered as a leading technology for real-time detection and studies of biological binding events [8–13].

Among the main types of biomolecular recognition elements used in SPR biosensors antibodies are used most frequently because of their high affinity, versatility, and commercial availability, but a steady increase in applying nucleic acids (including aptamers) can be observed recently. Biosensors based on nucleic acid are simple, rapid, and inexpensive. In contrast to enzyme or antibodies bioreceptors, nucleic acid recognition layers can be readily synthesized and regenerated [14–17]. A convenient and reliable method for immobilization of thiol-derivatized DNA molecules on the SPR sensor surface is based on the findings that upon exposure of a gold substrate to thiols, a quite strong bond between gold and sulfur (~ 44 kcal/mol) forms rapidly, typically within seconds to minutes [18–20].

This article is aimed at the development of novel plasmonic biosensing method for the detection of tuberculosis based on the specific gene sequences hybridization with complementary surface-bound oligonucleotide probes related to the *rpoB* gene of *M. tuberculosis*.

2. Materials and methods

2.1. Reagents

The single-stranded oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Germany). Urea and KH_2PO_4 were purchased from Fluka (Buchs, Switzerland). 6-Mercapto-1-hexanol and SSC buffer $20\times$ concentrate were obtained from Sigma (Oakville, Canada). All solutions were made with deionised MilliQ water.

2.2. SPR system set-up

A schematic diagram of the instrument design is shown in Fig. 1. A 10 mW He–Ne laser operating at a wavelength of 632.8 nm

was used as the light source. A polarizer is used to set linear p-polarization. The beam was then directed to a rotating diffusing disk in order to remove the laser speckles effects and to smooth the laser intensity profile. Then two lenses produce a convergent beam through a coupling prism on a sensing surface. The SPR sensor system includes a glass coupling prism BK7. A commercial biosensor chip (Biacore) with a 50 nm gold thin film was then placed in immersion contact with the prism. For the real time tests a flow injection measuring cell with 20 μL volume was developed. The entire system was placed on a rotating stage with a possibility of 2D linear translation for exact beam positioning. The SPR coupling at a specific angle of light incidence on the sensor head (SPR angle) made the excitation of surface plasmons possible over gold/adjacent medium interface. The SPR effect was accompanied with a drastic decrease of intensity of p-polarized component at the SPR angle. The spatial distribution of the reflected light intensity was then recorded by a CCD camera (Hamamatsu C4742-95) and examined by a proper software image treatment. Even if a linear CCD detector is sufficient for the SPR resonance dip recording, a drastic improvement in the reduction of signal noise and signal dispersion due to the non-ideality in the light beam propagation and uniformity of sensor surface modification was achieved when multiple lines provided by a CCD camera were employed. For numerical analysis of the obtained SPR curves, polynomial curve fitting method of forth order and the centroid method were used. Primary SPR response values were expressed in arbitrary units where 1 a.u. is equal to the $2.7 \times 10^{-3}^\circ$ (2.7 m $^\circ$) of angular shift.

Prior to modification, gold surface of the glass slides was cleaned with freshly prepared “piranha” solution (3:1 mixture of concentrated H_2SO_4 and 30% H_2O_2) at room temperature for 2 min, then rinsed thoroughly with MilliQ water, and dried in a nitrogen flow. The cleaned gold slide was placed on the SPR coupling prism using refractive index matching immersion liquid (Cargille Labs, USA). The flow rate (usually $50 \mu\text{L min}^{-1}$) was controlled by a peristaltic pump “Ismatec”, (IDEX Corporation, Switzerland). Immobilization of thiol-modified capture probes on the gold sensor was carried out in 0.5 M KH_2PO_4 , pH 3.8. As a running buffer on the stage of hybridization, a solution of $2\times$ SSC (30 mM Na citrate, 300 mM NaCl, pH 7) was used.

3. Results and discussion

3.1. Selection of oligonucleotide sequences

Sequence-specific hybridization biosensors rely on the detection of hybridization events between immobilized nucleic acid molecules with known sequences (capture probes) on the sensor surface and nucleic acid molecules in analyzed solution with unknown sequences (targets). The immobilized capture probe should be in a well-defined orientation and readily accessible to the complementary targets [21]. Selection of the most appropriate oligonucleotide capture probe is a key task for a successful DNA hybridization sensor development. Resistance to rifampin and related rifamycins is known to arise as a result of mutations occurring in a discrete region of the *rpoB* gene of *M. tuberculosis* that alters the sequence of a 27-amino-acid region of the RNA polymerase beta subunit [2,4]. Therefore, we decided to use this hot spot of the *rpoB* gene. The selection of the length and the sequence of the oligonucleotide probe were made based on its ability to intramolecular and intermolecular interactions. The formation of strong secondary structures within the potential probe is undesired, because it could be an obstacle for the efficient hybridization with complementary targets in the analyzed solutions. To assure uniqueness of polymerase chain reaction (PCR) primers, 18–30-mer oligonucleotides are usually applied [22]. We also checked

the properties of the oligonucleotides of this length range, which sequence includes the 526 and 531 codons of the *rpoB* gene (mis-sense mutations in these positions were found in ca. 80% of the resistant strains of *M. tuberculosis*). All studied oligonucleotide sequences contain the normal CAC codon 526 and the mutated TCG → TTG codon 531. The values of thermodynamic parameters of their self-hybridization were calculated using DINAMelt web server [23–25]. Among the oligonucleotides possessing the lowest ability to intramolecular interactions ($\Delta G = -0.39$ kcal/mol) we have chosen the 21-mer ACCCACAAGCGCCGACTGTG as a capture probe and have named it **P2**. For immobilization on a gold surface of the sensor chip 5'-end of the oligonucleotide was modified by SH-group via a spacer of 6 methylene groups. This spacer is necessary to decrease a probability of steric hindrances for hybridization near the sensor surface.

For hybridization selectivity tests, the following target oligonucleotides were used: **T2** (CAACAGTCGGCGCTGTGGGT) – complementary to **P2**, **TN** (CGACAGTCGGCGCTGTGGGT) containing single-base mismatch (complementary to the sequence containing normal TCG codon 531 of the *rpoB* gene) and noncomplementary oligonucleotide **TC** (GCTATCAGCCAGAACACCA). The calculated free energy changes for their self hybridization in solution (-0.61 , -1.11 and -0.95 kcal/mol, respectively) showed that formation of stable internal secondary structures of these oligonucleotides is hardly probable.

For a sandwich hybridization format, longer (extended) targets **T2E** (CAGTCAGTCAGTCAGTCAGTTTTCAACAGTCGCGCTGTGGGT) containing two complementary fragments (separated by TTTT insertion) to the capture probes **P2** and to the detection probes **DP** (ACTGAGTGACTGACTGACTG) were used. The calculated free energy changes for self-hybridization of **T2E** and **DP** (-1.35 and -1.37 kcal/mol, respectively) evidence the appropriateness of these oligonucleotides for the investigations because of a quite low probability of their stable internal secondary structures.

3.2. Immobilization

In a first series of experiments, calibration of the SPR system was carried out by flowing on the sensor chip different refractive index solutions of NaCl ranging from 1 to 50 mM. Known refractive index values associated to these solutions allowed us to calibrate the instrument as well as to determine the detection limit of the system to be close to 10^{-6} refractive index units (RIU).

An optimal probe density on the sensor surface is critical for efficient DNA hybridization and sensitive signal detection. The amount of immobilized thiol-modified capture probes on the surface can be controlled by varying the exposure time. Therefore, 1 μ M solution of **P2** in the immobilization solution (0.5 M KH_2PO_4 , pH 3.8) was injected into the measuring cell at the flow rate of about $50 \mu\text{L min}^{-1}$ during 20 min at room temperature and then stopped for 40 min for incubation. SPR monitoring demonstrated gradual increase of the sensor signal, and, consequently, in surface density of the immobilized capture probes. A short (1 min) additional pulse of **P2** solution resulted in an additional increase of the SPR response suggesting that saturation of the sensor surface by thiol-modified capture probes was not achieved during the 1 h-immobilization. Then, during washing by the same immobilization solution, partial decrease in SPR response was caused by removal of non-immobilized oligonucleotide molecules (Fig. 2).

The difference of 81 a.u. ($2.7 \text{ m}^\circ/\text{a.u.} \times 81 \text{ a.u.} \approx 220 \text{ m}^\circ$) between SPR signal before the injection of **P2** and after its incubation and the wash of the measuring cell is a result of the immobilization of the thiol-modified capture probes. The change in resonance angle can be converted into surface mass uptakes using a factor of 120 m° per 100 ng cm^{-2} [26,27]. The calculated **P2** surface den-

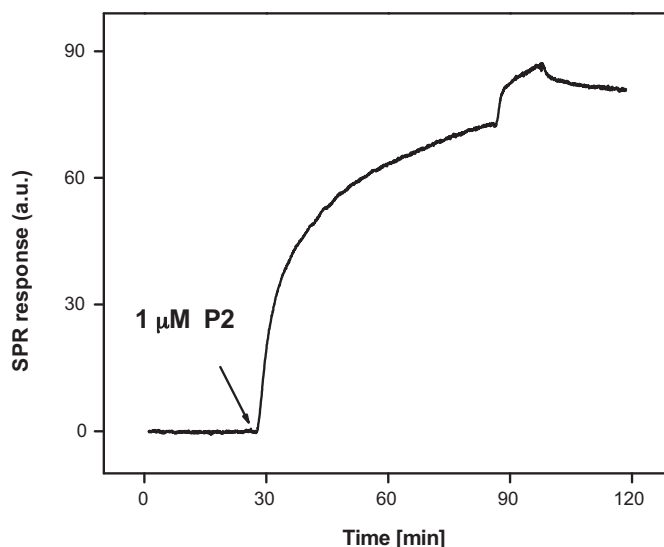


Fig. 2. SPR sensorgram for covalent immobilization of thiol-modified **P2** on the gold surface.

sity equals $\sim 180 \text{ ng cm}^{-2}$ (28 pmol cm^{-2} or 1.7×10^{13} molecules cm^{-2}). The theoretical coverage of a two-dimensional close-packed full monolayer of ssDNA is 1×10^{14} molecules cm^{-2} , assuming that the molecules are perfect cylinders 1.1 nm in diameter and are oriented perpendicularly to the plane of the surface [28]. Using this calculation, the coverage (Fig. 2) represents about 17% of a saturated monolayer for thiolated ssDNA. Herne and Tarlov assumed that very high surface coverage of ssDNA do not lead to optimal hybridization efficiency as the complementary sequence can be sterically prevented from hybridizing at high surface DNA density [18]. In this work we obtained an efficient, but far from saturated immobilization of **P2** on the gold surface of the SPR biosensor.

After the **P2** immobilization stage, the sites on the gold surface free from thiol-modified oligonucleotides should be blocked. For this purpose we used 1 mM aqueous solution of 1-mercapto-6-hexanol (MCH). Tarlov and co-workers have suggested that MCH can prop up DNA molecules that would otherwise lie flat on the Au surface. It can also displace non-specifically bound DNA [18,28]. MCH is thought to improve hybridization efficiency by occupying areas of the surface that are not coated by DNA thereby preventing interactions of DNA from analyzed sample with the gold surface.

3.3. Hybridization

The main process of DNA hybridization sensor performance is recognition of nucleic acid molecules contained in analyzed samples by surface-bound capture probes. Like the process in a solution, nucleic acid hybridization onto a transducer surface is stronger and more specific when the complementarity degree between two DNA chains increases. The specificity and stability of the linkage reach a maximum in the case of full (100%) complementarity. However, the hybridization and its efficiency over solid supports are still unpredictable and depend on many parameters and phenomena such as the analyte diffusion towards the surface of the sensor, its bidimensional diffusion, the adsorption and desorption, the size of probes and targets, the target concentrations and surface probe densities [12,29].

To clarify the situation, we compared the theoretical predictions based on thermodynamic parameters of hybridization between oligonucleotides **P2**, **T2**, **TN** and **TC** obtained for solution conditions (using DINAMelt web server) and experimental SPR sensor responses obtained at their hybridization on the solution-surface

Table 1Thermodynamic parameters of hybridization between the capture probe **P2** and the targets **T2**, **TN** and **TC**.

Oligonucleotides	Free energy change, ΔG (kcal/mol)	Enthalpy change, ΔH (kcal/mol)	Entropy change, ΔS (cal/mol/°K)	Melting temperature, T_m (°C)
P2 and T2	−34.2	−174.1	−473.8	69.1
P2 and TN	−31.9	−153.7	−412.8	70.3
P2 and TC	−4.3	−50.4	−156.2	−9.2

All calculations were performed for 22 °C, 0.33 M Na⁺, 0 M Mg²⁺, and 100 nM concentration of the oligonucleotides.

interface. The calculated thermodynamic parameters of hybridization shown in Table 1 between **P2** and complementary **T2**, **P2** and **TN**, containing single-base mismatch, predict efficient formation of double stranded structures and a low probability of strong hybridization between **P2** and noncomplementary **TC**.

Hybridization with surface-bound DNA could range over a period since a few minutes up to many hours [18,19,26,30,31]. Aiming to reduce the analysis time for sensor application, we have chosen a rather short hybridization time of 3 min of sample injection and 10 min of incubation at stopped flow. After that, the measuring flow-cell was washed by running buffer solution at a standard flow rate a 50 $\mu\text{L min}^{-1}$.

The experimental SPR responses shown in Fig. 3 and obtained at the injection of 100 nM solutions of oligonucleotides into the measuring flow-cell with surface-bound **P2** are in good agreement with the theoretical predictions mentioned above. Indeed, the complementary target **T2** shows the highest sensor response, the single-base mismatched oligonucleotide **TN** produces somewhat smaller response and no response was observed at injection of the noncomplementary oligonucleotide **TC**. It should be noted that the sensor signal reached after injection and incubation of **T2** and **TN** did practically not decrease during the washing by the running buffer. It confirms that stable duplexes are formed on the sensor surface.

Using the bioselective element for other samples analysis is possible only after its regeneration. For this purpose solutions of low or high pH, of some chaotropic agent were applied. Concentrated aqueous solution of urea (8 M) showed the desired result: injection of urea solution followed by the running buffer restores the SPR signal to its initial value. This procedure was repeated 5–6 times per day during two weeks, and no essential deterioration of the SPR signal had been observed.

Fig. 4 shows the dependence of SPR sensor response as a function of **T2** concentration. Over 500 nM, the SPR sensor response

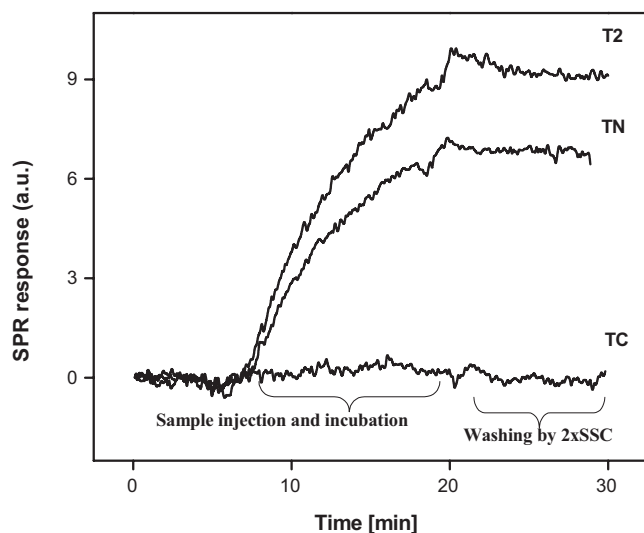


Fig. 3. SPR sensograms for hybridization between the surface-bound capture probe **P2** and the targets **T2** (complementary), **TN** (containing single-base mismatch) and **TC** (noncomplementary).

reaches a saturation and the maximal registered response is about 25 a.u. (66 m°) corresponding to a surface coverage of 55 ng cm^{−2} (8.5 pmol cm^{−2} or 5 × 10¹² molecules cm^{−2}). The hybridization efficiency which is defined as the percentage of surface-bound capture probes **P2** undergoing hybridization with the complementary target is ~30%. Even if this hybridization efficiency is lower than the 100% reported by Herne and Tarlov [18], it is much higher than those obtained in many other investigations, for example, for the two surface-bound probes (ACTG)₅ and C₂₀, Lee et al. reported that only 3.3% and 7.7% of the immobilized probes, respectively are available for hybridization [32]. It should be noted that Herne and Tarlov have obtained the high level hybridization efficiency at coverage of surface bound probes of 5.2 × 10¹² molecules cm^{−2}. It means that in our case, we have practically the same absolute quantity of hybridized target molecules but at a higher density of surface-bound oligonucleotides.

A linear response $\Delta\theta$ (m°) = 0.20 C (nM) + 0.64, with R² = 0.973 we observed up to 200 nM. A similar plot of the SPR responses versus DNA concentrations [$\Delta\theta$ (m°) = 0.204 C (nM) + 0.700, R² = 0.989] was obtained for the AutoLab SPR for a concentration lower than 150 nM [26]. From the noise of the signal measurements of our experimental set-up (0.7 m°) and assuming the necessary signal-to-noise ratio (S/N) of 3, the lowest detection limit is about 10 nM.

The obtained value of the lowest detection limit could be improved by using a sandwich hybridization format shown in Fig. 5: at first, an extended **T2E** consisting of a **T2** sequence and additional 24 nucleotides is allowed to hybridize with the capture probes **P2** immobilized on the sensor surface, then the detection probes **DP** interact with the exposed additional portion of **T2E**.

The calculated value of hybridization free energy changes between **P2** and **T2E** obtained for solution conditions (using DINAMelt web server) is −34.9 kcal/mol, very similar to that of **P2**–**T2** hybridization (see Table 1). However, as it was expected

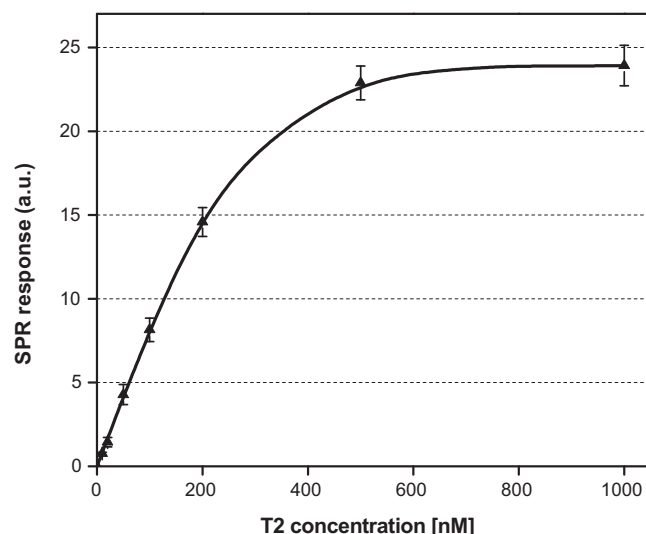


Fig. 4. Dependence of SPR response as a function of **T2** concentration in 2 × SSC.

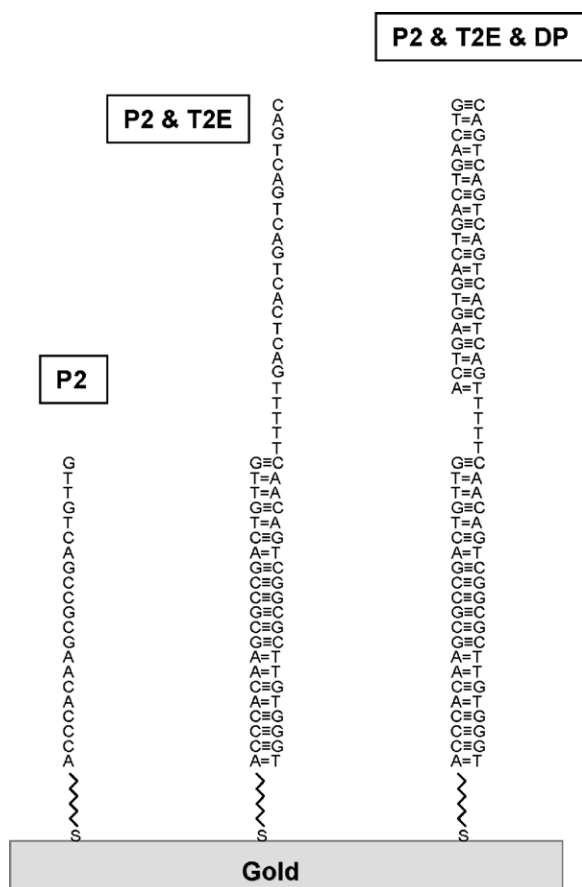


Fig. 5. Proposed scheme of sandwich hybridization format using **P2**, **T2E** and **DP**.

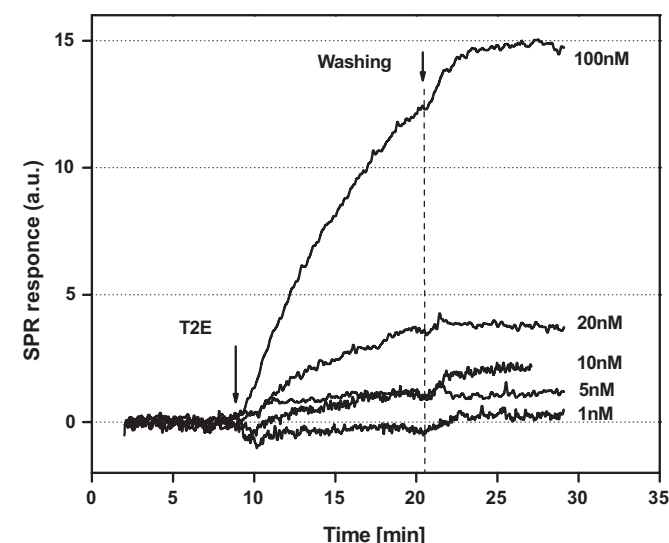


Fig. 6. SPR sensograms for hybridization between the surface-bound capture probes **P2** and the targets **T2E** at various concentrations.

(due to mass-dependent nature of SPR signal), the injection of the equimolar concentration of extended oligonucleotides **T2E** into the measuring cell led to a higher SPR response with a detection limit of 5 nM **T2E** (Fig. 6).

The values of free energy changes calculated using DINAMelt web server for hybridization between **DP** and **T2E** (−28.2 kcal/mol) and for **DP-P2** interactions (−4.7 kcal/mol) clearly demonstrates a very high probability of strong interactions in the first case and

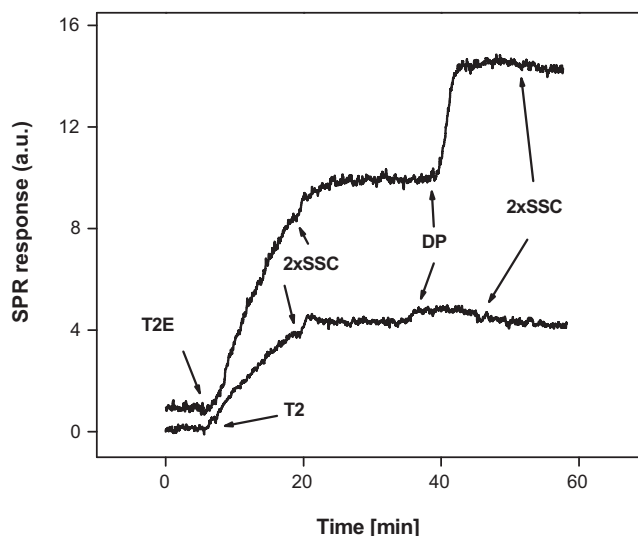


Fig. 7. SPR sensograms when adding DP after P2-T2 and P2-T2E hybridization.

rather low probability in the second one. Indeed, injection into sensor cell of the oligonucleotides **DP**, which is complementary to the free additional part of **T2E**, after **P2-T2E** hybridization results in a significant additional SPR response, as shown in Fig. 7. In control experiments, when **DP** was added into the sensor cell after **P2-T2** hybridization, no sensor signal was observed, confirming that **DP** specifically interacts with **T2E**, but does not interact with **T2** or with the capture probes **P2**, which could be in non-hybridized state. Thus, this suggests that further improvement of the SPR sensor sensitivity could be obtained by using a sandwich hybridization format. Taking into account that the SPR response on 1 nM T2E was observed but it was comparable with the noise level (Fig. 6), the further improvement of sensitivity will make possible to detect the target at subnanomolar quantities.

4. Conclusions

We have developed a method based on SPR for detecting single-stranded oligonucleotides related to the *rpoB* gene of *M. tuberculosis*. As far as our knowledge, the nucleotide sequence of the rifampicin resistance region of the *rpoB* gene was used in SPR sensor development in first time. This work demonstrates the feasibility of the prepared bioselective element for quick detecting in the low nanomolar range the target oligonucleotide molecules; when using sandwich hybridization format detection in subnanomolar range is possible. Once the capture probes are immobilized on the sensor chip, it can be used at least more than 50 times. We believe that further development of the present method would be particularly suitable for detecting specific genes in *M. tuberculosis* related to its drug resistance. Furthermore, this technology could be integrated into a sensor array to determine the antimicrobial resistance profile of *M. tuberculosis* thereby helping the doctors in their prescription of effective chemotherapy regimes and playing an important role in the efforts to prevent the threatening transmission of this dangerous disease.

Acknowledgements

The authors would like to thank North Atlantic Treaty Organization for financial support – Collaborative Linkage Grant PDD(CP)-(CBP.NUKR.CLG 983025) and the financial contribution from the Canada Research Chair. The work has been supported in part by complex program of National Academy of Sciences of Ukraine *Sensor systems for medical, ecological and industrial needs.*

References

- [1] B.R. Bloom, C.J.L. Murray, *Science* 257 (1992) 1055–1064.
- [2] J.M. Musser, *Clin. Microbiol.* 8 (1995) 496–514.
- [3] [3] Anti-tuberculosis drug resistance in the world. Report no. 4. WHO/HTM/TB/2008.394. Available at [http://www.who.int/tb.beijingmeeting/media/drs_report_executive_summary.pdf](http://www.who.int/tb/beijingmeeting/media/drs_report_executive_summary.pdf) (accessed September 2010)].
- [4] S. Ramaswamy, J.M. Musser, *Tuber. Lung Dis.* 79 (1998) 3–29.
- [5] L.B. Heifets, G.A. Cangelosi, *Int. J. Tuber. Lung Dis.* 3 (1999) 564–581.
- [6] S.-N. Cho, P.J. Brennan, *Tuberculosis* 87 (2007) S14–S17.
- [7] A. Van Rie, R. Warren, I. Mshanga, A.M. Jordaán, G.D. van der Spuy, M. Richardson, J. Simpson, R.P. Gie, D.A. Enarson, N. Beyers, P.D. van Helden, T.C. Victor, *J. Clin. Microbiol.* 39 (2001) 636–641.
- [8] A.K. Deisingh, M. Thompson, *Can. J. Microbiol.* 50 (2004) 69–77.
- [9] O. Lazcka, F.J. Del Campo, F.X. Muñoz, *Biosens. Bioelectron.* 22 (2007) 1205–1217.
- [10] J. Homola, *Chem. Rev.* 108 (2008) 462–493.
- [11] M.G. Manera, J. Spadavecchia, A. Leone, F. Quaranta, R. Rella, D. Dell'atti, M. Minunni, M. Mascini, P. Siciliano, *Sens. Actuators B: Chem.* 130 (2008) 82–87.
- [12] F.R.R. Teles, L.P. Fonseca, *Talanta* 77 (2008) 606–623.
- [13] S. Roh, T. Chung, B. Lee, *Sensors* 11 (2011) 1565–1588.
- [14] E. Luzi, M. Minunni, S. Tombelli, M. Mascini, *Trends Anal. Chem.* 22 (2003) 810–818.
- [15] J. Homola, *Anal. Bioanal. Chem.* 377 (2003) 528–539.
- [16] V. Velusamy, K. Arshak, O. Korostynska, K. Oliwa, C. Adley, *Biotechnol. Adv.* 28 (2010) 232–254.
- [17] S. Scarano, M. Mascini, A.P.F. Turner, M. Minunni, *Biosens. Bioelectron.* 25 (2010) 957–966.
- [18] T.M. Herne, M.J. Tarlov, *J. Am. Chem. Soc.* 119 (1997) 8916–8920.
- [19] F. Lucarelli, G. Marrazza, A.P.F. Turner, M. Mascini, *Biosens. Bioelectron.* 19 (2004) 515–530.
- [20] R.K. Smith, P.A. Lewis, P.S. Weiss, *Prog. Surf. Sci.* 75 (2004) 1–68.
- [21] J. Wang, *Nucleic Acids Res.* 28 (2000) 3011–3016.
- [22] R. Sommer, D. Tautz, *Nucleic Acids Res.* 17 (1989) 6749.
- [23] <http://mfold.bioinfo.rpi.edu/>.
- [24] N.R. Markham, M. Zuker, *Nucleic Acids Res.* 33 (2005) W577–W581.
- [25] N.R. Markham, M. Zuker, in: J.M. Keith (Ed.), *Methods in Molecular Biology*, Humana Press, Totowa, NJ, 2008, pp. 3–31.
- [26] X. Su, Y.-J. Wu, W. Knoll, *Biosens. Bioelectron.* 21 (2005) 719–726.
- [27] Q. Tang, X. Su, K.P. Loh, *J. Colloid Interface Sci.* 315 (2007) 99–106.
- [28] J.K.N. Mbindyo, B.D. Reiss, B.R. Martin, C.D. Keating, M.J. Natan, T.E. Mallouk, *Adv. Mater.* 13 (2001) 249–254.
- [29] V. Chan, D.J. Graves, S.E. McKenzie, *Biophys. J.* 69 (1995) 2243–2255.
- [30] S.D. Keighley, P. Li, P. Estrela, P. Migliorato, *Biosens. Bioelectron.* 23 (2008) 1291–1297.
- [31] K.A. Peterlinz, R.M. Georgiadis, T.M. Herne, M.J. Tarlov, *J. Am. Chem. Soc.* 119 (1997) 3401–3402.
- [32] G.U. Lee, L.A. Chrisey, R.J. Colton, *Science* 266 (1994) 771–773.