A Novel Platform Technology for the Detection of Genetic Variations by Surface Plasmon Resonance

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***Abstract*—We report on a novel approach to identify genetic variations based on the detection of specific polymerase-chain-reaction products by surface plasmon resonance. We use a recently developed, home-made spectrometer which exploits chips with integrated optics and microfluidics. The gold film at the chip surface is locally functionalized with single-stranded, thiol-modified probe DNA by applying a nanoliter dispenser. We discuss the chemical conditions for the achievement of maximum SPR signal strength and spot array density, and evaluate the SPR detection of selective binding of a set of 17 PCR products.**

I. Introduction

Straightforward and fast detection of genetic variations becomes increasingly important in medical diagnosis. Here we report on a novel approach where batch-synthesized polymerase-chain-reaction (PCR) products consisting of a DNA fragment with a single-stranded (ss) overhang are hybridized to complementary probe oligonucleotides immobilized at a gold surface (Fig. 1). Hybridization is detected by employing surface plasmon resonance (SPR) as signal transducer. SPR spectroscopy has recently gained attention as powerful tool for highly sensitive and label-free detection of specific biomolecular recognition events [1]. In our approach, the detection of the genetic defects is accomplished by a combination of multiplex PCR and SPR. In the PCR, primers specific for different mutations are used. As a result, different defects will lead to the generation of overhangs with different but well defined sequences – called tags. The latter allows to detect the specific binding of the PCR products to an universal set of anti-tag probe oligonucleotides.

SPR occurs at noble metal surfaces, e.g. gold, when light of a certain wavelength strikes the surface at a given angle through a prism. SPR sensors measure the change of refractive index in a ∼200 nm proximity of a ∼50 nm thick gold layer. The dielectric constant of the near-surface layer alters upon adsorption or binding of molecules, thus allowing detection of DNA hybridization on gold surfaces.

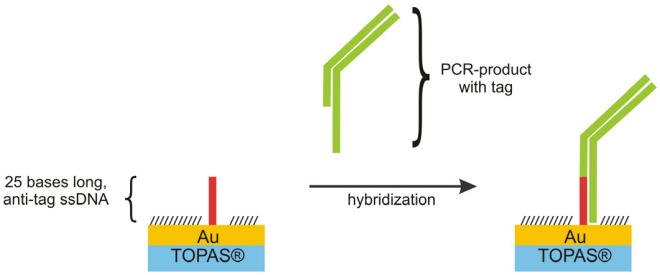

Fig. 1. Schematic viewgraph of the hybridization of PCR products (green) with immobilized single-stranded probe DNA (red). The black bars depict a self-assembled monolayer of mercaptohexanol.

In our investigations, the targets are double-stranded (ds) PCR products with a single-stranded tag. The tags are linked to the primer DNA. The ds part of the target has a length of 250-300 base pairs (bps). The tags are 25 bases long. The primer sequences are specific for genetic variations, and the tag sequences are chosen to be complementary to specific probe anti-tags that are immobilized on the sensor surface. In this way, PCR targets are synthesized which show a direct correlation between the particular genetic defect and tag sequence. The latter is probed by SPR. Here, we investigate the specific binding of 17 different model target PCR products to corresponding anti-tag sequences. The tag and anti-tag sequences are artificially chosen. Their binding efficiency is studied.

II. Instrumentation

A. Chip

Optically transparent TOPAS® chips [2] (76 x 26 x 4 mm^3) with integrated micro lenses, and a ∼50 nm thick gold layer

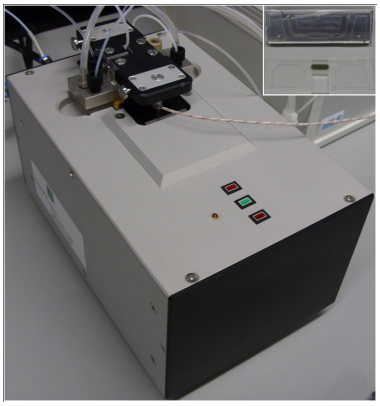

Fig. 2. SPR device with mounted channel plate. The inset shows the opened channel plate and the SPR chip.

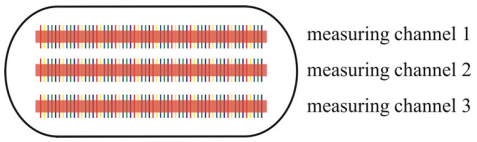

Fig. 3. Schematic viewgraph of the positions of the three optical measuring channels (red horizontal bars) on the gold surface together with linear spots of probe DNA (colored vertical lines).

(area: 3 x 12 mm^2) are used for the SPR investigations [3] (Fig. 2).

B. On-Chip Microfluidics

A channel plate is mounted onto the chip, consisting of a PDMS flow cell, a heat exchanger and corresponding ports for fluids and vacuum. The channel of the flow cell is 120 μm high and 3 mm wide. The cell is made by casting a corresponding silicon master with PDMS (Fig. 2 inset). It is connected to an aluminium plate, the temperature of which is controlled by means of a thermostatic water bath. The temperature at the aluminum block is measured using a thermocouple. The channel plate is fixed to the chip by applying vacuum to three cavities integrated into the PDMS replica. The analyte is pumped through the flow cell by a 500 μl syringe pump.

C. SPR Spectrometer

The gold film of the SPR chip is illuminated by three light-emitting diodes (LEDs; wavelength: 810 nm). Each LED illuminates about one third of the gold surface, further on referred to as measuring channel (Fig. 3). The light reflected from the gold film is measured by a CCD camera providing images with 1280 rows representing the spatial resolution of the spot array at one measuring channel, and 960 columns representing the angular intensity distribution of the individual SPR spectra. One pixel of the rows corresponds to $\sim$ 7.3 μm. The SPR signal is the result of averaging over the width of one measuring channel.

D. Evaluation of SPR Data

The SPR minima of the 1280 rows are determined by a mathematical fitting algorithm. The shifts of the SPR minima in so-called response units (r.u.) are taken as SPR signals. The development of the SPR signal in time is used to determine the local binding kinetics. The real-time data were corrected by referencing to control spots where no binding of target molecules takes place.

E. Fluorescence Microscopy

To check the successful hybridization of target PCR products to surface-bound probe DNA, fluorescence microscopy was used in addition to SPR. To this aim, the samples were stained with YOYO-1 (Invitrogen Y3601). Images of the spots were taken with a fluorescence microscope Axiovert 200M (Carl Zeiss).

III. Materials and Methods

A. Synthesis of PCR Products

Model PCR products with protruding ss tags were synthesized by using primer pairs (Biomers), in which the forward-primers were 5'-modified by different tag sequences separated by an internal hexaethylenglykol (HEG) spacer: 5'-tag-HEG-AGCCTGAATGGCGAATGG-3'. The reverse primer was in each case the same: 5'-GACATTAACCTATAAAACTAGGCGTATCA-3'. PCR was performed using the pUC19 Vector (Invitrogen) as the template to generate the 300-bp product. The PCR products were cleaned by applying a PCR purification kit (Qiagen). Individual PCR products were checked for consistency on a 2% agarose gel, and subsequently, quantified by UV-absorbance (BioPhotometer; Eppendorf).

B. Thiol-Modified Probe DNA

As the anti-tags, thiol-modified ssDNA (HS-ssDNA) was used. The thiol group is linked to the 3' end of the oligonucleotide via a $(CH_2)_3$-T_8 (T_8 – eight thymine bases) spacer. Thiols form covalent bonds to gold surfaces, and thus, are suitable for immobilization of probe DNA. The probes (Biomers) are complementary to the 17 tag sequences listed in Table I. In addition, three non-complementary control sequences were used for the formation of reference spots.

C. Plotting of Probe DNA Arrays

The gold surface was cleaned by rinsing it with ethanol (absolute; Merck) and deionized water. Then, the chips were blown dry with nitrogen. The probe solutions were spotted into lines at the gold surface using a nanoliter dispenser NanoPlotter 2.1 (GeSiM). Our optimized immobilization solution contained 10 μM HS-ssDNA, 20 mM $MgCl_2$, and 5% (w/w) glycerol (Sigma) in an aqueous solution. The droplet volume of the dispenser was $\sim$50 pl. The droplets were deposited with an in-line pitch of 36 μm. The distance between the lines was 140 μm. Sixty lines were spotted in parallel onto the gold surface. In order to compare between the results obtained for the three measuring channels, continuous lines were spotted

TABLE I
TAG AND ANTI-TAG SEQUENCES. HAIRPIN SEQUENCES ARE UNDERLINED.

Name	Sequences (5'-3')
tag 1	ATTGCGTGCACTTGCTTCGGTAGGA
tag 2	TCGGTATGAGATGCCGCCAATTCGA
tag 3	GAACGGACGTCGGTTAGCAACAACA
tag 4	ATGCGTACGTGTTGGAGGACGTAAC
tag 5	TGCACTATGACTGAATGTGCGGTGT
tag 6	GCCGCTTCGACCGTATCTGGATAAT
tag 7	TATTCGGTTGAGCCATGTCGACTGT
tag 8	AACACAATCGAACACATCAGTCGGC
tag 9	TTGACGAGGAATGCGGTAGACTAGG
tag 10	CCGTGCGAATACAGAGCTTACGATG
tag 11	CGCGTTCTATAATCGGATCTGCGAC
tag 12	AATGGTACTCAACATACGCCGTAGC
tag 13	GGTGTCTAGCCGTACATCGATCAAG
tag 14	CCAAGTAGTTAACAACGCGAGCATG
tag 15	AGCATCACGAGAGTAGGATCTACCT
tag 16	GGCAACACACTCTAGCGATAATTGG
tag 17	CGAGACCTTATTCTGGAGAGCCTAT
control 1	ACCGTGAATGGTTGCAGTCCAACCATT TTTTTT-$(CH_2)_3$-SH
control 2	CTTATCTAACGTCGTTACTCTGGCC TTTTTTTT-$(CH_2)_3$-SH
control 3	CTATCTAGCATGCCTATCAGCGTTG TTTTTTTT-$(CH_2)_3$-SH

ranging over all three channels. The atmosphere in the spotting chamber was adjusted to 80% rel. humidity at 23°C. In the experiments for optimizing the glycerol concentrations, the in-line pitch size was 50 μm, and the distance between lines was 100 μm. In this case, the solutions had standard composition but varying glycerol content up to 5% (w/w). After plotting the gold surface was rinsed, and treated with a mercaptohexanol (MCH) blocking solution for 30 min, containing 1 mM MCH (Sigma) in 10 x TE buffer (100 mM Tris, 10 mM EDTA, in deionized water, pH 10). Finally, the DNA arrays were rinsed with deionized water, dried in nitrogen flow and stored at 4°C.

D. Hybridization Procedure

The channel plate, connected to the sample reservoir and to the pump by teflon tubes, was mounted to the functionalized chips, and the system was fitted into the SPR device. Then, the flow channel was rinsed with a 120 mM $MgCl_2$ aqueous solution. Then 90 μl of the hybridization solution, containing 5 ng/μl PCR product in 120 mM $MgCl_2$ aqueous solution, was pumped into the flow cell above the spotted gold surface. The volume flow was adjusted to $\sim$ 6 μl/s during all pumping steps, and the temperature of the channel plate was kept at 40°C. To accomplish a fast hybridization, the target solution was pumped back and forth over the chip. After the hybridization step, the system was rinsed with 500 μl rinsing solution. The whole procedure was repeated for each PCR product in ascending order from tag 1 to 17.

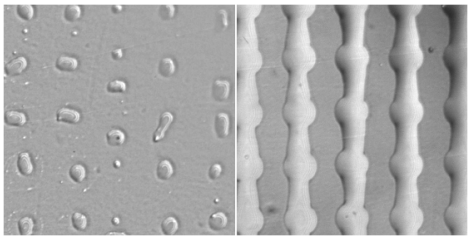

Fig. 4. Bright-field optical microscopy images of the residues after plotting immobilization solutions containing 0% (left) and 5% (right) (w/w) glycerol. The in-line pitch size is 50 μm. The line pitch is 100 μm.

IV. RESULTS AND DISCUSSION

A. Optimizing the Spotting Parameters

Controlling the concentrations of probe DNA, Mg^{2+} and further additives is decisive for the achievement of a proper probe density on the sensor surface, and thus, for the achievement of reasonable SPR signals. The chemical conditions during spotting are further crucial for a precise local spotting, and consequently, for the achievement of dense spotted arrays.

Glycerol as a viscose additive, e.g., improves the adherence of the spotted droplets at the gold surface, and therefore, the stability of the generated spotting lines. Different glycerol concentrations were tested to optimize the spotting parameters. These investigations revealed that adding glycerol to the immobilization solution has a large effect on both the geometrical quality and the homogeneity of the spotted lines (Fig. 4). Without glycerol, the spotted droplets are initially mobile at the gold surface, leading to inhomogeneous spotted arrays. This is different with 5% (w/w) glycerol, where the droplets remain in the position where they are deposited. In this case, each droplet has a contact diameter of $\sim$ 70 μm which allows to spot lines with a minimum distance of 100 μm.

Divalent cations like Mg^{2+} are known to have an impact on the surface probe density, and thus, on the hybridization efficiency. Very recently, we have shown by X-ray photoemission spectroscopy (XPS) that the probe density can be improved by increasing the $MgCl_2$ concentration up to 20 mM [4].

MCH forming self-assembled monolayers (SAMs) on gold is used for blocking the free surface between the spots. Simultaneously, it removes DNA unspecifically adsorbed to the gold surface [5], [6]. Thus by applying MCH, the free sensor surface is protected from unspecific adsorption of target DNA, and probe DNA is better accessible during hybridization [7].

B. Selectivity of the Tag and Anti-Tag sequences

The data of real-time measurements were corrected by referencing them to the signals of the spots "control 1" where specific hybridization cannot take place. Thus, signal fluctuations due to pressure and temperature changes are minimized. Fluorescence microscopy (Fig. 5) gives clear evidence that the spots of the control sequences stay dark compared to the other spots where selective binding is detected by SPR. Fig. 6

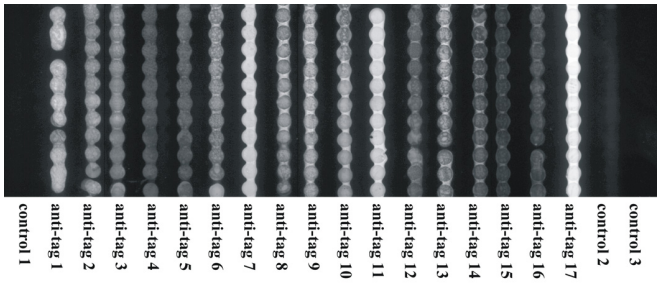

Fig. 5. Fluorescence image of the spot-line array, stained with YOYO-1 after hybridization. Only the spots with PCR products bound to complementary probe DNA appear bright. The control spots with non-complementary probe DNA stay dark.

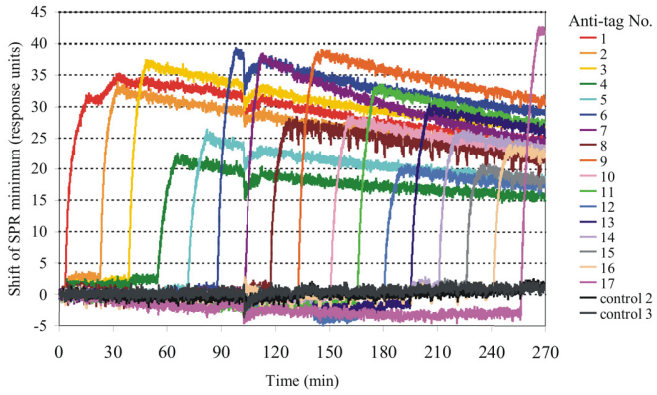

Fig. 6. Time dependence of the SPR signals during hybridization of the 17 investigated PCR products. The signals of the middle measuring channel are presented. PCR products were pumped over the DNA array in a serial, ascending order from tag 1 to 17.

clearly shows that SPR signals emerge only at spots of anti-tags complementary to the tag sequences of the PCR products. The signals develop within a few minutes. Only small cross hybridizations (below 3 r.u.) take place, e.g. between the tags and anti-tags 1 and 2 as well as 11 and 12. These cross hybridizations have to be minimized in future work by either rising the hybridization temperature or improving the stringency by lowering the salt concentration of the hybridization solution. Further, the SPR signals observed on the anti-tag spots 4, 5, 12 and 15 are notable lower ($<$21 r.u.) than the signals obtained on the other spots. We suppose that this is the result of possible hairpin formation of the tags and anti-tags 4, 5, 12 and 15 (cf. Table I) leading to a reduced hybridization efficiency. However, further investigations are necessary to confirm this hypothesis. In future, this problem has to be avoided by further optimization of both stringency of binding and sequence design.

Nonetheless, the presented SPR investigations clearly show that it is possible to differentiate between perfectly matched sequences and cross hybridizations by our developed platform technology.

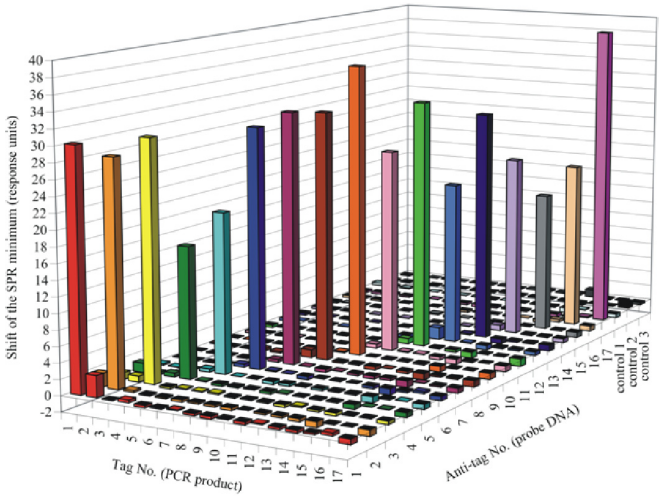

Fig. 7. Three-dimensional bar chart of the maximum SPR signals obtained (cf. Fig. 6). The signal values present averages of signals from nine spots (three spots on every of the three measuring channels). All signals were corrected by subtracting the averaged signal of the three control spots after every hybridization step. The standard deviations are $\pm$3.2 r.u. for the hybridization and $\pm$0.8 r.u. for the control signals. Same color code as in Fig. 6.

V. Conclusions

The presented SPR platform is a powerful tool for real-time detection of PCR-product hybridization on arrays with thiol-modified oligodeoxynucleotides. So far, up to 180 different spots can be analyzed simultaneously. The use of microfluidics enables us to detect the PCR products selectively within minutes.

Acknowledgment

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