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ORIGINAL PAPER

Single nucleotide polymorphism detection by optical DNA-based sensing coupled with whole genomic amplification

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Abstract The work presented here deals with the optimization of a strategy for detection of single nucleotide polymorphisms based on surface plasmon resonance imaging. First, a sandwich-like assay was designed, and oligonucleotide sequences were computationally selected in order to study optimized conditions for the detection of the rs1045642 single nucleotide polymorphism in the gene *ABCB1*. Then the strategy was optimized on a surface plasmon resonance imaging biosensor using synthetic DNA sequences in order to evaluate the best conditions for the detection of a single mismatching base. Finally, the assay was tested on DNA extracted from human blood which was subsequently amplified using a whole genome amplification kit. The direct detection of the polymorphism was successfully achieved. The biochip was highly regenerable and reusable for up to 20 measurements. Furthermore, coupling these promising results with the multiarray assay, we can foresee applying this biosensor in clinical research extended to concurrent analysis of different polymorphisms.

Keywords Surface plasmon resonance imaging · Whole genome amplification · DNA-based sensing · Pharmacogenomics · Single nucleotide polymorphism · *ABCB1*

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Introduction

The detection of single nucleotide polymorphisms (SNPs) in target genes is an attractive goal for diagnostics and pharmacogenomics. A SNP is a DNA sequence variation of a single nucleotide. SNPs represent 90 % of all human polymorphisms and they are present in the population with allelic frequencies of 1 % or greater [1]. Most of these variations in the human genome are neutral (i.e., they have no effect on gene expression, regulation, or function). In some cases, SNPs that are present in coding sequences or gene regulatory regions can alter gene functions and change individual characteristics. For example, they could confer increased susceptibility to a particular disease or might elicit different responses to drugs [2]. For this reason, applied research on SNPs may be of relevance for clinical practice.

Different approaches concerning DNA-based sensing for detection of SNPs based on different transduction principles have been reported in the literature [3–8]. Interesting strategies have been reported for optical sensing and, in particular, surface plasmon resonance (SPR) transduction. Both nucleic acids and peptide nucleic acids have been used as a recognition element, either immobilized on a chip or used in

a sandwich-like assay [9]. In some works, specific proteins such as DNA mismatch repairing proteins [10–14] or enzymes [15] were used to achieve mismatch recognition. In the methods developed, attention was focused on sample pretreatment, which consists of extraction of DNA, in the case of amplification of the target fragment, and its subsequent dissociation to obtain single-stranded DNA that is able to hybridize the probe immobilized on the sensing surface. In some cases, denaturing agents, such as formamide, were used [16]. To increase the SPR signal intensity, many works attempted signal-enhancing strategies, aiming to achieve the detection of the target sequence directly in nonamplified genomic DNA, where the target analyte is found at femtomolar to attomolar concentrations.

In most of the published works, nanostructures were applied in the design of sandwich-like assays, using nanoparticles as mass enhancers or to obtain plasmon coupling. Zanoli et al. [17] recently reported interesting advances in DNA-sensing strategies using functionalized gold nanoparticles (AuNPs).

More recently, the application of SPR imaging (SPRi)-based sensing has been reported for the detection of target sequences in real samples of genomic DNA from plant and human blood samples in sandwiched AuNP-based assays [18]. In both cases, streptavidin-modified nanoparticles carrying specific biotinylated nucleotide sequences were used. In particular, marker sequences of transgenosis in genomic DNA samples carrying different amounts of genetically modified (GM) sequences (Roundup Ready soybean [18] were detected. The system was able to selectively identify the GM target sequence down to zeptomolar concentrations in solutions containing GM and GM-free genomic DNA at attomolar concentrations, even in the presence of a large excess of noncomplementary DNA [18].

The same approach was used for the detection of SNPs in human genomic DNA samples extracted from lymphocytes. In particular, the mismatch studied occurs in the globin gene and leads to thalassemia. The detection of targets or control DNAs was obtained by a sandwich hybridization strategy, using AuNPs conjugated to an oligonucleotide sequence complementary to the final tract of the DNA target not involved in the hybridization on the sensor surface. As sensing probes, peptide nucleic acids [19] were used and discrimination between fully matched and single-base-mismatched sequences was achieved down to 1 fM.

This challenging investigation opens up new possibilities for real applications of SPRi-based sensing. However, some room is left for SPRi-based sensitive detection of SNPs by exploring new assay designs by coupling an in silico computationally assisted approach for probe design, i.e., in silico selection, with suitable sample pretreatment.

In this report we aim to demonstrate the applicability of SPRi to the detection of SNPs in DNA extracted from human lymphocytes. We used a combination of an optimized computer-assisted design of the probes and sample pretreatment, consisting in the enrichment of the whole genomic material, which was extracted from the human lymphocytes to be analyzed. Usually in affinity-based sensing, when dealing with samples with a limited amount of DNA derived from a source, a standard PCR preamplification of the target gene or sequence is commonly performed before bioanalysis. In particular, so-called whole genome amplification (WGA) is an interesting alternative to conventional gene amplification approaches, and may potentially bypass some limiting factors when using genomic samples directly. It basically consists in a nonenzymatic fragmentation of the genetic material of the sample, followed by an amplification using universal primers. WGA is able to perform the amplification of the whole genome contained in a real sample, thus increasing the amount of DNA. A high-quality sample can be amplified one-million-fold while maintaining accurate loci and allele representation. Thus, it is evident that WGA is a promising tool to obtain a sufficient quantity of DNA from samples with a limited starting amount [20]. It is possible to apply WGA to samples collected from several years in order to study archived patient DNA. WGA can be useful when access to patient cells is limited, as in the case of samples obtained by needle biopsies and by laser capture microdissection from cancer patients [21].

As a consequence, WGA is expected to find new applications in biomedical research as a combined process of total nucleic acid amplification.

The SNP used here as a case study was rs1045642, which occurs in the gene *ABCB1* [ATP-binding cassette, subfamily B (MDR/TAP), member 1] at position 87138645 of chromosome 7. *ABCB1* is a key gene in pharmacogenomics and the rs1045642 SNP is related to the incidence of many diseases: aspirin resistance ischemic stroke and its subtypes [22], cancer risk [23], response to paroxetine in Japanese major depression [24], multidrug resistance in plasma cell myeloma [25], response and toxic effects of drugs in patients with advanced renal-cell carcinoma [26], and susceptibility to viral hepatitis A infection in Mexican Americans [27]. This wide spectrum of diseases indicates the reason for the interest in its detection.

In particular, here computationally selected probes were immobilized on an SPRi biochip surface in order to study optimized conditions for the detection of the rs1045642 SNP in the *ABCB1* gene. The SPRi biosensor was first developed using standard solutions to evaluate the best conditions for the detection of a single mismatching base. Probe characteristics were studied and optimized for the position of the polymorphic site along the DNA sequence, the length of the DNA probe, and its functionalization. First, synthetic DNA sequences were used to test and optimize the

assay, and then the strategy was tested on human DNA extracted from human blood and amplified by WGA.

Materials and methods

In silico analysis: rational probe selection and assay design

The DNA probes that were used for all the steps of the assay were selected both for immobilization on the biosensor surface and for polymorphism detection. Expected performances were evaluated and chosen by OligoWiz 2.0 [28], a software program with verified capacities for predicting the performances of probes in biosensing applications [29]. A score between 1 (maximum) and 0 (minimum) was assigned to nucleic acid sequences to represent the computationally estimated performances in hybridizing the target. This protocol provided recommendations on the design and offered the possibility to evaluate probe characteristics, allowing optimization of the working conditions of the assay.

Solutions, reagents, probes, and targets

Immobilization solution was used for immobilization of the probes on the gold chip surface; it was a 1 M aqueous solution of KH_2PO_4, pH 3.8. Hybridization solution was used as a running solution; it was an aqueous solution of 300 mM NaCl, 20 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.4 with 0.05 % poly (ethylene glycol) sorbitan monolaurate (TWEEN® 20). Thiolic solution was used to passivate the biochip surface and it consisted of a 1 μM 11-mercapto-1-undecanol and 1 μM 6-mercapto-1-hexanol solution. All reagents were purchased from Sigma-Aldrich (Milan, Italy). All solutions were prepared in Milli-Q water (Millipore, Billerica, MA, USA) and filtered using vacuum filter cups (Millipore E express plus, 0.22 μm) and syringe filters (Puradisc, cellulose acetate, 0.2 μm from Whatman, Dassel, Germany).

Probes and targets were purchased from Eurofins MWG Operon (Ebersberg, Germany) and the corresponding sequences are reported in Table 1.

SPRi instrumentation and biochip preparation

An SPRi-Lab$^+$ instrument, from Genoptics-Horiba Scientific (Orsay, France), was used to test hybridization between probes and targets. Interactions were followed in real time by an optical apparatus, and a digital image of the biochip surface was shown displaying the interactions occurring on it. The whole instrumentation setup was previously reported [30].

Thiolated DNA probes were covalently immobilized on the gold chip with the help of a polydimethylsiloxane mask to obtain a removable microwelled support for deposition of the probes.

Probes (0.8 μL, 10 μM in immobilization solution) were incubated for 72 h after deposition. DNA targets were prepared in hybridization solution. Hybridization solution was also used as a running buffer for the SPRi instrument. Thiolic solution was used to saturate the gold surface.

Whole genome amplification

Genomic DNA was extracted from blood using a DNeasy 96 blood and tissue kit from QIAGEN. WGA was performed with Illustra GenomiPhi HY DNA amplification kits from General Electric, and genotyping was performed with TaqMan® SNP genotyping assays from Life Technologies/Applied Biosystems. Each injection of WGA samples was 100 μL of 6 ppm of amplified genome in hybridization solution, preheated for 7 min at 95° C. The genotype selected to assess the validity of the method was the C/C allele (the hypothesized ancestral allele).

The WGA sample was quantified using the fluorescent dye PicoGreen® (Molecular Probes, Eugene, OR, USA) with a TD-700 fluorimeter (Turner Biosystems, Milan, Italy). To calibrate the fluorimeter, five known concentrations of a standard double-stranded DNA from lambda phage (lambda DNA from Fermentas, Milan, Italy) were prepared and used as standards. Their fluorescence intensities were measured in the calibration range from 0 to 500 ppb. For quantification, the WGA sample was diluted in a buffer composed of 10 mM tris(hydroxymethyl)aminomethane hydrochloride and 1 mM EDTA, pH 7.5.

Results and discussion

The work presented here describes the optimization of a strategy for detection of SNPs in DNA sensing based on SPRi technology. First, the assay was designed by choosing DNA sequences by a computationally assisted approach; then the detection strategy was optimized for synthetic DNA sequences for the position of the polymorphic site along the DNA sequence, the length of the DNA probe, and its functionalization. The strategy developed was then tested on WGA samples obtained from human blood.

In silico analysis: rational probe selection and assay design

The first and crucial aspect to take into account when designing a DNA biosensor is the selection of the probe. In this work, the in silico design of the probes for SNP rs1045642 in the gene *ABCB1* was achieved. Probes were designed on a 511-mer fragment of the *Homo sapiens ABCB1* gene on chromosome 7 (from position 87138390 to position 87138900 of chromosome 7, from position 3673 to position 4183 of the coding sequence of the gene).

Table 1 Probes and target sequences

CProbe(5′thiol) (22-mer)	5′ HS-$(CH_2)_6$-GTCACTGCCTAATGTAAGTCTC 3′
CProbe(3′thiol) (22-mer)	5′ GTCACTGCCTAATGTAAGTCTC-$(CH_2)_6$-SH 3′
CTarget (22-mer)	5′ GAGACTTACATTAGGCAGTGAC 3′
Target 84 (84-mer)	5′GAGACTTACATTAGGCAGTGACTCGATGAAGGCATGTATGTTGGCCTCCTTTGTGCCCTCACAATCTCTTCCTGTGACACCAC 3′
DProbe END 21 (21-mer)	5′ HS-$(CH_2)_6$-GTGGTGTCACAGGAAGAGATT 3′
DProbe INT 21 (21-mer)	5′ HS-$(CH_2)_6$-TCACAGGAAGAGATTGTGAGG 3′
Target END 21 FM (21-mer)	5′ AATCTCTTCCTGTGACACCAC 3′
Target END 21 BIO FM (21-mer)	5′ BIO-AATCTCTTCCTGTGACACCAC 3′
Target END 21 MM (21-mer)	5′ GATCTCTTCCTGTGACACCAC 3′
Target END 21 BIO MM (21-mer)	5′ BIO-GATCTCTTCCTGTGACACCAC 3′
Target INT 21 FM (21-mer)	5′ CCTCACAATCTCTTCCTGTGA 3′
Target INT 21 BIO FM (21-mer)	5′ BIO-CCTCACAATCTCTTCCTGTGA 3′
Target INT 21 MM (21-mer)	5′ CCTCACGATCTCTTCCTGTGA 3′
Target INT 21 BIO MM (21-mer)	5′ BIO-CCTCACGATCTCTTCCTGTGA 3′
DProbe END 15 (15-mer)	5′ HS-$(CH_2)_6$-TCACAGGAAGAGATT 3′
DProbe END 12 (12-mer)	5′ HS-$(CH_2)_6$-CAGGAAGAGATT 3′
DProbe END 9 (9-mer)	5′ HS-$(CH_2)_6$-GAAGAGATT 3′
UProbe (18-mer)	5′ HS -$(CH_2)_6$-GAGGGCGATGCCACCTAC 3′
DProbe END 15 BIO MM (15-mer)	5′ TCACAGGAAGAGATT -BIO 3′
DProbe END 15 BIO FM (15-mer)	5′ TCACAGGAAGAGATC –BIO 3′

CProbe (5′ thiol) and CProbe(3′ thiol) are the same sequences used with 5′ and 3′ immobilization sites, respectively

Underlined polymorphic site, *END* polymorphic site in terminal position, *INT* polymorphic site in internal position, *DProbe* discriminating probe (sequence used for single nucleotide polymorphism detection), *UProbe* unspecific probe, *BIO* biotinylated

Considering the prediction by the software, we designed the assay as reported in Fig 1.

Three DNA sequences were chosen as follows: a capturing probe (score 0.88) estimated to be highly specific for the *ABCB1* gene. This probe was modified with a thiol group at the 5′ end CProbe(5′thiol) or at the 3′ end CProbe(3′thiol). Two other sequences were selected on the polymorphic site in order to find the best discriminating probe for SNP recognition. These discriminating probes mapped the polymorphic site within the sequence (DProbe INT 21, score 0.48) and at the 3′ end (DProbe END 21, score 0.55). They were chosen to be as similar as possible in terms of estimated binding performances (evaluated in silico) in order to ensure that any difference in the SPRi signal could be ascribed to the position of the polymorphism.

Mimicking WGA fragment hybridization with Target 84: primary response

Target 84 (Table 1) was used as a "simulator" fragment to mimic the target sequence of the *ABCB1* gene containing the SNP of interest. This target had the first 22 bases (5′ end) fully complementary to the capturing probe (selected with OligoWiz 2.0) and the polymorphic site at position 64 (see the assay scheme in Fig. 1).

CProbe(5′thiol) and CProbe(3′thiol) (same sequence but functionalized with a thiol group at the 5′ end and 3′ end, respectively) were preliminarily tested in order to establish which one provided the highest SPRi signal in hybridizing Target 84. Their performances were evaluated by testing a 250 nM solution of Target 84 in hybridization solution (see Fig. S1). This concentration was chosen for the good performances and reproducibility in terms of the SPRi signal (see Fig. 2), in particular using CProbe(5′ thiol). Figure S1 shows that the best signal was recorded for CProbe(5′thiol); thus, this was chosen for further hybridizations and for calibration of Target 84 on the SPRi biosensor. Signals obtained from hybridizations of different concentrations of Target 84 were recorded for CProbe(5′thiol) and the relative calibration curve was plotted (Fig. 2a).

The measurements were highly specific and no interaction was recorded on the gold surface (passivated by thiolic solution) or on blank reference spots (treated with immobilization solution and without probes). This is evidenced by the differential image of the interacting biochip shown in Fig. 2b. All modifications that occurred at the biochip surface are visualized with a color scale, with red corresponding to the highest signal. Target 84 hybridized with immobilized CProbe (five bright spots) but not with the control probe (UProbe, spot U in Fig. 2b). No interaction was recorded with the blank spot (control spot, spot B in Fig. 2b) or with the gold surface (blue area surrounding the spots). The reproducibility, expressed as

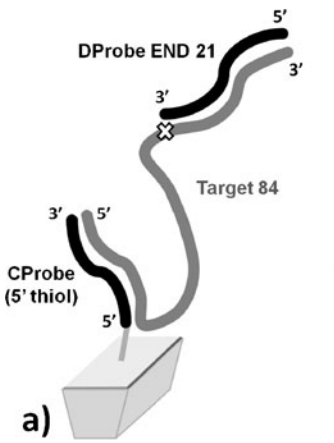

Fig. 1 The designed assay in the case of a discriminating probe carrying the polymorphic site at the end of the sequence (DProbe END 21, **a**) and in the case of the discriminating probe carrying the polymorphic site inside the sequence (DProbe INT 21, **b**). The capturing probe, designed to selectively capture the Target 84 fragment and designed for the *ABCB1* gene, was immobilized on the biochip surface

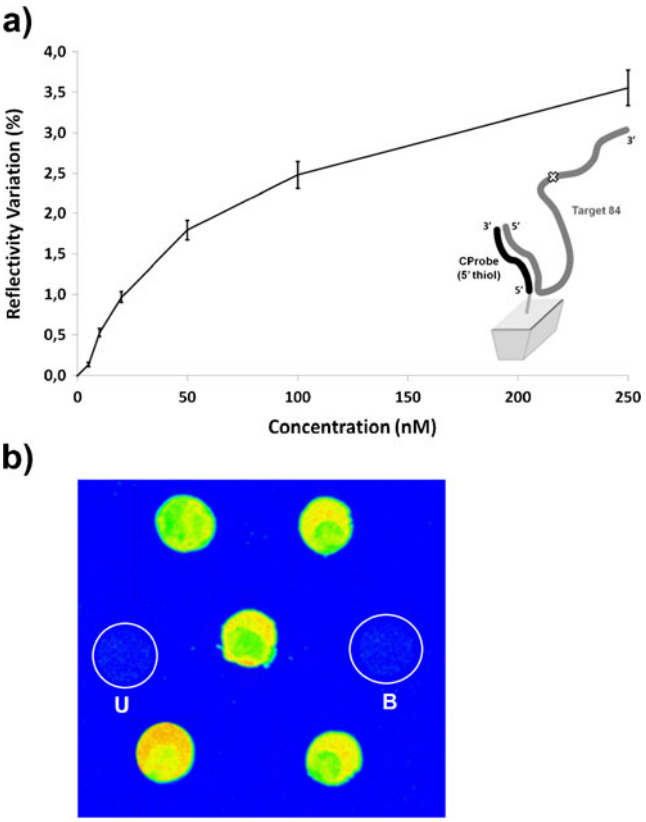

Fig. 2 **a** Variation of surface plasmon resonance imaging (SPRi) signals for CProbe(5′thiol) versus different Target 84 concentrations between 5 and 250 nM. For each point of the curve, the standard deviation was calculated and reported, resulting in a coefficient of variation of less than 9 %. **b** A differential image of the interaction between Target 84 (250 nM) and CProbe is shown. The *dark-blue area*, i.e., the area where no interactions occurred, is the gold on the chip surface (passivated by thiolic solution). *Bright areas* represent the spotted surface where immobilized CProbe interacts with Target 84. *U* indicates the unspecific probe spot and *B* indicates the blank spot for reference

the percent coefficient of variation (CV) was excellent, being less than 9 %.

Optimization of discriminating probe features for SNP detection

Step 1: influence of the position of the polymorphic site along the discriminating probe sequence

The aim of this step was to verify whether the position of the polymorphic site along the probes could influence the ability of the probe to discriminate the SNP. We chose two probes carrying the polymorphic site in a position within the sequence (DProbe Int 21) and at its 3′ end (DProbe End 21), respectively. Both probes were immobilized on the biochip surface. Calibration curves of both probes with the corresponding fully complementary targets are shown in Fig. S2.

In this optimization step, SPRi signals relative to the hybridization of probes with both the fully complementary targets (Target INT 21 FM and Target END 21 FM) and the single base mismatching targets (Target INT 21 MM and Target 21 END MM) were evaluated, using both biotinylated and nonbiotinylated target sequences. All combinations tested are reported in Fig. 3a. No significant differences in signals were recorded between matching and mismatching targets with nonbiotinylated sequences (details are provided in Fig. S3a). This finding is probably because the presence of one mismatching base did not significantly modify the stability of the duplex formed. Conversely, in case of biotinylated targets, significant differences between fully complementary targets (Target INT 21 BIO FM and Target END 21 BIO FM) and single base mismatching targets (Target INT 21 BIO MM and Target END 21 BIO MM) (Figs. 3, S3b) were found.

Considering all the combinations performed (summarized in Fig. 3a), we noticed that only one probe, carrying the mutation at the end of the sequence, could differentiate between fully matching and single base mismatching hybridization. Furthermore, biotinylated target sequences played a key role. Only by hybridizing biotinylated targets with an internal polymorphic site (Target INT 21 BIO FM and Target INT 21 BIO MM) on the probe carrying the terminal polymorphic site (DProbe END 21) was it possible to have significant discrimination for the SNP. To explain these findings, we believe that here, where fewer nucleotides (15-mers) are involved in the hybridization, the stability of the hybrid can be more easily affected by the presence of one mismatch than in a longer sequence. Furthermore, it seems that in our assay, biotin could play a key role in mismatch discrimination. Hence, from these results, biotin seems to enhance the influence of a single base mismatch on the stability of the duplex structure.

Step 2: influence of the length of the discriminating probe

The influence of probe length on the ability to detect a single mismatching base was also studied. Probes of

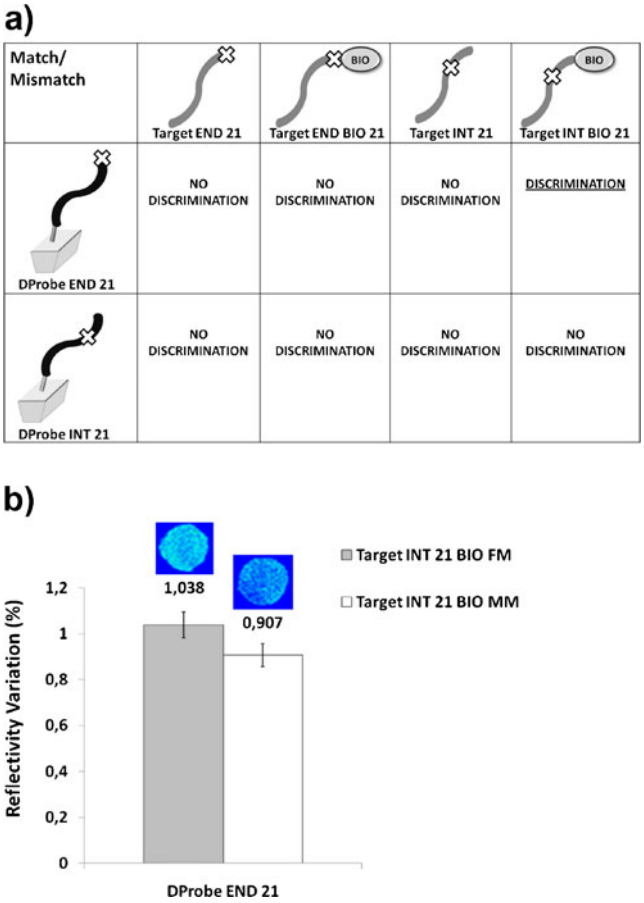

Fig. 3 Optimization of discriminating probe features for single nucleotide polymorphism detection: influence of the position of the polymorphic site along the sequence and the presence or absence of the biotin modification. **a** The ability to discriminate between a fully matching target and a single base mismatching one is reported for each probe–target combination tested. **b** SPRi values and differential images recorded from the only combination able to differentiate fully matching hybridization from mismatching hybridization, i.e., DProbe END 21 hybridized with Target INT BIO 21

different length were immobilized on the gold biochip surface: DProbe END 21 and other three probes, i.e., DProbe END 15, DProbe END 12, and DProbe END 9, obtained by shortening DProbe END 21 at the 3′ end.

Biotinylated and unmodified targets of 21-mers with the polymorphic site within the sequence were tested for hybridization, and SPRi signals were evaluated both for match and mismatch conditions. The results are shown in Fig. 4.

No signals were recorded with DProbe END 9. The other probes, 21-mers, 15-mers, and 12-mers, were all able to discriminate between matching and mismatching targets. Shortening the sequence can help to enhance the effect of the mismatching base, but a too short sequence is not able to hybridize, i.e., DProbe END 9. Very low signals were recorded from DProbe END 12, and the best ratio between full matching and mismatching signals was found for DProbe END 15. From these results we concluded that DProbe END

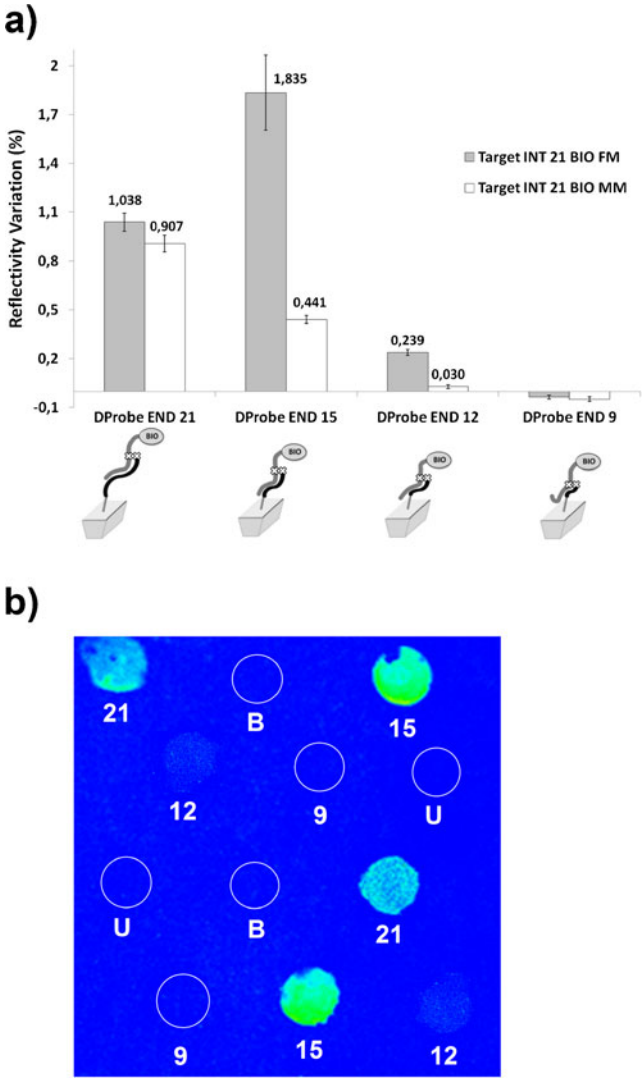

Fig. 4 **a** Reflectivity variation recorded for probes differing in length when they hybridize the fully complementary biotinylated target (*gray bars*) and the single base mismatching one (*white bars*). **b** Differential image for the fully matching target. The numbers *21*, *15*, *12*, and *9* indicate spots for DProbe END differing in length (21-mers, 15-mers, 12-mers, and 9-mers, respectively), *U* indicates the unspecific probe spots and *B* indicates blank spots for reference

15 was, among the probes tested, the one that could ensure good hybridization with targets and the highest difference in signals between the fully matching target and the mismatching one, giving the best performance in discriminating the SNP.

Mimicking the complete assay for the discrimination of SNP on Target 84

After the optimization of the probe, we found that the best conditions were ensured by a 15-base sequence modified with biotin at one end. We proceeded further to test the complete assay on the SPRi sensor (Fig. 1). DProbe END 15, with its optimal features, was tested on Target 84, which mimics the genomic sample in the assay. In this case we mimicked a sample of homozygote DNA with both alleles with the same sequence in the *ABCB1* gene. CProbe(5′thiol) was immobilized on the sensor surface and DProbe END 15 was used in a secondary hybridization for the discrimination of the SNP. The ability of the system to discriminate between match and mismatch was evaluated. Then, the results with the optimized probe were compared with those with an unmodified one (no biotin modification) to confirm if the biotin could play a key a role in the discrimination of the SNP on Target 84 (Fig. 5).

Biotinylated probes gave a signal difference corresponding to $\Delta\Delta R$=0.508 %; no signal was recorded from DProbe END 15 BIO MM on Target 84 (Fig. 5). Unmodified DProbe END 15 FM (ΔR=0.397 %) and DProbe END 15 MM (ΔR= 0.286 %) gave a lower but still significant difference in signals ($\Delta\Delta R$=0.111 %). Hence, biotinylated DProbe END 15 BIO FM and DProbe END 15 BIO MM were chosen for SNP discrimination on WGA samples.

Discrimination of SNPs in WGA samples from human blood

The assay, optimized as described already, was finally tested on WGA samples obtained from blood with the aim of

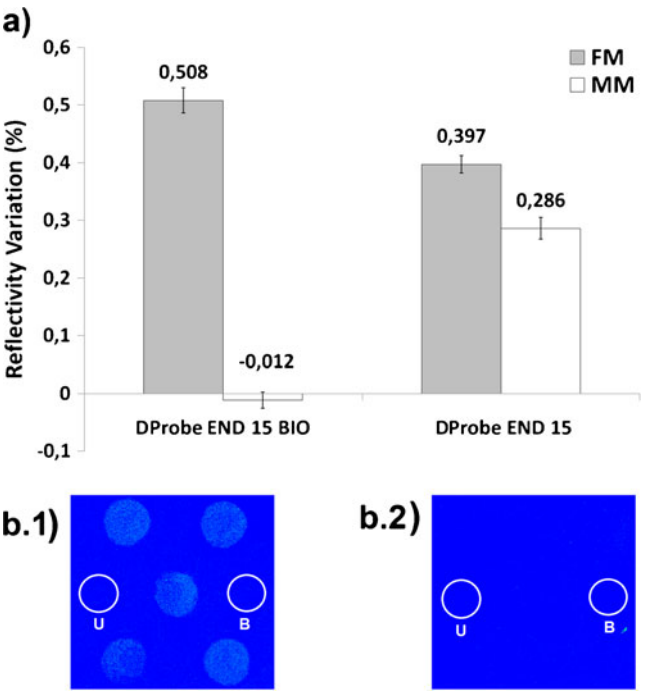

Fig. 5 SPRi signals from the secondary hybridization between the biotinylated discriminating probe (DProbe, *left*) and the unmodified one (*right*) on precaptured Target 84 (*a*). Signals for the fully matching (*FM*) DProbe (*gray bars*) are compared with those for the single base mismatching (*MM*) DProbe (*white bars*). The relative differential images of the interaction between captured Target 84 and DProbe END 15 BIO are reported for fully matching DProbe (*b.1*) and for single base mismatching DProbe (*b.2*)

verifying the possibility to discriminate the presence of the SNP also in samples containing a whole human genome. In fact, WGA allowed us to enrich a genomic sample, human in this case, from nanograms to micrograms. The final product corresponds to the whole starting genome and was composed of long fragments of random length. As proof of principle, we chose to analyze the putative ancestral allele, i.e., the homozygote C/C genotype of the SNP rs1045642. This sample was obtained from blood and genotyped as reported in "Materials and methods." This genotype was used here as a positive standard to assess the assay developed. Therefore, in this case the strands that will bind CProbe on the biochip will carry a guanine residue (complementary to the ancestral C nucleotide). On this basis, the assay was based on two hybridization steps, as represented in Fig. 6: in the first step the direct binding of the WGA sample and its detection was accomplished; then, by injecting the two discriminating probes (DProbe END 15 BIO FM and DProbe END 15 BIO MM) on the prehybridized WGA, we achieved discrimination. In particular, genotype detection was realized by comparing signals relative to the fully complementary sequence, DProbe END 15 BIO FM, containing a terminal guanine residue, with those relative to the mismatching one, DProbe END 15 BIO MM, containing a terminal thymine residue.

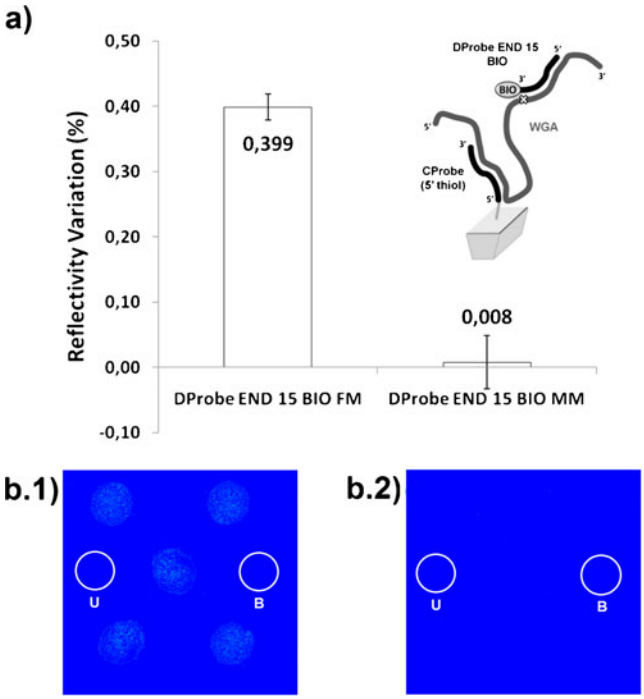

Fig. 6 SPRi signals recorded from secondary hybridization on the captured whole genome amplification (*WGA*) sample. Signals were compared for DProbe END 15 BIO FM and, after regeneration and the new binding of the WGA sample, DProbe END 15 BIO MM. The relative differential images of the interaction are reported for the fully complementary (*b.1*) and for the mismatching discriminating (*b.2*) probe, respectively

Before injection, the WGA sample (6 ppm) was thermally pretreated to dissociate the double-stranded DNA strands, allowing surface hybridization of the appropriate strand to the CProbe. The first result obtained was the direct detection of the WGA sample, giving a very good SPRi signal of ΔR=0.809 % with an excellent reproducibility among spots (CV=2 %). Moreover, the specificity of the detection on the genomic sample was confirmed by control spots, i.e., no significant signal was observed with the WGA sample: ΔR=(0.022±0.043)%. This result was relevant per se, considering the nature of the sample tested, i.e., a whole genome sequence. Furthermore, it confirms the goodness of the selected capturing probe (CProbe) selected by the in silico approach applied.

After the WGA hybridization, the secondary amplification was recorded by injecting in series the fully matching DProbe END 15 BIO FM and, after regeneration and the new binding of the WGA sample, the mismatching DProbe END 15 BIO MM.

As reported in Fig. 6, the averaged signals of the secondary hybridizations with the discriminating probes clearly gave the expected result: SPRi signals were recorded only when the full match was formed by addition of DProbe END 15 BIO FM, giving ΔR=0.399 %, with CV=5 %. In contrast, when DProbe END 15 BIO MM was injected on the prebound WGA, no signal was recorded. Moreover, the specificity of this recognition was confirmed by testing the unspecific probe (UProbe), designed with the purpose of not interacting with the WGA sample, which gave no significant signal: ΔR=(0.015±0.022)%. Finally, the sandwich-like structure obtained was highly regenerable and the sensor was able to work for up to about 20 measurement cycles (Fig. S4).

With this final assay we successfully tested the ability of the strategy developed to discriminate a single base mismatch in a genomic sample, but also confirmed the genotype of the sample, i.e., C/C. In fact, since the discriminating probe (DProbe END 15 BIO MM), containing a terminal thymine residue, did not bind the sample, the presence of an adenine residue can be excluded. This result opens up the possibility of recognizing the ancestral genotype in a fast and single-sample analysis in a real-time and label-free manner.

Conclusions

We have reported SPRi-based sensing applied to detection of SNPs. In particular, as proof of principle, the rs1045642 SNP was selected on the *ABCB1* gene. The measurement conditions were optimized in terms of the rational selection of probe sequences and their length, the position of the polymorphic site, and functionalization (with biotin in the

case of the discriminating probe). We first used a model system based on a synthetic DNA fragment, Target 84, to develop the strategy to be used further on WGA samples derived from human blood. The analytical parameters were evaluated in terms reproducibility, and resulted in an averaged CV<9 %. Finally, the sensor was applied to genomic samples extracted from blood and enriched by the WGA procedure. We clearly demonstrated, using a genotyped sample, that this method allows one to discriminate between full match and mismatch samples, allowing successful detection of the polymorphism on the *ABCB1* gene, homozygote C/C in this case. This approach has general validity and can be transferred to other genotypes of the rs1045642 SNP, as well as other SNPs.

In conclusion, these promising results can be seen as the starting point for future work oriented toward the direct detection of SNPs in real samples. Moreover, the possibility to perform measurements in a multiarray assay by the SPRi technique allows us to foresee the possible simultaneous analysis of several SNPs in the same genomic sample. To this aim, different probes specific for a number of genes could be immobilized on the sensor to screen different SNPs. Furthermore, the heterozygosis degree at the polymorphic site could be evaluated by the system, making use of sequential analysis with suitable discriminating probes. The system is label-free and regenerable, which are two key features for application of biosensors in clinical diagnostics. These findings, combined with the WGA technique, will allow the application of SPRi-based sensing also to analytical research fields for which the limiting factor is the scarce amount of DNA samples for direct biosensing assays.

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