Label-free discrimination of single nucleotide changes in DNA by reflectometric interference Fourier transform spectroscopy

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ABSTRACT

Phenotypic variation – such as disease susceptibility and differential drug response – has a strong genetic component. Substantial effort has therefore been made to identify causal genomic variants explaining such variation among humans. Point mutations (PMs), which are single nucleotide changes in the genome, have been identified to be the most abundant form of causal genomic variants, making them useful, reliable diagnostic markers. Methods developed to genotype PMs have moved towards solid-phase assays, which not only show greater sensitivity and specificity, but also enable scalability and faster processing time. Most current assays are, however, based on fluorescent probes, which makes them relatively expensive. To develop a more cost-effective label-free genotyping method, we used a porous silicon (PSi) base as an efficient support for DNA biosensing and coupled it with reflectometric interference Fourier transform spectroscopy (RIFTS). To assess the versatility of this approach, we tested both a single nucleotide substitution in VKORC1 (-1639G > A; rs9923231) and a single nucleotide insertion in BRCA1 (5382insC; rs80357906). We demonstrate that the PSI-RIFTS method can efficiently detect both PM types with high sensitivity where hybridization of complementary DNA can be quantifiably differentiated from mismatch and non-complementary hybridization events. In addition, we show that the PSI base with immobilized DNA not only can be re-used to type further samples, but it also remains stable for 14 days, suggesting its potential for high-throughput applications.

1. Introduction

Point mutations, which create single nucleotide changes in the genome, are the most abundant form of human genetic variation [1]. These genetic variants are responsible for most phenotypes including various traits and diseases. For instance, point mutations in BRCA1, a gene encoding a tumor suppressor protein involved in DNA repair, lead to an increased risk of up to 50-fold for breast cancer development [2]. Initially thought as an Ashkenazi Jewish-specific mutation – reach polymorphic frequencies in many populations worldwide. Specifically, in Iran, the 5382insC allele appears to be common among breast cancer patients [3,4]. Single nucleotide variants may also be related to hidden traits such as inter-individual differential drug response (i.e. pharmacogenetic trait). Among the many genes involved in drug response, VKORC1, encoding the vitamin K epoxide reductase, is a key gene which is strongly associated with sensitivity to warfarin pharmacotherapy with the -1639G > A variant being recommended by FDA to be genotyped for warfarin dosage adjustment (FDA drug label) [5]. Interestingly, this polymorphic variant is included in multiple FDA-approved in vitro human genetic drug metabolism tests such as the INFINITI 2C9 & VKORC1 Multiplex Assay for Warfarin sensitivity [6].

Hence, the sensitive detection of point mutations in trait- or disease-implicated genes is of utmost significance in efficient clinical molecular diagnosis. A wide range of methods have thus been developed to screen...
such genetic variants including but not limited to single-strand conformation polymorphism assay, hetero duplex analysis, high resolution melting and direct DNA sequencing [7-11]. In recent years, solid-phase DNA-based genotyping arrays have appeared as one of the most powerful methods for clinical screening of point mutations due to their high processing speed and high multiplexity.

Essentially, the sensing surface of a DNA-based biosensor consists of an immobilized single stranded oligonucleotide which binds to its complementary target DNA sequence via hybridization. The strength of this is due to the ease and speed of fabrication, remarkable optical and morphological properties (including tunable pore size and porosity), large internal surface area and versatile surface [13].

Among different PSI optical biosensors [14], those based on reflectometric interference Fourier transform spectroscopy (RIFTS) have received a lot of attention due to its applicability as a label-free biosensor. In this approach, light is illuminated on the surface of a PSI single/multi-layer and the interference pattern of all reflected beams from all interfaces is recorded. After exposing the PSI surface to mobile complementary DNA molecules, these penetrate the pores of PSI and hybridize to their immobilized counterparts, causing variation in the refractive index of the layer. As a consequence, a shift in the interference pattern is observable, which can then be used as a key parameter for biosensing applications. Multiple studies have, on this basis, been able to detect complementary DNA from non-complementary DNA by fabricating different PSI surface structures including single layer [15-19], Waveguide [20,21], photonic crystal [22,23], resonant mirror [24] and microcavity [25]. However, to the best of our knowledge, no study to date has used PSI-RIFTS, as a label-free solid-phase genotyping method to detect point mutations. We therefore aimed to examine the applicability of PSI-RIFTS with a single layer to detect two structurally different point mutations with diagnostic value, namely a single nucleotide substitution in VKORC1 (−1639G > A; rs9923231) and a single nucleotide insertion in BRCA1 (5382insC; rs80357906). We demonstrate that the PSI-RIFTS method not only can efficiently detect both point mutations, but it also showed reusability and was stable for 14 days, suggesting its potential for scaling up.

2. Materials and methods

2.1. Preparation of the optimal PSI surface

P-type silicon wafers with resistivity of 0.5 Ω cm and thickness of 800 μm were used in the (100) crystallographic orientation. For optimal DNA biosensing, the cleaning process and the parasitic layer elimination procedure was undertaken as previously reported by us [26]. The PSI surface was then electrochemically processed in an electrolyte solution with 35:50:15 proportions of 38–40 wt. % HF: 97 wt. % ethanol: DI water, with a current density of 70 mA/cm² for 300 s.

2.2. Functionalisation of the PSI surface

2.2.1. Oxidation

In the electrochemical process of PSI surface optimisation, the hydrofluoric acid (HF) reaction causes the PSI surface to become extremely active and forms hydrogen bonds (H-bonds) such as Si–H, Si–H₂ and Si–H₃. These H-bonds are not stable in environmental conditions due to the exchange of hydrogen bonds with oxygen groups, resulting in surface oxidation [23]. To stabilize and also hydrophilicise PSI surfaces, which is an essential criterion for biosensing applications, samples were exposed to hydrogen peroxide (35%, v/v; Merck, Germany) in a dark room at room temperature (RT) for 90 min [26,27]. The hydrogen peroxide-treated PSI wafers were then rinsed thrice with deionized water and subsequently dried.

2.2.2. Linker addition

To couple oxidized PSI with bio-molecules, 3-aminopropyl-triethoxysilane (APTES) and glutaraldehyde (GA) are commonly used as efficient linking agents [22,25,27-31]. Since both APTES and GA have sub-nano sizes (0.8 nm and 0.7 nm respectively), they can readily penetrate into the pores and produce uniform thin layers on the internal surface of PSI [27,32]. Oxidised PSI samples were thus immersed in 5% APTES (Merck, Germany), in a water/methanol mixture (1:1, v/v) for 20 min at RT. The samples were then rinsed with deionized water and then baked in the oven at 110 °C for ten minutes to maximize crosslink between the Si group of APTES and Oxygen groups on the PSI surface. Next, the PSI samples were immersed in 2.5% GA (Merck, Germany), diluted with 20 mM HEPES buffer (pH 7.4), for 30 min and were finally rinsed thrice with deionized water to remove excess GA [33].

2.3. Genotyping

2.3.1. Selection of genomic variants

For the PSI chip to be functionally relevant we sought representative variants which were not only common in frequency in the human population, but were also shown to have an established causal effect on complex genetic traits. Among the many common complex diseases, we chose breast cancer given its high rate of fatality [34]. For this, we chose BRCA1 as the most high-penetrance gene in breast cancer development and among the many functionally deleterious variants reported in this gene, we identified a loss-of-function insertion variant (c.5302insC; rs80357906) resulting in a frameshift which is found to be common in many populations including Iran [3,4]. To represent a functionally important common trait, we analysed pharmacogenetically-relevant variants and identified a common variant in VKORC1 (−1639G > A; rs9923231) which has been approved by FDA as a key variant in adjusting warfarin dose administration [35]. To obtain appropriate oligonucleotide (oligo hereafter) sequences carrying these variants, we used the oligo design of most commercial DNA chips (e.g. Roche) [36] which are 30–31 nucleotides in length and the variant is placed at the median nucleotide position. Accordingly, we designed two oligo pairs with each pair representing the ancestral and derived alleles at each locus. To check the specificity of the designed oligos, BLASTn was used to check their level of complementarity with other regions of the human genome. All four oligos were obtained in a modified form carrying an amine (NH₂) at the 5’ end essential for immobilization.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligo modification</th>
<th>Ancestral allele</th>
<th>Derived allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1</td>
<td>NH2-modified</td>
<td>5NQ2GACAGGAAGAAATGGCCAAGAGAAAGGTA3</td>
<td>5NQ2GACAGGAAGAAATGGCCAAGAGAAAGGTA3</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>5TAACTTCTTGCTGGATTCCTTGCTC3</td>
<td>5TAACTTCTTGCTGGATTCCTTGCTC3</td>
</tr>
<tr>
<td>VKORC1</td>
<td>NH2-modified</td>
<td>5NHTGTGCAGGCACAGAGATGGCTCTGGGCTC3</td>
<td>5NHTGTGCAGGCACAGAGATGGCTCTGGGCTC3</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>5GGGAGGCCCCAGACACCAGTGCTCTGGCTGACAC3</td>
<td>5GGGAGGCCCCAGACACCAGTGCTCTGGCTGACAC3</td>
</tr>
</tbody>
</table>
details of the oligos, see Table 1. An additional four unmodified oligos complementary to each of the four modified oligos were also obtained. All oligos were HPLC purified and synthesized by Macrogen (Seoul, South Korea).

2.3.2. Immobilization of modified oligo probes

All modified oligo probes representing the ancestral and derived alleles of both genes were immobilized by linkers on the surface of the functionalised PSI surface. Briefly, 5 μl of each probe (50 μM) was placed on the post-GA PSI surface. Samples were then incubated at 37 °C and 40% relative humidity for 2 h to allow covalent attachment of modified oligos without significant evaporation of the probe solution. Finally, the prepared samples were thoroughly rinsed with deionized water thrice to remove excess mobile DNA [25].

2.3.3. Allelic detection and genotype calling

The unmodified target oligo probes were allowed to hybridize on the surface of PSI with immobilised probes (PSI-IP hereafter). Briefly, PSI-IP samples were exposed to 5 μl of target probes (50 μM) and incubated at 37 °C for 20 min in humidified conditions. After the incubation period, samples were rinsed thrice with deionized water to remove unhybridized target probes. To create a mismatch hybridization, the unmodified probe of the ancestral allele was hybridised with the immobilised derived allele probe and vice versa. To ensure that the signals detected using the PSI-RIFTS method were true positives and representing the interference signal from the hybridization of complementary target probes, the unmodified ancestral probe of one gene was used as a fully non-complementary negative control (i.e. non-target probe) for both alleles of the other gene.

2.3.4. Test of PSI-IP reusability

Given that the PSI-IP are covalently attached to the base and hybridized target oligo can potentially be removed without any change to the base, we tested the reusability of the PSI-IP base with 50μM fresh target molecules. For this purpose, PSI-IP samples were incubated at 97 °C for 30 s, 2 min and 4 min to identify the optimal incubation time for complete separation of target molecules, and then rinsed thrice with deionized water and subsequently dried.

2.4. Characterization of the PSI surface

2.4.1. Field emission scanning Electron microscopy (FE-SEM)

A field emission scanning electron microscope (FE-SEM, Hitachi S-4160, Japan) was used to characterise the PSI surface and estimate both the pore size distribution and the porous layer thickness. The diameter and distribution of the pores were computed by the ImageJ software (ImageJ; National Institutes of Health, Bethesda, MD, USA).

2.4.2. Contact angle measurement

The contact angle (CA) was measured to evaluate the level of hydrophilicity of the surface of the PSI samples by using water droplets (volume of each droplet is 4μl) and a charged couple device (CCD) camera connected to a computer (SharifSolar, Iran). For each sample, initially three droplets were placed on different parts of the PSI surface. Next, the contact angle of each droplet was measured and the mean angle for each sample was reported as the representative CA of a given PSI sample.

2.4.3. Reflectometric interference Fourier transform spectroscopy (RIFTS)

RIFTS was implemented by using a tungsten lamp illuminating the PSI surface via an optical fibre. A collimator was then used to collect the reflected beams using an objective lens coupled with a multimode fibre directed into a spectrophotometer (EPP2000-HR, StellarNet, USA) at a spectral resolution of 0.5 nm. The value of effective optical thickness (EOT), which is equal to 2 nl (i.e. the product of the refractive index (n) and thickness (d) values of the porous layer [15], was calculated by the Fourier transformation of the reflectance spectra and computed with the IGOR software (Wavemetrics Inc., Oregon, USA).
2.4.4. Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectra of samples were obtained using a FTIR spectrometer (Bruker Optics, USA) equipped with a horizontal attenuated total reflectance (ATR) accessory (MIRacle, PIKE Technologies, USA) to characterize the surface chemical groups of PSi samples. The transmittance spectra were recorded in the wavenumbers ranged between 550 and 4000 cm⁻¹.

3. Results and discussion

3.1. Surface morphology and pore diameter distribution of fresh PSi

Since surface roughness and pore diameter distribution are two important variables for efficient biosensing detection [26], we took FE-SEM images of the fresh PSi samples. The images of the surface (Fig. 1a) and the cross section (Fig. 1b) of the porous layer showed that uniform straight pores were generated on the surface. The pore diameter distribution (estimated based on Fig. 1a) is depicted in Fig. 1c. Diameter of pores was in the range of 5–40 nm with mean 16.15 ± 5.95 nm. However, the majority (76%) of the generated pores were 10–20 nm wide, displaying a satisfactory level of uniformity.

3.2. Functionalization

Given that the generated fresh PSi met the essential criteria for biosensing detection, we subsequently took three steps to functionalize the surface (Fig. 2a) [33,37]. A common characteristic of Fresh PSi surfaces is hydrophobic behaviour. This hydrophobicity is mainly due to the presence of Si-Hx bonds on the PSi surface [14]. Prior to functionalization, we therefore examined surface wettability of the generated fresh PSi by contact angle (CA) measurement. The water contact angle was 120°, confirming the hydrophobicity of the generated PSi surface. Next, we used the CA method as an indirect, convenient approach to verify each functionalization step (see Fig. 2b). Initially, the surface was oxidized using hydrogen peroxide. CA analysis showed a substantial decrease in the water contact angle to 35°, consistent with

Fig. 2. a) The schematic view of PSi surface functionalization, b) water contact angle (CA) c) RIFTS and d) FTIR spectra of fresh PSi surface and surface functionalization in a stepwise manner, Inset in c: Changes in EOT magnitude after each functionalization step.
efficient oxidization of the surface which gives it its hydrophilic nature. The surface was next treated with APTES to salinize the surface, increasing CA slightly to 48° due to the presence of long hydrocarbon chains on the surface. Finally, after GA binding in the last functionalization step, the contact angle decreased to 43° mainly because of the presence of long hydrocarbon chains on the surface. CA analysis not only detected the effect of each functionalisation step, but it also showed that the functionalized surface is sufficiently hydrophilic and thus suitable for biosensing.

We also undertook RIFTS to examine the effect of the functionalization steps on the surface of the fresh PSi. As an example, Fig. 2c (main plot) shows the RIFTS spectra of fresh and stepwise functionalized of one of PSi sample and the inset provides the corresponding EOT values after each functionalization step. Post-oxidation decreases EOT values significantly (from 31,579 ± 323 nm to 30,218 ± 293 nm) due to the decrease in the refractive index of the PSi layer, which is a consequence of the oxidation of Si atoms to silicon oxide. However, after stepwise treatment of the surface with APTES (EOT: 30,984 ± 81 nm) and GA (EOT: 30,976 ± 94 nm), we observed an increase in EOT. This EOT shift is likely to be due to the substitution of air molecules in the pores with APTES or GA, which in turn increases the refractive index of the PSi layer. Overall, these observations, in agreement with other studies [13,15], showed consistency with the results of CA analysis, further confirming that the surface has been successfully functionalized.

In parallel to CA measurements and RIFTS analysis, Fourier-transform infrared spectroscopy (FTIR) was performed to identify the chemical bonds as well as functional groups of PSi samples. According to Fig. 3, the peak at 2110 cm$^{-1}$ in freshly etched PSi related to Si–H groups and was removed with oxidation process. The peaks around 3400 cm$^{-1}$ is assigned to the O–H stretching vibration. APTES functionalization disappeared the O–H stretching vibration and appeared the CH$_2$-stretching bands at 2930 cm$^{-1}$. It seems that the weak peaks between 1300 cm$^{-1}$ and 1450 cm$^{-1}$ belongs to the CH bending bands. In addition, peaks at 1550 cm$^{-1}$ and 1630 cm$^{-1}$ belongs to NH$_2$-stretching bands after APTES functionalization. Finally, glutaraldehyde conjugated of PSi showed the appearance of a new broad peak at about 1560 cm$^{-1}$, which corresponds to N=C stretching group and aldehyde carbonyl group in 1720 cm$^{-1}$. Moreover, functionalization by GA amplified the CH$_2$-stretching and bending bands.

3.3. DNA detection

Based on RIFTS, an observed shift in EOT ($\Delta$EOT) can be attributed to DNA hybridization and may therefore be used for DNA detection on a functionalized PSi surface. After immobilizing the target amine-modified oligos, $\Delta$EOT was calculated as a function of the concentration of complementary DNA for the oligos representing the ancestral alleles of both $BRCA1$ and $VKORC1$ (see Fig. 3). The function of both genes showed a strong fit to a logarithmic growth curve ($R$ = 0.9204 and 0.9473 for $BRCA1$ and $VKORC1$ respectively). It seems that after the 50 M complementary DNA for both gen, the sensor response curves approached a plateau and became asymptotic, which indicates that almost all of the active sites have been saturated. Interestingly, when we removed the gene labels, the function based on all data points showed a highly similar trend and fit well to a logarithmic growth curve ($R$ = 0.9332). This suggests that $\Delta$EOT is a function of complementary DNA concentration, regardless of the sequence composition of the target DNA. Given the saturation pattern for both genes and our aim to achieve maximum $\Delta$EOT for DNA detection, we selected the concentration of 50 M for allelic detection and genotyping.
3.4. Genotyping

For genotyping, it is essential that the DNA detector can differentiate between the ancestral and the derived alleles by measuring a response variable. Here, we examined whether $\Delta$EOT could be used not only to detect DNA with different sequence composition, but to also detect different sequences with single nucleotide changes. Interestingly, both the 5302C insertion in BRCA1 (Fig. 4a) and the -1639G > A SNP in VKORC1 (Fig. 4b) were differentially detected from the complementary DNA. Also, importantly, the mismatch sequence was differentially detected from a totally non-complementary DNA. This demonstrates the power of this label-free PSI-RIFTS method in detecting common single nucleotide variants. This suggests the high sensitivity of this method in detecting complete hybridization as quantified by $\Delta$EOT. However, even though hybridization with a non-complementary probe is differentiable from both mismatch and full-complementary probes, the specificity of this approach needs to be rigorously assessed based on a large set of samples before it can be potentially used for diagnostic purposes. The negative $\Delta$EOT observed for NTC is expected since dissolution or oxidation of the PSI surface results in a decrease in the thickness of the porous layer [38]. Although, this negative value is small, we believe that this can be eliminated or become infinitesimal by more carefully controlling the surface functionalization steps.

The refractive index of DNA layer is given by [39]:

$$n^2 = K = \sum \frac{\varepsilon - \varepsilon_0}{E} = \frac{N\mu}{E}$$

(1)

where $n$, $K$, $\varepsilon$, $\varepsilon_0$, $\vec{E}$, $\vec{F}$ and $N$ represent the refractive index, dielectric constant, permittivity, permittivity of free space, electric polarization, applied electric field, induced dipole moment and the number of dipole moment in volume respectively. Any changes in DNA molecules (e.g., linear vs. supercoil, single vs. double-stranded, mutant vs. not mutant) redistributes the electric charge over molecule and changes its dipole moment. According to Eq. (1), this revision alters the refractive index,dielectric constant and, permittivity of DNA molecules. The observations of higher dielectric constant [40,41] or higher permittivity [42] for double-stranded DNA compare to the single-stranded DNA are acceptable by foregoing discussion. In addition, it is reasonable to detect the contrast in dispersion curve (dielectric constant vs. frequency) for partially denatured DNA compare to double helix or supercoil DNA [43]. As the same way, it is expected that the refractive index of our samples which contains not mutant DNA differ from samples with mutant ones (Fig. 4).

3.5. Multiple sample reusability

To examine the reusability of the functionalized PSI chip to genotype multiple genomic samples, which is essential for an efficient DNA detector, we first allowed hybridization of target DNA as in the genotyping step and measured EOT before and after hybridization step. We then completely denatured the double stranded DNA by heating in a furnace at 95 °C for several PSI chips for a range of different time periods (30 s – 240 s). The reflection measurements showed that a denaturation time of four minutes gives optimal results which EOT’s reaches to initial values with an approximation of less than 1%. By using this optimal denaturation process, several reusability test (50 μM complementary BRCA1, 50 μM complementary VKORC1, 50 μM SNP, 50 μM non-complementary VKORC1 and 6.25 μM complementary VKORC1) were performed and $\Delta$EOT (EOT’s before and after the second hybridization process) in each case were measured. Fig. 5 compares the change in effective optical thickness for the first time and reuse tests in each case. As illustrated, the reuse tests did not show any significant change in the level of observed AEOT. In addition, we investigated the reusability of the PSI chips after being refrigerated for two weeks for the both genes given that no differential pattern was observed between the two genes in the previous test. The AEOT measurements showed that the PSI chips retain their activity.

3.6. Advantages of the PSI-RIFTS method for label-free biosensing

Hitherto, label-free point mutation detecting biosensors have been reported by different techniques in the literature. Biosensors based on field-effect transistor (FET) [44,45], quartz crystal microbalance (QCM) [46], surface plasmon resonance (SPR) [47] and nano-gap array [48] are examples of such techniques with high sensitivity and selectivity. However, the fabrication process of FET devices and nano-gap arrays is costly, and the SPR method requires expensive signal detection equipment, making these approaches less attractive. In addition, QCM is highly sensitive to environmental conditions (especially temperature) and is relatively complex to construct and operate. Although, the sensitivity of our method falls behind some of the aforementioned methods, further refinement of the current PSI base by constructing porous silicon photonic crystals (i.e. a multilayer periodic structure, which has a narrow peak in its reflection spectrum) will allow higher sensitivity and selectivity [22,49]. Moreover, as these structures reflect specific light wavelengths often yielding bright and vivid colours, they can be used in colorimetric sensing which is a more convenient and cost-effective method than RIFTS. We thus believe that such improvements on the PSI base will result in a biosensor which can not only efficiently detect point mutations, but is also simple and cheap to run, and has the potential to be used for numerous samples. Specifically, with respect to the latter, the PSI-IP may be re-used several times to genotype different samples, making this method an attractive platform for genotyping.

4. Conclusion

We report a PSI-RIFTS method for genotyping point mutations in multiple samples. We suggest that the genotyping an array of insertion and substitution variants across the genome should be undertaken to examine whether consistent results are obtained regardless of the sequence composition of probes. Also, importantly, the PSI base should be designed to detect the alternate alleles simultaneously as an efficient genotyping platform. It would therefore be essential to test it on sheared extracted genome samples of mutation-positive individuals to validate the method. Such a validation will be pivotal for its use as a standard genotyping method especially for molecular diagnostic purposes.

Acknowledgments

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References