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Biosensing of BCR/ABL fusion gene using an intensity-interrogation surface plasmon resonance imaging system

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ABSTRACT

In this work, a custom-made intensity-interrogation surface plasmon resonance imaging (SPRI) system has been developed to directly detect a specific sequence of BCR/ABL fusion gene in chronic myelogenous leukemia (CML). The variation in the reflected light intensity detected from the sensor chip composed of gold islands array is proportional to the change of refractive index due to the selective hybridization of surface-bound DNA probes with target ssDNA. SPRI measurements were performed with different concentrations of synthetic target DNA sequence. The calibration curve of synthetic target sequence shows a good relationship between the concentration of synthetic target and the change of reflected light intensity. The detection limit of this SPRI measurement could approach 10.29 nM. By comparing SPRI images, the target ssDNA and non-complementary DNA sequence are able to be distinguished. This SPRI system has been applied for assay of BCR/ABL fusion gene extracted from real samples. This nucleic acid-based SPRI biosensor therefore offers an alternative high-effective, high-throughput label-free tool for DNA detection in biomedical research and molecular diagnosis.

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1. Introduction

In the past decade, radioactive, enzymatic, and luminescent compounds have been widely used as sensing probe. However, these labeled probes suffer from different disadvantages coming from radiation, storage, and disposal [1]. Alternatively, the probes used in surface plasmon resonance (SPR) do not need any more special treatment before using. Hence, SPR sensor has received much attention as a label-free method for the biological molecules detection, including DNA [2,3], low molecular weight proteins [4] and antibody/antigen [5]. However, most experiments based on SPR biosensors are single sample analysis or single channel analysis, which is not possible for multi-component analysis. Therefore, the development of a sensitive, label-free and high-throughput detecting technique based on SPR is highly desirable [6].

SPRI is an advanced SPR technique, where the biological interactions can be monitored in the formation of array-based detection of change in pixel intensity. Parallel analysis of many

interactions can be analyzed simultaneously from the spatially resolved functionalized metallic surface [7,9]. The variation in the reflected light intensity is proportional to the change in the refractive index due to molecular binding [10,11]. In this way, both sensorgrams (i.e., resonance signal vs time) and real-time images of the sensor chip can be simultaneously recorded for high-throughput analysis [12]. Many studies have been done to improve the SPRI performance, such as nano-structured SPR biosensor [13] and SPR phase detection techniques [14]. Various SPRI devices have been developed to implement hybridization-based sensing, including miRNA/DNA detection [15], bacterial genotyping and SNP identification [16,17]. However, their detection sensitivity is not as high as the traditional angle-interrogation SPR biosensor while maintaining high-throughput detection. Thus, it is necessary to further explore highly sensitive biosensing strategy for detection of DNA by improving a custom-made intensity interrogation SPRI system performance.

Chimeric BCR/ABL fusion gene is involved and plays a crucial role in the evolution of chronic myelogenous leukemia (CML). Determination of BCR/ABL fusion gene is important for early diagnosis, prognosis, and even better for CML patients during the treatment [18]. Nowadays, several methods have been applied to recognize BCR/ABL fusion gene, such as flow cytometry (FCM) [19], fluorescence in situ hybridization (FISH) [20] and real-time

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quantitative reverse transcription polymerase chain reaction (RT-PCR) [18]. These attractive methods are able to efficiently and accurately detect BCR/ABL fusion gene. However, specialized facilities and highly trained personnel are usually required for these techniques. Therefore, direct detection of BCR/ABL fusion gene with the aim to develop a fast SPRI screening method is of great significance for clinical diagnosis.

We here report a sensitive SPRI biosensor with gold arrays chip and its application for the detection of BCR/ABL fusion gene. The incident angle used to excite surface plasmon resonance was optimized to improve the homogeneity of pixel intensity. Under optimum conditions, the nanomolar target BCR/ABL fusion gene could be distinguished from the non-complementary DNA. This SPRI biosensor exhibited an excellently distinguishing capability and was applied for assay of longer DNA chain at about 600 bp in PCR real samples with satisfactory results. By adopting LED light source, high resolution CCD system and appropriate sensing chip, we have achieved high sensitivity detection of ssDNA without sensitivity compromise. The detection limit was much better than other custom-made SPRI biosensors [21,22]. However, there is still much room for improvement, compared with these using high-precision piezo ceramic motor and laser [23]. More fundamental insights could be provided by this cheap and flexible method during the analysis of proteomics studies, biomarker screening and clinical diagnosis at clinically low levels in sample matrices [24,25].

2. Experimental

2.1. Reagents

DNA oligonucleotides were synthesized by Sangon Biotech (Shanghai, China). The sequences are listed in Table 1. All oligonucleotides were dissolved in TE buffer (10 mM, Tris-HCl, 1 mM EDTA, pH 8.0) to attain stock solutions of 10 μ M, and diluted to the desired concentration with appropriate buffer prior to use. 6-Mercapto-1-hexanol (MCH) was purchased from Sigma-Aldrich (St Louis, MO, USA). All other reagents were of analytical grade. All aqueous solutions used in the experiments were prepared with Milli-Q water (≥ 18 M Ω , Milli-Q, millipore).

2.2. Fabrication of gold island array SPRI microchip

Prior to vapor deposition, the gold substrate-BK7 glass slides were thoroughly cleaned in a piranha solution ($\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2=3:1$), extensively washed with deionized water and ethanol, and dried with nitrogen. BK7 glass slides were then patterned with a photomask, which was made up of 800 μ m diameter circles with 1.6 mm center-to-center spacing. Then 2 nm chromium was deposited on the patterned substrate as a basic adhesion, followed by deposition of a 51 ± 2 nm thickness gold film via e-beam

evaporation and removal of photomask. Finally, the glass substrate immobilized with an array of gold cylinder, whose diameter is 800 μ m and height is about 51 nm, was used as sensor chip. The microarray chips of gold cylinder substrates shown in Fig. 1 (a) were stored in a vacuum desiccator until use.

2.3. SPRI setup

A single channel SPRI sensor platform (Fig. S1), based on the Kretschmann geometry of attenuated total reflection method, was developed to investigate the ssDNA hybridization. In this setup, an expanded and polarized beam with a central wavelength of 650 nm from an InGaAlP LED (Thorlabs, USA) is collimated and reflected from the base of an equilateral BK7 triangular prism ($n=1.616$, Thorlabs, USA) where an SPRI chip is attached. A flow-cell (50 μ L) with a separated flow chamber mounted on a 360-degree rotation stage (Thorlabs, USA), is interfaced with the sensor chip to confine the sample during experiments. A rotatable sheet polarizer (Oriel, USA), which can select the polarization of light between p and s, is utilized to excite the surface plasmons. A KDS-200 syringe pump (KD Scientific, Holliston, MA, USA) is used to deliver liquid samples over sensing channel. The light reflected from the sensing channel on the sensor chip surface is collected and delivered to a cooled 24-bit CCD camera (Retiga 2000 R from QImaging) with a resolution of 1600×1200 pixels, $7.4 \mu\text{m} \times 7.4 \mu\text{m}$ pixel size. The acquired real-time images are streamed to computer for grayscale analysis by lab-developed software written in Labview. In order to minimize the effect of background and dust adsorption, a differential image was introduced (Fig. 1(d)) by subtracting s-polarized (Fig. 1(b)) from p-polarized (Fig. 1(c)) images for further analysis. Regions of interest (ROI) corresponding to individual gold cylinder were defined. The SPRI signal was monitored with the CCD camera, and the reflectivity changes (ΔR) of each ROI were recorded and plotted upon time.

2.4. Immobilization of capture probes

Prior to capture probe immobilization, the bare gold chip was cleaned with fresh piranha solution for 10 min to eliminate adsorbed organic impurities. Then, the pretreated gold chip was washed thoroughly with Milli-Q water and pure alcohol, followed by drying under nitrogen. Immobilization solution was injected into the flow cell at the flow rate of 0.05 mL min^{-1} . When a steady state baseline was reached, 200 μ L 1.0 μ M solution of thiolated DNA probe in immobilization solution (1 M KH_2PO_4 , pH 3.8) was injected in the flow cell by the fluid delivery system at a flow rate of 0.1 mL min^{-1} . They were then incubated for 5000 s at room temperature. The chip was undocked and washed with milli-Q water, then incubated in 1.0 mM MCH blocking solution at room temperature for 2 h in the dark. After washed with ethanol and deionized water, the chip was dried by nitrogen gas. Finally, the chip was remounted on SPRI system and ready for hybridization analysis.

2.5. SPRI real-time monitoring

Fig. 2 is a cartoon illustration of real-time monitoring process and the increase of pixel intensity when ssDNA is adsorbed onto the gold microarray. The SPRI biosensor was measured at an angle with the highest sensitivity and images were collected at a rate of one frame per 10 s. The gold array chip functionalized with thiolated capture probe/MCH was used for DNA detection. Hybridization solution (30 mM Na_3PO_4 , 450 mM NaCl, 3 mM EDTA, 0.25% Triton $\times 100$, pH 7.4) was used as the running buffer in the experiment. After the signal of sensor reached a steady state, the

Table 1
DNA sequences employed in this work.

Oligonucleotide	Sequence (5'–3')
Capture probe	SH-(CH ₂) ₆ -AAAAAAGAGTTCAAAAGCCCTTC
Detection probe	GAAGGGCTTTTGAAGTCT
Non-complementary sequence	GCAAAGAAACAGATCCCATG
Primer (forward)	GCAAGCTTACCATGGACATCCGTGG
Primer (reverse)	GTCGACCTTGCCATCAGAAGC
β -actin primer (forward)	CTGAAGTACCCATCGAGCACGGCA
β -actin primer (reverse)	GGATAGCACAGCTGGATAGCAACG

The italic portion of capture probe represents complementary sequences to detection probe.

Fig. 1. Raw and differential SPRI images of microfabricated gold cylinder arrays chip. (a) Image of the fabricated microarray chips. (b) SPRI images of chip immersed in water illuminated by s-polarized lights. (c) SPRI images of chip immersed in water illuminated by p-polarized lights. (d) The differential image of chip processed by subtracting s-polarized from p-polarized images.

solution of DNA diluted with hybridization buffer was injected in the flow cell and flowed for 20 min. Then the chip surface was rinsed with the running buffer to wash away the free DNA. The sensor surface was regenerated with the regeneration solution (50 mM NaOH) to remove the binding analytes on the gold film for the next measurements. Three replicate tests were carried out to the same sample and the average value was calculated. The binding/regeneration processes on the chip was repeated 20 times to assess the reusability of the sensor chip. All experiments were carried out at a flow rate of 0.01 mL min^{-1} at room temperature about 25°C . The noise of SPRI sensor was measured to be 0.7, as shown in Fig. S2.

2.6. Preparation of DNA sample and PCR amplification

K562 cells, the mostly used cell strain of CML, were kindly donated from the First Affiliated Hospital, Chongqing Medical University. Total RNA was extracted using mini DNA-free total RNA extraction kit and cDNA was obtained by applying a reverse transcription method from total RNA [26]. PCR was performed in a final volume of $50 \mu\text{L}$ containing $5.0 \mu\text{L}$ of cDNA, $1.0 \mu\text{L}$ of $20 \mu\text{M}$ each primer, $25 \mu\text{L}$ of Premix Taq (1.25 U of DNA polymerase,

$2 \times$ Taq buffer, 0.4 mM of dNTPS) and $18 \mu\text{L}$ of water. BCR/ABL DNA was initially denatured at 94°C for 5 min, followed by 34 cycles of 94°C for 30 s (denaturation), 51°C for 30 s (annealing), 72°C for 40 s (extension), and final 5 min extension. PCR products were identified by running 10 mL of PCR mixture in 1.5% agarose gel for 15 min and observed under ultraviolet light. PCR products were denatured by heating at 100°C for 7 min in a water bath, and immediately chilled in ice for 5 min to obtain denatured ssDNA for the next detection.

3. Results and discussions

3.1. Homogeneous pixel intensity

Homogeneous pixel intensity can improve the image quality and data reliability. A fixed angle monitoring scheme can eliminate the bias produced by the shift of the imaging beam projecting on the camera [27], but it is not easy to obtain high horizontal resolution image even a high resolution image acquisition device or a micro sensor chip is employed [8]. As shown in Fig. 3(a), the images of gold islands become more and more blurry along the direction of

Fig. 2. Cartoon representation of ssDNA hybridization onto gold microarray spots and image of pixel intensity increase.

arrow. Brighter circles in the regions of gold islands edge from A to H were generated by light diffraction, which could be seen in the image (Fig. 3(a)) and their corresponding line profiles (Fig. 3(b)) [28,29]. Black arrow indicates the increase of luminance.

At the beginning of optical alignment, CCD camera was focused on the center of gold film at point O shown in Fig. S3. However, focus difference between point A (B) and point O would decrease the image quality and thus the inhomogeneous distribution and interference fringe was slightly magnified, which made the choice of ROI for analysis difficult. According to the refraction law, the focus difference (l_1) from point A (B) to O is calculated as

$$l_1 = l_{AB} \times \sin \theta, \quad (1)$$

where θ is the incident angle, l_1 is the focus difference, l_{AB} is distance between the point A and point B on sensor chip. Hence, a smaller incidence angle θ must be adopted to reduce the focus difference and approach a higher image quality.

Fig. 4(c) is 3D SPRi image of the gold island. Each projection represents a gold island, and the bump height represents gray value of each island. The differences of gray values among these 48 gold islands are less than 9.7%. Hence, this image is acceptable to be regarded as homogeneous.

3.2. Selection of optimum incident angle

Angular interrogations methods are able to be implemented on this SPRi sensing system to measure the changes in reflectivity (ΔR) when the material adsorbs upon or detaches from the gold film surface. The reflected light intensity recorded at the incident angle ranging from 55° to 70° is plotted in Fig. 4 under the condition of running buffer. A strong dip at 63.5° , called SPR angle, is

observed in the reflectivity curve. Due to the adsorption of capture probe onto the gold film, the shift of resonance angle to a higher angle is the basis of detection [5]. The sensitivity S of this the SPRi instrument is defined as

$$S = \Delta R / \Delta \theta, \quad (2)$$

where ΔR is the change in the reflectivity and $\Delta \theta$ is the shift of angle. The incident angle at the sharpest slope of reflectivity curve can provide the maximum sensitivity [30]. However, the smaller incident angle means a larger noise because the fluctuations of light source intensity would dominate the noise. So, strong noise is usually the side product at this location and eventually leads to a dissatisfactory limit of detection (LOD).

The above described optimization procedure provides a significant compromise between sensitivity and image quality. Therefore, considering signal-to-noise ratio (S/N), the optimum monitored angle was chosen not in the steepest part of the reflection curve but near to the resonance angle at about 63° [31,32].

3.3. Optimization of experimental parameters

Probe concentration, probe immobilization time and hybridization time are the most valuable factors to affect DNA hybridization detection. In order to investigate their effects on the detection efficiency, 100 nM ssDNA was used for the parameters optimization process.

Fig. 5(a) shows the effect of probe concentration on SPRi signal response. With the increasing of probe concentrations from 0.4–1.0 μM , more probes were immobilize on the gold film surface, and the maximum response was obtained at 1.0 μM . The continuous increase of probe concentration more than 1.0 μM had

Fig. 3. (a) SPRi image of the microfabricated arrays. (b) Representative SPRi image and the line profile for the third row in the array. The length of X coordinate is corresponding to the position of the spots along the third row of gold islands shown in the a. (c) 3D SPRi image representation of gold islands shown in a.

negative effect on the SPRi signal, due to two-dimensional crowding, which leads to steric hindrance and probe–probe interactions [33]. Therefore, $1.0 \mu\text{M}$ was chosen as the optimized probe concentration.

In order to shorten measurement time while maintains the detection efficiency, the influence of probe immobilization time on the SPRi response was studied as shown in Fig. 5(b). SPRi signals increased upon the increasing immobilization time from the moment when capture probe was injected into the flowing cell to 4500 s. Longer the time for probe immobilizing on the gold surface, higher the SPRi signal could achieve. However, the SPRi signal no longer improved when immobilization time was longer than 5000 s. Therefore, 5000 s was selected as the optimal parameter for probe immobilization.

Hybridization time is another time parameter to affect the detection efficiency. The SPRi response increased gradually within

800 s at a flow rate of 0.01 mL min^{-1} once the hybridization began (Fig. 6). It approached a saturated plateau when 20 min had past. Considering the hybridization efficiency and the time required for the flow cell was filled, 20 min was selected as the appropriate hybridization time for the following experiments.

3.4. SPRi detection of the ssDNA

Different concentrations of target ssDNA were investigated under optimal experimental conditions. The measurements of target ssDNA were repeated three times on the same chip after regeneration. The sensorgrams obtained from SPRi detection are shown in Fig. 6. ΔRU shows the response of ssDNA hybridization with capture probe. After each test, the surface was completely regenerated with the NaOH (50 mM) solution to make the dynamic curve go back to the baseline for the next detections. The

Fig. 4. Reflectivity curves for the gold island chip in running buffer. The blue solid and red dashed lines are the reflectivity curve before and after the adsorption of material upon the gold film. Inset: Magnified region near the minimum of reflectivity curve minimum.

introduction of ssDNA led to a proportional increase in the SPRI signal. Fig. 7 illustrates the real-time images recorded at different ssDNA concentrations. SPRI signal increases with the increase of concentration from 0 nM to 500 nM. The relationship between the concentrations and the SPRI signals of the target ssDNA that hybridized with the probe is shown in Fig. 8. The error bars represent the standard deviation among the results of the parallel measurements. Based on the 3σ rule, the LOD for target DNA were estimated to be 10.29 nM for the direct detection. The specificity of the SPRI sensor reported here was evaluated by comparison of non-complementary DNA sequence (NC DNAs). As shown in Fig. 8, SPRI signal for target DNA gives a remarkable increase compared to that of non-complementary DNA sequence. The signal for NC DNAs is as small as that of blank. These results indicate that the SPRI sensor system exhibits excellent specificity for the determination of target DNAs.

In addition, the reproducibility of this SPRI sensor system was also investigated. Five replicate measurements of the target DNA at the concentration of 50 nM were performed, and the relative standard deviation was calculated as 12.6%, which demonstrated good reproducibility. The evaluation of reusability of this system was operated on the same chip. After 20 regenerations, the change of SPRI signal was less than 10%, suggesting the same sensor chip could be reused at least 20 times.

Fig. 5. Effect of (a) probe concentration, (b) probe immobilization time on DNA hybridization detection (1000 nM). When one parameter changed, the others under their optimal conditions.

Fig. 6. Typical sensorgram of target miRNA hybridization and regeneration.

3.5. Detection of PCR real samples

The SPRI sensor system reported here was then employed to detect real samples to prove its practicability. K562 cells and negative real sample were collected as the detecting targets. Total RNA from K562 cells and negative real sample was firstly extracted, then cDNA was obtained by applying a reverse transcription method. After PCR amplification procedure, the PCR products of K562 cells and negative real sample (lane 2 and 3) were detected by agarose gel electrophoresis showed in Fig. 9. The PCR products of K562 cells presented the light bands at about 600 bp which were corresponded to the characteristic position of BCR/ABL fusion gene. DNA concentration of K562 cell was measured by nanodrop 1000 as $699.4 \text{ ng } \mu\text{L}^{-1}$. After hybridization with BCR/ABL fusion gene from K562 and negative real sample, the SPRI signals gave the mean average signals of 1362.3 and 1326.2. These results indicated that the expression level of BCR/ABL fusion gene in K562 were up-regulated compared with that in negative real sample, which were in good agreement with result obtained from the gel electrophoresis.

4. Conclusions

In summary, a custom-made SPRI sensor system has been developed to analyze the BCR/ABL fusion gene of CML on gold island array microchips. The monitoring parameters have been optimized

Fig. 7. 3D SPRI image representations of gold arrays after hybridization. (a) 0 nM (b) 10 nM (c) 50 nM (d) 100 nM (e) 250 nM (f) 500 nM.

Fig. 8. SPRi signals recorded from capture probe and non-complementary DNA vs. different concentrations of the synthetic target (from 10 nM to 500 nM).

Fig. 9. Gel electrophoresis photo of PCR amplified products: (lane 1) DL 2000 DNA marker (the bands from top to bottom: 2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp and 100 bp), (lane 2) K562 cells, (lane 3) negative real sample + β -actin.

to improve the data quality and measurement efficiency. The detection limit of 10.29 nM was achieved by direct ssDNA immobilization on the sensing surface without amplification. This SPRi sensor system showed an excellent selectivity for different DNA sequences and had been successfully used for real-time detection of BCR/ABL fusion gene in PCR products from real sample. The simple sensing chip fabrication method allows customized sensor chip to be used for rapid, label-free and high-throughput analysis of DNA. More clinical diagnostics could be carried out on this SPRi sensor system to further extend and quantify a wider application range.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.optcom.2016.05.035>.

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