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RESEARCH ARTICLE

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Circulating microRNA breast cancer biomarker detection in patient sera with surface plasmon resonance imaging biosensor

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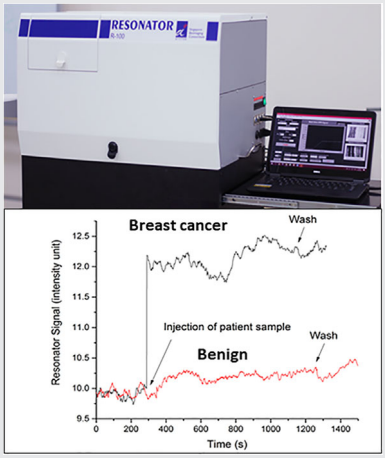
Abstract



In this article, we report for the first time, the detection of circulating miRNA as a breast cancer biomarker in patient sera using surface plasmon resonance imaging biosensor. The advantage of this approach lies in the rapid, label-free and sensitive detection. The sensor excites plasmonic resonance on the gold sensor surface and specific DNA-miRNA molecular bindings elucidate responses in the plasmonic resonance image. Experiments of detecting synthetic miRNA molecules (miR-1249) were performed and the sensor resolution was found to be 63.5 nM. The sensor was further applied to screen 17 patient serum samples from National Cancer Centre Singapore and Tan Tock Seng Hospital. Sensor intensity response was found to differ by 20% between malignant and benign cases and thus forms, a potential and an important metric in distinguishing benignity and malignancy.

KEYWORDS

breast cancer biomarker, circulating miRNA, clinical study, SPR imaging, surface plasmon resonance biosensor

1 | INTRODUCTION

World Health Organization (WHO) reports that breast cancer is the most common form of cancer among women, with 2.1 million being diagnosed with breast cancer annually. It is the cause of 627 000 deaths in 2018, making up 15% of all cancer-related deaths among women [1]. That being said, detection of breast cancer at an early stage can tremendously improve patient outcome, enabling effective treatment and reducing mortality rate [1]. Currently, mammography is the most widely used screening method for breast cancer [2, 3]. However, the rate of false-positive mammograms is shown to be high. The cumulative probability of false-positive measurement was 61% for women undergoing 10 years of routine mammography screening [4].

In the United States, the rate of false-positive mammograms stands at 22% to 31% [5], while an over-diagnosis rate of 22% is reported in Canada [6]. A study conducted by Harvard Medical School [4] shows that the economic impact of false-positive mammogram is $4 billion annually in the United States. Undoubtedly, there is an unmet clinical need for an accurate, affordable and rapid detection alternative for distinguishing between malignant and benign breast lesions for patients.

Detection of circulating biomarkers in patient body fluid is a promising alternative approach for cancer diagnosis [2]. However, to date there is no blood-based biomarkers for early breast cancer diagnosis in clinical practice [2]. Cancer antigen 15-3 (CA15-3), carcinoembryonic antigen (CEA) and cancer antigen 27-29 (CA27-29) are two currently known circulating biomarkers for breast cancer, but have been reported to lack sensitivity for early breast cancer detection [7]. Recently, clinical relevance of circulating miRNAs for breast cancer has been discussed [8] and the correlation of cancer development with expression levels of miRNAs have been found [9, 10]. In a previous study [11], miR-1249 was identified as one of the circulating biomarkers for early breast cancer detection.

In recent years, increasing research interests have been on optical sensors [12–24] and the applications in biomolecular detection [25–35]. In localized surface plasmon resonance (LSPR) biosensing [36], metallic nano-particles interact with excitation electric field and produce LSPR resonance peak shift for molecular binding interaction detection. Miti et al [37] applied gold nanoparticles based LSPR for synthetic miR-17 detection, which was the molecular biomarker for lung, breast and gastric cancers. However, hybridization chain reaction process was required for signal amplification and the detection time was near 1 hour. Bimetallic nanoparticles of gold and silver were also used for synthetic miRNA-21 molecule detection [38], however, relatively complex detection process with exonuclease III (Exo III)-assisted target recycling and hybridization chain amplification were required. Portela et al [39] recently demonstrated direct detection of lung cancer biomarker, synthetic miRNA-210 molecules. Another promising biosensing approach is surface plasmon resonance (SPR). It measures the molecular binding through plasmonic resonance excitation at a gold sensor surface. Schmieder et al [40] combined SPR biosensor and microfluidic chip for synthetic miRNA-93 measurement, but RNA-DNA-hybrid antibodies based amplification was needed. Xue et al [41] further applied a 2D material sensing layer, antimonene, on gold layer for synthetic miRNA-21 and miRNA-155 biomarkers detection. In recent years, SPR imaging has become an emerging method for real-time biomolecular binding monitoring [26, 36]. Charge-coupled device (CCD) camera is used to capture the SPR image produced by the biomolecular bindings associated refractive index changes at the sensor surface [26, 42]. The major difference between SPR imaging sensor and conventional SPR sensor is the camera based detector and plasmonic contrast based SPR imaging signal. Linear detector array is commonly used at SPR sensors, such as Biacore systems [26]. SPR imaging sensors based on intensity [42], angular [43], spectral [15], phase [44], polarization contrast [45] and spectral-phase interrogation [30, 31] have been reported. However, the reported applications in miRNA detection were limited to synthetic samples [46] and the only clinical study was on influenza detection [47]. Currently, no clinical study on cancer diagnosis has been reported to date with SPR imaging biosensor and circulating miRNA biomarkers [46].

In this article, we demonstrate for the first time the detection of circulating breast cancer biomarkers in patient samples using SPR imaging biosensor. The detection time of the sensor is clocked at 25 minutes. Specific DNA probe molecules were immobilized on gold sensor surface to capture the circulating breast cancer biomarker (miR-1249) molecules. The specific molecular binding influences the plasmonic resonance condition on the sensor surface, producing an increase at the SPR intensity signal. Such a phenomenon allows for label-free, real-time monitoring of DNA-miRNA molecular bindings. The optical design captured plasmonic contrast images from p- and s- polarization for differential SPR imaging measurement, which was different from conventional angular SPR sensors with linear array detector.

2 | EXPERIMENTAL

2.1 | Optical design of the SPR imaging biosensor

Figure 1 shows schematic of the optical sensor. He-Ne laser (633 nm) was used as the excitation light source

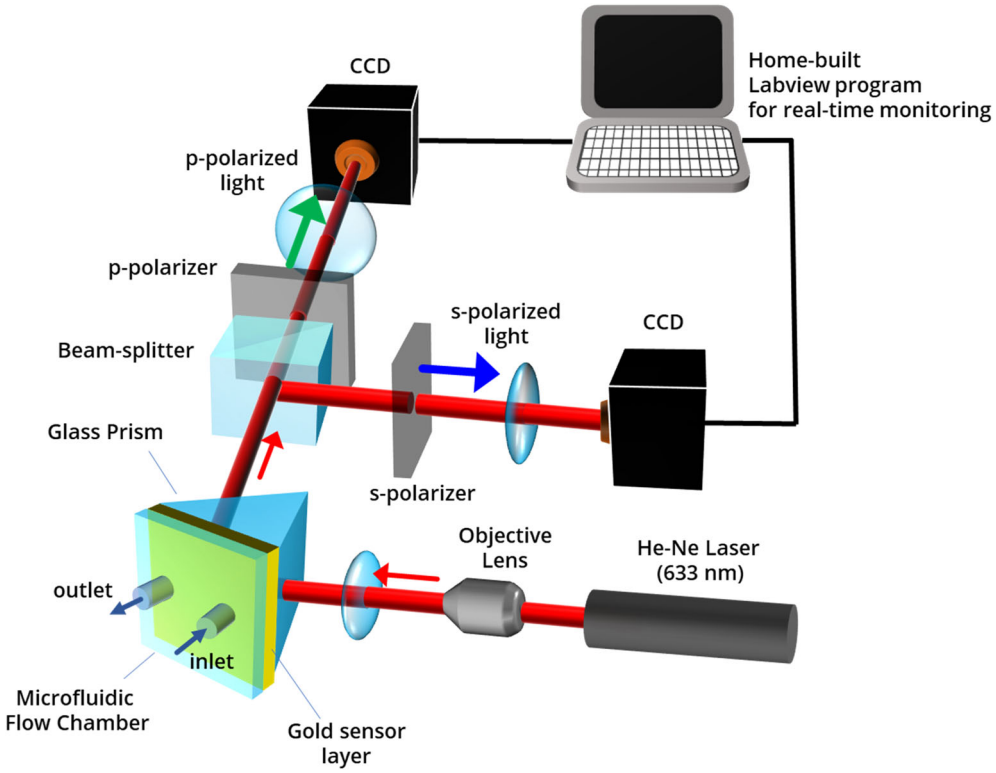


FIGURE 1 The optical schematic diagram of the surface plasmon resonance imaging biosensor

and the laser beam was collimated to 5 mm by lens. It was incident on the attenuated total reflection (ATR) surface of a glass prism, which was coated with 50 nm gold thin film for surface plasmon wave excitation. In addition, a micro-fluidic flow cell was attached for sample injection. The specific bio-molecular binding changed the plasmonic resonance condition at the gold-dielectric interface, which produced subsequent intensity variation in the reflected light. Due to the oscillation direction of the surface plasmon wave, only the p-polarized light was excited at SPR and the s-polarized light could serve as measurement reference for common noise cancelation. In addition, a beam splitter cube and two polarizers were used to split the optical beam into p- and s-polarized images, which were captured by two CCD cameras simultaneously. Real-time subtraction between the p- and s-polarization image intensities was performed with a self-coded Labview program, which also provided real-time monitoring for the sensor signal. Figure 2 shows image of the device prototype of the SPR imaging biosensor.

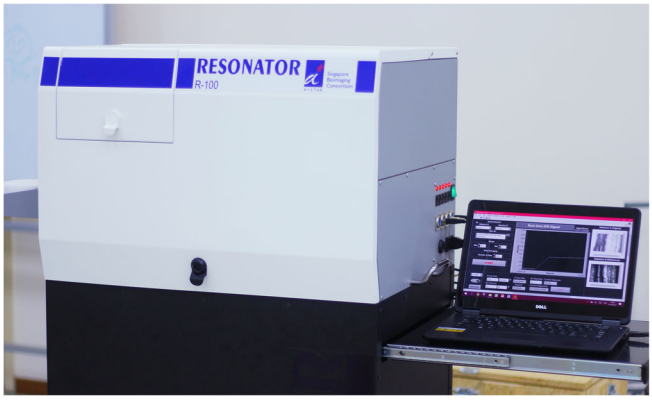


FIGURE 2 Device prototype of the surface plasmon resonance imaging biosensor

2.2 | Synthetic miRNA biomarkers

In synthetic miRNA molecules detection, surface probe DNA molecules with thiol group modification (5′-TGA AGA AGG GGG GGA AGG GCG T/5ThioMC3-D/−3′, Integrated DNA Technologies) were immobilized onto the gold sensor surface to capture and detect synthetic miR-1249 (5′-ACG CCC UUC CCC CCC UUC UUC A-3′, Integrated DNA Technologies). In addition, nonspecific miRNA, miR-3162-5p (5′-UUAGGGAGUAGAAGGGUGGGGAG-3′, Integrated DNA Technologies) was used as control.

2.3 | Study subjects

For the patient samples, total sample number was 17. Ten of them were obtained from women with malignant

breast lesions (patients with DCIS or invasive carinomas) and seven were from patients with benign ones. These were preoperative peripheral blood samples obtained from patients with abnormal mammograms from National Cancer Centre Singapore and Tan Tock Seng Hospital between 2015 and 2017. Ethics approval was obtained from Centralized Institutional Review Board (CIRB) of SingHealth (CIRB Reference number: 2018/2874).

2.4 | Serum samples processing

Patient blood samples were obtained before or during surgery in Becton Dickinson Vacutainer SST tubes. Within 50 to 60 minutes of venipuncture, the samples were centrifuged for 20 minutes (at 1300 rcf) and the serum samples were stored at −80°C. Before SPR imaging detection, total RNA including miRNA were extracted from all patient serum samples using the miRNAeasy Serum/Plasma Advanced Kit (Qiagen) according to the manufacturer's protocol.

2.5 | Serum samples for Next Generation Sequencing

The serum samples were processed with the HTG EdgeSeq miRNA Whole Transcriptome Assay protocol (HTG Molecular Diagnostics, Inc., Arizona). Next Generation Sequencing (NGS) was performed to determine the presence of miRNA in the serum samples and to examine the association between the measurement of miRNA levels from SPR and NGS. NGS is a quantitative method, it can provide quantitative read count for specific miRNA level at patient serum sample. The purified PCR products were then used for sequencing, while the control were Human Brain Reference RNA samples (Ambion, Texas). After that, the Illumina NextSeq platform was utilized for the sequencing of data libraries and 5% PhiX was spiked into the library during the process. It generated the demultiplexed FASTQ files, which were also aligned with the miRNA reference sequences from the miRbase20 database using the HTG EdgeSeq Parser. Finally, raw data counts for each miRNA were recorded.

3 | RESULTS AND DISCUSSION

3.1 | Detection of synthetic miR-1249 biomarker

The sensor was first applied for synthetic circulating breast cancer biomarker (miR-1249) detection. During measurement, sensor surface functionalized with DNA probe molecules was kept in RNase free water buffer for baseline recording. miR-1249 sample (5 μM in RNase free water) was then injected. After 15 minutes of immobilization, the sensor surface was washed with RNase free water buffer to remove nonspecific bound molecules. The measurement result is shown in Figure 3. The figure shows clear signal increase after the injection of miR-1249 sample, while nonsignificant sensor signal is recorded for nonspecific control miRNA sample (miR-3162-5p, 5 μM). During detection, there were miRNA molecules bonded on the sensor surface, while some miRNA molecules left at the same time, which produced signal variation. In addition, the minor signal drop after wash was believed to be caused by the fluidic turbulence after the washing process.

Homola et al [48] reported the equation for biosensor resolution estimation:

$$\text{Detection limit} = \frac{\text{Biomolecule concentration}}{\text{Sensor response}} \times \text{Stability} \quad (1)$$

As shown in Figure 3, the injection of 5 μM miR-1249 molecules produces 4.88 intensity unit sensor response. Figure 4 further shows the measurement standard deviation (SD) of RNase free water within 700 measurements, which is 0.062 intensity unit. According to Equation (1), the sensor resolution is found to be 63.5 nM. This value is higher than that of the miRNA-based clinical study for influenza patient at 1 nM in the literature [47]. However, our method is a label-free single step detection approach for circular miRNA biomarkers, which improves the

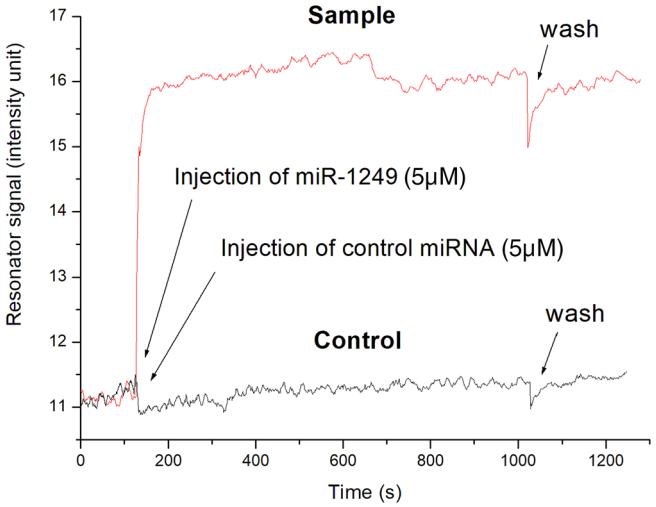


FIGURE 3 Detection results for synthetic specific and control miRNA molecules

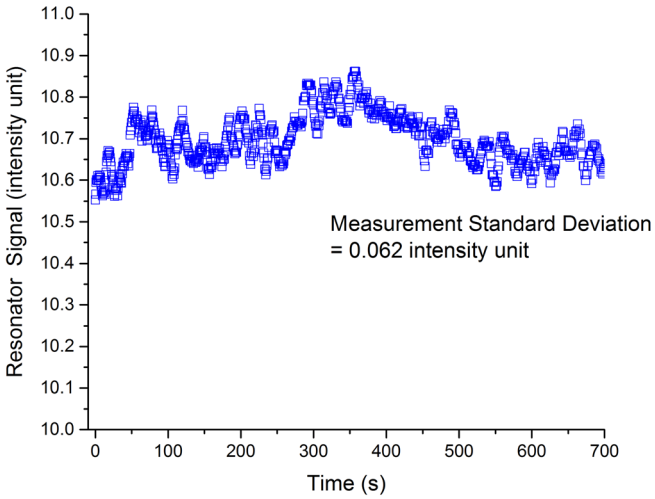


FIGURE 4 Sensor response of 700 measurement data points with RNase free water buffer

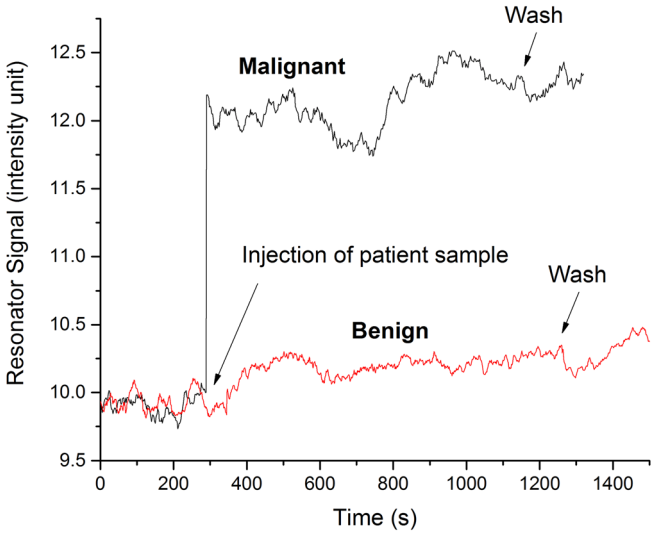


FIGURE 5 Detection results for malignant and benign patient serum samples

practical implementation for clinical diagnosis. In addition, it is the first demonstrate of SPR biosensor for clinical cancer patient sample diagnosis. Further measurement results for different concentrations of miR-1249 molecules are shown at Figure S1.

3.2 | Detection of clinical malignant and benign patient serum samples

The SPR imaging biosensor was further applied for the detection of clinical patient samples. A total of 17 preoperative peripheral blood and serum samples were from women with abnormal mammograms from National Cancer Centre Singapore and Tan Tock Seng Hospital. Ten were malignant cases, and seven samples were from women with benign breast lesions and taken as benign controls. Prior to measurement, probe DNA molecules (5′-TGA AGA AGG GGG GGA AGG GCG T/5ThioMC3-D/-3′) were immobilized onto the gold sensor surface for 1 hour, followed by a wash process. During detection, the sensor surface was kept in RNase free water buffer for baseline recording for 5 minutes, with malignant or benign serum sample injected thereafter, and the resultant DNA-miR-1249 molecular binding interactions were monitored in real-time with the SPR imaging biosensor. Figure 5 shows the measurement results for a malignant patient sample where a significant increase in sensor response (2.39 intensity unit) was exhibited after the sample injection, 86% higher than that of the benign sample. As shown in Figure 5, no significant increase in sensor signal (0.32 intensity unit) was recorded after the injection of a benign patient sample. The results demonstrate the application of SPR imaging biosensor for circulating

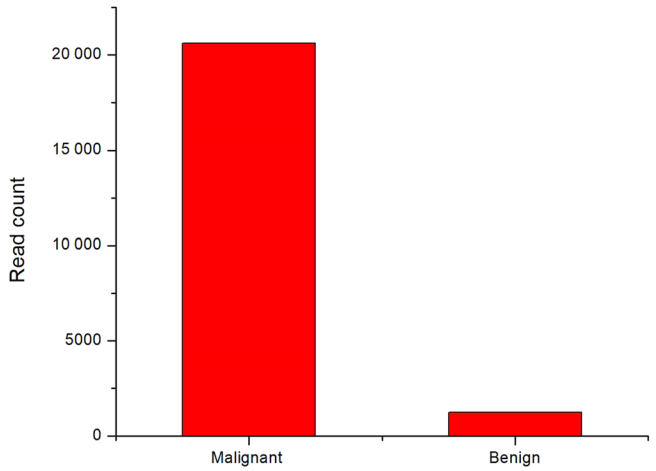


FIGURE 6 Next generation sequencing result for the same patient samples

breast cancer biomarkers (miR-1249) detection in patient serum samples. Figure 6 shows the measurement results of the same samples with the NGS method, which correlates with the findings of SPR imaging biosensor.

Further measurements were conducted for all 17 patient samples using a similar detection process. Baseline was established with 5 minutes of measurement with RNA-free water. It was followed by the injection of patient samples. DNA-miR-1249 molecular bindings were recorded for 15 minutes. The sensor intensity difference between the baseline and signals after bindings gave the response of the samples and the results are shown in Table 1. In addition, all the molecular binding curves are plotted at Figure S2. The average signal value of malignant cases is found to be 20% higher than that of the benign cases. The identification accuracy for breast

TABLE 1 Measurement results for 17 malignant and benign patient samples

	Malignant patient	Benign patient
Average sensor signal (Intensity unit)	2.92 ± 1.92	2.32 ± 1.55
Number of case	10	7

malignant patient samples can further be improved through considering multiple types of circulating miRNAs as biomarkers in future experiment. Study in [49] showed that three-miRNA signatures from serum potentially can distinguish between breast cancer patients from healthy individuals. In addition, another research on a seven-miRNA model [50] also shows positive results on early breast cancer detection.

4 | CONCLUSION

In conclusion, the detection of circulating miRNA as a breast cancer biomarker in cancer patient samples using SPR imaging biosensor was demonstrated for the first time. The sensing modality is capable of rapid, label-free and highly sensitive detection. Experiments with the synthetic biomarker molecules miR-1249 were performed, where the sensor resolution was found to be 63.5 nM. The sensor was employed for detecting circulating miRNA in 17 patient serum samples from National Cancer Centre Singapore and Tan Tock Seng Hospital. A 20% difference in sensor intensity response between malignant and benign cases was found, marking a new metric for distinguishing the two cases from each other. The detection accuracy can be further improved by detecting multiple types of circulating miRNA in future experiments.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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