A high sensitive SH-SAW biosensor based 36° Y-X black LiTaO3 for label-free detection of Pseudomonas Aeruginosa

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ABSTRACT

A label-free and high sensitive shear horizontal surface acoustic wave (SH-SAW) biosensor for the nucleic acid detection of Pseudomonas Aeruginosa was developed in this work. An effective detection cell was fabricated by dint of acrylic material. The base frequency of the SH-SAW biosensor fabricated on 36°-Y-X rotated black LiTaO\textsubscript{3} piezoelectric substrate was 210 MHz. The self-assembled monolayer was formed on the gold surface of the sensitive area, and the increase of mass loading in the sensitive area leads to phase shift of SH-SAW. In this work, the detection limit has been improved by 3–4 times than that in our previous works, which is 1 nmol/L for the nucleic acid of the Pseudomonas Aeruginosa. And the phase shifts related to detection sensitivity are also elevated to 4° after the specific binding of the ss-DNA probes and the complementary sequences. In this study, the plot of phase shift against the logarithm of concentration of Pseudomonas aeruginosa nucleic acid was found to be linear over the range from 0.1 nmol/L to 1000 nmol/L with a correlation coefficient 0.98777. The detection limit as low as 0.28 nmol/L was achieved for Pseudomonas aeruginosa nucleic acid in this paper.

1. Introduction

Pseudomonas Aeruginosa is one of the most common clinical opportunistic pathogens, ranking the top of clinical gram-negative pathogens [1]. This kind of bacteria is widely distributed in nature, which can also be found in the skin, respiratory tract and intestinal tract of the normal human beings [2]. The infection of the Pseudomonas Aeruginosa can occur in such cases as surgery, tumor and immunosuppressed patients [3]. Pseudomonas aeruginosa is also the main pathogen that causes infection in the ICU and post-burn infection. Serious infection is likely to develop into sepsis, which has a high mortality.

Since the Pseudomonas Aeruginosa has attracted much attention in the clinical situations, many techniques have been developed for its detection [4]. Both separation and purification culture methods are approved standard methods, but normally it takes 3 days to obtain the results. PCR method is also an effective way to detect pathogens. However, DNA extraction is unavoidable and necessary means need to be adopted to avoid false positives. These techniques afore mentioned are generally time-consuming, and various factors have to be taken into consideration during the experiment process [5]. Other than these traditional methods, biosensors have been used to detect bacteria [6] and it has several superior properties, such as rapid, label-free and real-time detection.

A few biosensor detection methods have been developed, for instance Quartz crystal microbalance (QCM), Film bulk acoustic resonator (FBAR), and surface acoustic wave. The sensitivity of the traditional QCM biosensor strongly depends on the thickness of the quartz chip, and QCM biosensor have low detection resolution due to low operating frequency, besides a thick substrate (0.5–1 mm) and large surface area (> 1 cm\textsuperscript{2}) are typically inevitable, leading it difficult to scale down. SAW love-mode biosensors have high sensitivity, but with the increase of film thickness insertion loss of the biosensor increases significantly, and it is difficult to set up a layer optimizing. As for the FBAR biosensors, which also have disadvantages including high fabrication cost, and difficult to combine with microfluidic techniques [7]. In contrast, SH-SAW biosensors are particularly capable of detection in
liquid environment and relatively easy to use. Moreover, it is a low-cost and low power consumption method, and wireless control can also be applied to SH-SAW biosensors. In summary, SH-SAW biosensors are designed to detect pathogen bacteria in this paper.

SAW devices are generally used in two propagation modes: Rayleigh propagation mode and shear horizontal mode. As for the Rayleigh propagation mode, particle displacement includes two components: one is the vertical component that is normal to the substrate and the other is the horizontal component that is parallel to the SAW propagation direction [8], so the movement trail of particles near the surface layer is elliptical. When the biosensors are used in the liquid phase, however, due to the normal component, most of the SAW energy dissipates into the liquid when the substrate gets contact with the surrounding liquid [9], so in this case it is not suitable for the liquid environment. In terms of the SH-SAW propagation mode, the particle displacement is parallel to the substrate, so very small portion of SAW energy can dissipate into the liquid when the liquid are added to the surface of the substrate [10]. Hence, the utilization of the SH-SAW is a better solution for biological detection in the liquid phase.

In this manuscript, a high sensitive biosensor based on SH-SAW technology is developed, which consists of high pure gold(Au) IDTs, sensitive area, electrodes, and a 36° Y-X black LiTaO$_3$ (BLT) piezoelectric substrate. For the first time, SH-SAW biosensor is applied for the detection of Pseudomonas aeruginosa nucleic acid. Compared to the congruent LiTaO$_3$ [11] that is usually used to stimulate the SAW, the BLT can obviously alleviate the pyroelectric effect of the wafers for the elimination of the generation of the electrostatic field, thus improving the yield significantly [12–14]. Additionally, a homemade PMMA portable cell including the design of the inlet/outlet ports and the liquid reservoir is selected to assist the tests. The whole system is used to detect the presence of the Pseudomonas Aeruginosa in the liquid phase, by monitoring the phase changes of SH-SAW signal when it interacts with the conjugated substance.

2. Theory

In this study, the delay line structure with a single channel was designed, which generally consists of input and output IDTs that were mounted on the 36° Y-X-LiTaO$_3$ substrate. The schematic illustration of SH-SAW biosensor chip is shown in Fig. 1 and the fabricated SH-SAW biosensor chip is shown in graphical abstract. As is shown in Fig. 1A, the SH-SAW is stimulated by the alternating electrical signal applied to the input IDTs, and then propagates along the surface of piezoelectric substrate through the delay line which lies between the input IDTs and the output IDTs [15]. The amplitude and phase velocity will vary with the change of the mass loading on the propagation path (Fig. 1B) induced by bio-probes immobilization and its reaction to the target sequence in the sensitive area [16]. The output signals of the SH-SAW biosensor are eventually obtained from the output IDTs [17], and the data is collected and processed with the aid of the network analyzer and LabVIEW software. The SH-SAW resonant frequency can be calculated by the following equation:

$$f_0 = V_s/2(a + b)$$  \hspace{1cm} (1)

Where $f_0$ is the resonant frequency of the SH-SAW, $V_s$ is the SH-SAW propagating velocity on the piezoelectric substrate, $a$ is the width of the IDTs’ fingers and $b$ is the gap between the IDTs’ fingers. Generally, a simple situation $\lambda = a = b$ is adopted, so $f_0$ can be further expressed as:

$$f_0 = V_s/4\lambda$$  \hspace{1cm} (2)

The mass sensitivity can be expressed as [18]

$$\Delta f = k \cdot \frac{\Delta m f_s^2}{A}$$  \hspace{1cm} (3)

Where $k$ is constant of the sensing system; $\Delta m$ is the mass loading; $A$ is the area.

Fig. 1 shows the working principle of the designed biosensor. Initially, the droplet is loaded in the sensitive area, and followed by the bio-probes immobilization on the surface of the bare gold through Au-S group. This leads to the change of the SH-SAW propagation velocity between the input and output IDTs, as well as phase shifts or frequency shifts of the acoustic signal. These shifts can be identified to quantify the specific hybridization of the bio-probes and its target sequences, and a pair of spare electrodes is also designed in the biosensor chip in case of the electrode damage. The image of fabricated biosensor chip is shown in Fig. 1C.

The self-assembled monolayer film is formed on the surface of the sensitive area after the F1-SH solution is pumped into the reaction cell, and then the hybridization reaction between F1-SH and F1-C starts when F1-C solution is pumped into the reaction cell.

3. Experimental

3.1. Materials and reagents

ss-DNA probe (F1-SH) and complementary sequence (F1-C) are provided by TSINGKE Biotech co. LTD (Chengdu, China), and the sequence of the PNA probe was CTRGACGCCATCACCGGCCCCGAGGA-GGAAG. The 5’ of F1-SH is modified with thiol. DNase/RNase-Free Water is purchased from TIAGEN Biotech co. LTD (Beijing, China), Phosphate buffer saline (PBS, 1 M, pH 7.4) is purchased from Sigma (St. Louis, MO) and Acetone and absolute ethyl alcohol are obtained from...
XINGHUAGUANGBO Chemical reagent co. LTD (Chongqing, China). F1-SH and F1-C are dissolved with the concentration of 1 nmol in DNase/RNase-Free Water.

3.2. Fabrication of SH-SAW biosensor

The SH-SAW biosensor with delay line structure is fabricated by a typical MEMS process, including lithography development, vacuum magnetron sputtering, and lift-off on a 36° Y-X black LiTaO₃ with thickness of 0.5 mm and diameter of 4 inches. Prior to the photoresist deposition, the wafer is cleaned by ultrasonic to wipe off the organic matters and metal ions, followed by the drying procedure for 30 min at 90°C. The photoresist is deposited on the LiTaO₃ piezoelectric substrate with a spin coater, and then the wafer is baked at 90°C for 3 min. Next, the wafer is exposed to the UV light, followed by the immersion in the developing solution, and then it is cleaned and dried. And a Cr/Au film (40/50 nm) is sputtered over the entire wafer surface by magnetron sputtering. Then, the IDTs and the sensitive area are formed by immersing the wafer into acetone to lift off excessive metal. Besides, the ultra-sonication is used to remove residual metal between IDTs fingers, and then the wafer is dried in N₂. At last, the SH-SAW biosensor chips are cut off from the wafer using dicing saw.

As shown in Fig. 2, the SH-SAW biosensor chip has 60 pairs of input IDTs and output IDTs (Fig. 2A), and the width of the fingers is 5 μm, which signifies the wave length is 20 μm (Fig. 2B). Taking the amount of liquid and energy loss into account, the size of the sensitive area is designed to be 6 × 5 mm. Since the SAW propagation velocity on the 36° Y-X LiTaO₃ piezoelectric substrate is 4212 m/s, the resonant frequency of the SH-SAW biosensor is about 210 MHz, the design parameters are shown in Table 1. To investigate the vibration and the SH wave characteristics, FEA was performed using COMSOL 5.3a software based on a 3D piezo-plane strain mode. Fig. 3 shows the characteristics of shear horizontal surface acoustic waves excited by input IDTs and the particles displacement. According to the spectrogram of the SH-SAW biosensor chip shown in Fig. 4B, the attenuation is less than 2 dB from air to liquid phase. Compared to the rayleigh mode (Fig. 4A), it demonstrates that the SH-SAW mode is more suitable for the substance detection in the liquid phase.

3.3. Fabrication of detection cell with micro-channels

The detection cell is designed by SolidWorks software and made of acrylic material, which consists of inlet/outlet micro-channels. The capacity of the reaction cell (Fig. 5B), with the diameter of 4 mm and the height of 1.5 mm, is about 20 μL. To guarantee the no-leaking property of the sensitive area of the SH-SAW biosensor, a sealing ring made of silicon rubber (external diameter: 7 mm; inner diameter: 4 mm) and Nd-Fe-B magnets (diameter: 8 mm; height: 2 mm) are used (Fig. 5A). A spindle is used for ease of operation, and the liquid inlet/outlet is also designed to introduce the solution tested (Fig. 5C). Six gold-tipped electrical spring probes are used to contact six pads of the SH-SAW biosensor respectively through the spring probe holes so that the signal of the SH-SAW biosensor can be detected. Then they are welded onto the PCB and the SMA cables are utilized to connect the vertical network analyzer (Fig. 5D).

Compared with our previous studies concerning the test of cell structure and setup [19] (Fig. 5E), the newly developed micro-channel detection cell overcomes several shortages. In our previous work, the reaction chamber is normally exposed in air, which potentially introduces several uncertain factors such as: humidity, liquid evaporation, human factors and so on, which are all likely to influence the experimental results. Furthermore, in this experiment, a peristaltic pump (Ismatec, Switzerland) is utilized to pump the liquids into the detection cell, which is conducive to reduce the artificial errors.

3.4. Stability test of the fabricated SH-SAW biosensor in liquid phase

To verify the stability of the biochips in liquid phase, phosphate buffer saline is pumped into the reaction cell, prior to that, the biochips should be cleaned and kept dry. To ensure the biochips are clean and dry, photoreist covered the surface of biochips before use. And then the photoreist is wiped off by immersing it into acetone for 3 min, followed by cleaning using absolute ethyl alcohol to dissolve the residual acetone and drying with nitrogen gas. When a biochip is placed under the detection cell, the PBS is pumped into the reaction cell by the peristaltic pump, after which the PBS solution is pumped out of the reaction cell to test the stability of the SH-SAW biosensor chip in the liquid phase.

3.5. Preparation of single-stranded DNA

In preparation for the ss-DNA probe (F1-SH) and complementary

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAW velocity</td>
<td>4160</td>
<td>m/s</td>
</tr>
<tr>
<td>IDT finger width d=λ/4</td>
<td>5</td>
<td>μm</td>
</tr>
<tr>
<td>Sensitive area</td>
<td>6 × 5</td>
<td>mm</td>
</tr>
<tr>
<td>Thickness of the Cr/Au film</td>
<td>40/50</td>
<td>nm</td>
</tr>
<tr>
<td>Number of the input and output fingers</td>
<td>60 × 60</td>
<td>pair</td>
</tr>
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Table 1: Parameters of the SH-SAW device.

![Fig. 2. Microscopy images of the Au IDTs (A), SEM images of the Au IDTs (B), showing that he width of the fingers is 5 μm and the wave length is 20 μm.](image-url)
sequence (F1-C), the end of the ss-DNA probe is modified with sulfhydryl groups, so the stable and ordered self-assembled monolayer can be formed on the gold surface of the sensitive area through Au-S bonds (177 KJ/mole) [20]. The fixed probes can have specific binding to the complementary sequence when they are introduced into the reaction cell. In the first step, the F1-SH and F1-C stored at 4 °C are diluted in deionized water to a concentration of 1 nmol/L. Then the solution is heated to 95 °C for 10 min cooled down to room temperature and pumped into the detection cell by the peristaltic pump.

3.6. Experiment setup

The experiment setup is shown in Fig. 6. In this experiment, the liquid is pumped into the reaction cell using a peristaltic pump (IS-MATEC-E4, Switzerland) (Fig. 6), and an Agilent 5080 A (∼9 GHz) network analyzer (Fig. 6) is optimized to measure the phase response (S21) of the SH-SAW biosensor chip.

The whole reaction is observed and recorded using a biosensor monitor system based on LabVIEW™ application, and the data is obtained and saved for the purpose of further processing with Origin™.

3.7. Detection procedures

The SH-SAW biosensor chip is always cleaned and dried before detection, and then is placed in the sensor groove of the micro-channel detection cell. The tests are conducted under the condition of fixed temperature because fluctuation will result in the property change of the bio-chips. In the first step, the phosphate buffer solution is pumped into the reaction cell, and later the phase and attenuation of the SH-SAW biosensor chip begin to change, which is displayed on the screen in real time and the data can be obtained by LabVIEW. During this process a liquid phase environment is created for the biological reactions. Next, the ss-DNA probe (F1-SH) solution is pumped into the detection cell with the peristaltic pump, and then the phase signal of the SH-SAW biosensor chip starts to drift due to the binding of sulfhydryl groups on gold electrodes. At last, different concentrations of the complementary sequence (F1-C) solution (0, 0.1 nmol/L, 1 nmol/L, 10 nmol/L, 100 nmol/L, 1000 nmol/L) are separately pumped into the reaction cells, and the phase signals of the SH-SAW biosensor chip start to drift again until the specific binding is achieved.

3.8. Specificity test

To assess the specificity, the SH-SAW biosensor chips are employed to detect staphylococcus aureus and E.coli. Initially, the ss-DNA probe of the pseudomonas aeruginosa is immobilized on the sensitive surface of the SH-SAW biosensor chip, and then the nucleic acid of staphylococcus aureus and E.coli are injected into the reaction cells respectively to verify the specificity.

4. Results and discussion

4.1. The stability of the SH-SAW biosensor chip

As is shown in Fig. 7, the phase shift is about 6 ° after the PBS solution is pumped into the reaction cell. The phase returns to stable state immediately and lasts for quite a long time after the PBS solution is pumped out of the reaction cell (Fig. 7A). It indicates that the phase stability of the biochips fabricated by our team is quite good. At the same time, the attenuation changes within the range of 0.1 Db (Fig. 7B),

Fig. 3. 3D simulated SH vibration displacement distribution. SH-SAW excited by input IDTs (A), and the particles displacement (B), (C).

Fig. 4. Attenuation changes in Rayleigh mode(A) and shear horizontal mode(B) from air to liquid phase respectively, the attenuation change in Rayleigh mode is much higher than that in SH mode, thus the SH mode is appropriate for the bio-reaction in liquid phase.
which indicates the acoustic wave energy attenuation can be ignored and the SH-SAW biosensor is suitable for the biological detection in liquid phase [21].

4.2. The analytical performance and characteristics of the SH-SAW biosensor

Under the stable environment, the quantitative behavior of SH-SAW biosensor is evaluated through detecting different concentrations of nucleic acid from Pseudomonas aeruginosa (blank, 0.1 nmol/L, 1 nmol/L, 10 nmol/L, 100 nmol/L, 1000 nmol/L), and real-time phase signals are shown in Fig. 8A, the histogram of specific binding of F1-SH to F1-C of Pseudomonas aeruginosa at different concentrations is shown in Fig. 8B. As is portrayed in the picture, the phase drifts increase with the rising concentration of Pseudomonas aeruginosa nucleic acid. The average phase drifts are in a linear relationship to the logarithm of concentration of Pseudomonas aeruginosa nucleic acid from 0.1 nmol/L to 1000 nmol/L with a correlation coefficient of 0.98777 (Fig. 8C). The linear regression equation used to calculate the phase drift is $3.06046 + 3.84858 \log C$, in which $C$ represents the concentration of

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**Fig. 5.** Structure of the micro-channel detection cell: the chip layer (A), the flow channel layer (B), the circuit layer (C), the whole device (D) and the previous testing cell (E).

**Fig. 6.** Diagram of the experimental setup. The blue solid lines represent the flow direction of the probe solution, the red solid lines represent the electrical connection of the instruments and devices (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).
Pseudomonas aeruginosa nucleic acid. The detection limit is as low as 
0.28 nmol/L, which is the blank concentration plus the 3-fold standard 
deviation. The results in this study reveal that the detection limit was 
lower than that of the traditional QCM biosensor [22]. The outcome of 
the experiment is also compared with some other reported results in 
Table 2.

As is shown in Fig. 9A and B, the gold particles on the gold electrode 
are almost in the same size and arranged compactly, and the bare gold 
chip surface is relatively smooth. Fig. 9B indicates that the tapping 
phase of bare gold surface is 2.7°. After the formation of self-assembled 
monolayers on the sensitive surface, the images (Fig. 9C, D) indicate 
that the surface morphology in the sensitive area has remarkably 
changed compared with bare gold chip surface, and the tapping phase 
increases to 5.9° due to the ss-DNA probes. After binding with F1-C, the 
tapping phase increases from 5.9° to 25.3°. Fig. 9E, F shows that 
change of the surface characteristic is obvious, which indicates that 
specific binding of complementary sequences and ss-DNA probes occurs 
in the sensitive area.

4.3. The specificity of SH-SAW biosensor

The specificity is especially important in nucleic acid detection. The 
non-target ss-DNA may make it possible that the phase shift can be 
higher than the actual value, which undoubtedly affects the specificity 
of SH-SAW biosensor. Thus it is required that the experiments should be 
performed to estimate the specificity of SH-SAW biosensor by detecting 
other two kinds of bacterial nucleic acid, E.coli and Staphylococcus 
aureus. Initially, the F1-SH of Pseudomonas aeruginosa is immobilized 
on the sensitive area of SH-SAW biosensor chip, and then the 
Pseudomonas aeruginosa nucleic acid, E.coli nucleic acid and 
Staphylococcus aureus nucleic acid are detected respectively under the 
same experiment condition with the concentration of 1 nmol/L. The 
results are shown in Fig. 10. Seen from Fig. 10 (A), a significant black 
curve represents the complementary sequence of Pseudomonas aeru-
ginosa, and its phase shift is about 4°, while that of E.coli and Sta-
phylococcus aureus are almost the same as blank solution. The corre-
spanding histogram is shown in Fig. 10(B). The results reveal that the 
SH-SAW biosensor in this study demonstrates its advantage in
discriminating Pseudomonas aeruginosa from other kinds of bacterial nucleic acid.

5. Conclusions

In this work, a SH-SAW biosensor with high sensitivity is successfully designed and fabricated on a 36° Y-X LiTaO3 piezoelectric substrate. An effective homemade detection cell with micro-channel is developed by dint of acrylic material. To avoid leaking, four magnets are placed in four corners of the detection cell. The inlet ports of the cell is used to pump liquid into the liquid reservoir and the outlet ports to pump liquid waste out. In addition, a silicon rubber O-ring seal is put on the surface of sensitive area to ensure that the bio-reaction runs smoothly.

The feasibility and sensitivity of the SH-SAW biosensor are demonstrated through detecting the nucleic acids of Pseudomonas Aeruginosa, and the phase drifts are more than 4° after the formation of self-assembled monolayer and the specific binding of ss-DNA probes to complementary sequences, which occurs in the bio-probes solution with 1 nmol/L concentration. The SH-SAW biosensor in this work shows its higher sensitivity compared with the previous studies. SH-SAW biochips with higher sensitivity and repeatability will be further investigated; besides, miniaturized detection system with multichannel array to detect various biological samples at the same time is another significant research topic.

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Fig. 10. The real-time phase signals of different nucleic acid from different bacteria. The Pseudomonas aeruginosa, E.coli, and Staphylococcus aureus were analyzed at a concentration of 1 nmol/L for three times each.

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