

Streptavidin-enhanced surface plasmon resonance biosensor for highly sensitive and specific detection of microRNA

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Abstract We are presenting a method for sensitive and specific detection of microRNA (miRNA) using surface plasmon resonance. A thiolated capture DNA probe with a short complete complementary sequence was immobilized on the gold surface of the sensor to recognize the part sequence of target miRNA, and then an oligonucleotide probe linked to streptavidin was employed to bind the another section of the target. The use of the streptavidin-oligonucleotide complex caused a ~5-fold increase in signal, improved the detection sensitivity by a factor of ~24, and lowered the detection limit to 1.7 fmol of miR-122. This specificity allowed a single mismatch in the target miRNA to be discriminated. The whole assay takes 30 min, and the surface of the sensor can be regenerated at least 30 times without loss in performance. The method was successfully applied to the determination of miRNA spiked into human total RNA samples.

Keywords Surface plasmon resonance · Biosensor · MicroRNA · Singal amplification · Streptavidin

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Introduction

MicroRNAs (miRNAs) are a class of small (~22 nucleotides), endogenous, non-protein-coding RNA molecules. These highly conserved single-stranded RNAs regulate gene expression by binding to the 3'-untranslated regions (3'-UTRs) of target mRNAs through the dual mechanism of mRNA degradation and translation inhibition. However, the exact mechanism to impair translation is still in debate [1]. In the past decade, the study of miRNA biology has attracted tremendous attention. The research results demonstrate that abnormal expression of miRNAs is associated with various diseases, especially cancers [2, 3], which makes them be promising biomarker candidates for early cancer diagnosis [4]. Therefore, to develop an accurate and simple assay for miRNA expression analysis is an urgent need.

MiRNAs present a particular challenge in detection because of the extremely limited size, significant sequence homology among family members and the low expression levels [5]. Traditionally, miRNAs are predominantly detected with Northern blot [6, 7], polymerase chain reaction (PCR) [8], and microarray analysis [9, 10]. However, these methods require extrinsic labels (e.g., fluorophores or radiolabels) for detection of probe-target hybridization, which ultimately increases the cost and complexity of the detection assay [11]. Several new miRNA detection methods have been reported, including sequencing technology [12], electrochemical approaches [13, 14], nanoparticle-based assays [15], rolling circle amplification (RCA)-based methods [16, 17], bioluminescence technology [18], and surface enhanced Raman spectroscopy [19]. These methods show high sensitivity, but they are relatively complex and tedious. This work used surface plasmon resonance (SPR) technique to present a novel simple method for miRNA detection.

SPR biosensing is a highly sensitive and in situ label-free detection technique [20]. This optical technique can monitor small changes in the refractive index induced by molecular adsorption on a noble metal film [21]. It exhibits various advantageous features, such as versatility, speed of analysis, giving real-time binding results, and no label required [22–24]. In addition, SPR biosensors have also made substantial advances toward high-throughput screening [25] and has been used for detection of a wide range of chemical and biological analytes [26], including short oligonucleotides at subnanomolar levels [27]. Some signal amplification strategies such as PCR, enzymatic reactions and nanoparticle enhancement have been adopted to improve the sensitivity of SPR DNA biosensing [28–31]. A SPR method using monoclonal antibody to recognize DNA/RNA duplexes for signal amplification has been evaluated to achieve sub-femtomole detection of miRNA [32]. Recently, a streptavidin-enhanced SPR assay has also been developed for detecting small DNA fragments at the lowest concentration of ~40 pM [33]. Herein, this amplification strategy was further used for highly sensitive and specific detection of miRNA. MiR-122 was selected as a model miRNA, which can regulate intrahepatic metastasis of hepatocellular carcinoma [34]. Under optimal conditions, the detection sensitivity was greatly improved, and the detection limit was obtained as low as a few femtomole in 100 μ L. This biosensor was capable of discriminating single oligonucleotide mismatch of the miRNA, showing a promising platform for miRNA expression analysis.

Experimental

Materials and apparatus

6-Mercapto-1-hexanol (MCH) and streptavidin from *Streptomyces avidinii* were purchased from Sigma-Aldrich (USA, <http://www.sigmaaldrich.com>). Diethylpyrocarbonate (DEPC) was from Solarbio (China, <http://www.solarbio.cn>). All other reagents were of analytical grade. All aqueous solutions were prepared using ultra-pure water (≥ 18 M Ω , Milli-Q, Millipore, www.millipore.com).

The buffer solutions employed in the experiment were as follows. Oligonucleotide dissolving buffer (TE buffer, pH 8.0) contained 10 mM Tris-HCl and 1 mM EDTA. Probe immobilization solution was 1 M KH₂PO₄ (pH 3.8). MiRNA hybridization buffer was 30 mM sodium phosphate containing 450 mM NaCl, 3 mM EDTA, and 0.25 % Triton $\times 100$ (pH 7.4). Regeneration solution was 50 mM NaOH. In order to protect miRNA from RNase degradation, all buffer solutions and water were treated with DEPC by mixing 0.1 % of DEPC

with the solution, and the mixed solutions were stored overnight prior to autoclaving. All solutions prepared for experiments were filtered (pore size 0.22 μ m) and degassed before use.

HPLC-purified miRNAs and thiol or biotin modified DNA oligonucleotides were synthesized by TaKaRa Biotechnology Co., Ltd. (Dalian, China, <http://www.takara.com.cn>) and Invitrogen Biotechnology Co., Ltd (Shanghai, China, <http://www.invitrogen.com>), respectively. The base sequences are described below:

MiR-122: 5' UGG AGU GUG ACA AUG GUG UUU G 3'

MiR-29: 5' UAG CAC CAU CUG AAA UCG GUU A 3'

P1: 5' TGT CAC ACT CCA AAA AAA-(CH₂)₆-SH 3' (capturing probe for miR-122)

P2: 5' bio-AAA AAA CAA ACA CCA T 3' (probe for miR-122)

P3: 5' TGT CAG ACT CCA AAA AAA-(CH₂)₆-SH 3' (capturing probe for miR-122 with one nucleotide mismatch)

P4: 5' bio-AAA AAA CAA AGA CCA T 3' (probe for miR-122 with one nucleotide mismatch)

All the miRNAs and DNA oligonucleotides were dissolved in TE buffer, which were diluted in appropriate buffer prior to use. For all the experiments, the SPR device Biacore XTM analytical system and a bare gold sensor chip (SIA kit Au) were used (Biacore AB, Uppsala, Sweden, <http://www.biacore.com>). All experiments were conducted at an operating temperature of 25 °C.

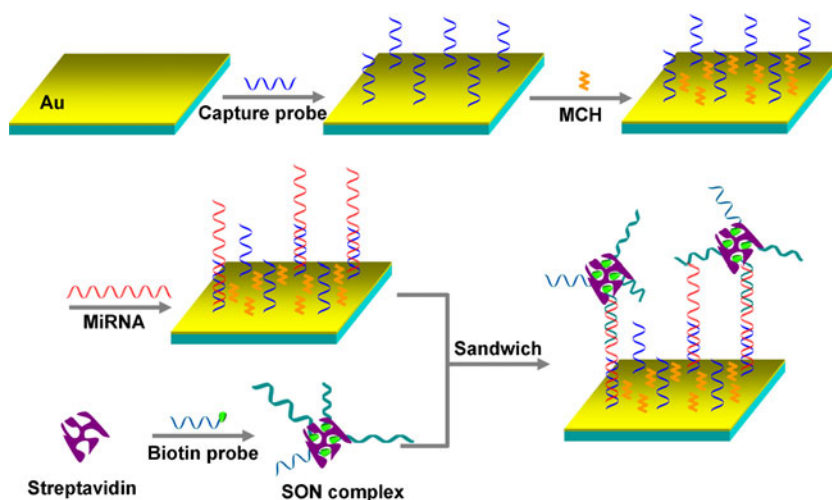
Immobilization of thiolated DNA probe

The gold sensor chip was cleaned with fresh piranha solution (30 % H₂O₂, 70 % H₂SO₄) for 10 min, then washed thoroughly with Milli-Q water and allowed to dry. The chip was docked into the Biacore XTM instrument. 1 μ M solution of thiolated DNA probe in immobilization solution was injected in the flow-cell and flowed through at a constant rate of 2 μ L min⁻¹ for 30 min. The chip was undocked and washed with Milli-Q water, then incubated in 1 mM MCH blocking solution at room temperature for 4 h in the dark. After washed with ethanol and deionized water, the chip was left to dry. Finally, the chip was docked and ready for hybridization.

MiRNA detection protocol

Sensor chip functionalized with the thiolated DNA probe/MCH was used for miRNA assay. As shown in

Scheme 1 Schematic representation of the miRNA assay using SPR biosensor and SON complex for signal amplification



Scheme 1, prior to the detection, a regeneration solution (50 mM NaOH) was injected for 1 min, followed with hybridization buffer. After sensor response was stabilized, the solution of miRNA in hybridization buffer was injected and flowed through the flow-cell for 14 min. The sensor surface was then flushed with hybridization buffer for 5 min. Preparation of streptavidin-oligonucleotide (SON) complex was performed *ex situ* by mixing $10\ \mu\text{L}$ 500 nM of biotinylated DNA probe and $5\ \mu\text{L}$ $0.1\ \text{mgmL}^{-1}$ of streptavidin into $85\ \mu\text{L}$ of hybridization buffer. This mixture was shaken for a few seconds and then let to rest for 5 min. Subsequently, the solution of SON complex was injected and flowed through the flow-cell for 10 min. After hybridization reaction, the chip was automatically washed with hybridization buffer to remove the unbound material. Finally, the sensor surface was regenerated with regeneration solution for 1 min and washed with hybridization buffer. All the hybridization experiments were carried out at a flow rate of $5\ \mu\text{L}\ \text{min}^{-1}$. The Biacore XTM displays results as a time course of resonance units (RU). The analytical signal was derived from the RU difference between the final value and the reference value recorded before sample injection.

Results and discussion

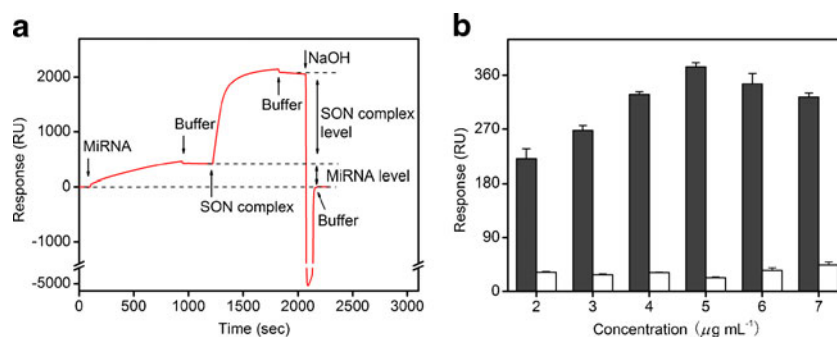
Assay principle

The miRNA detection was based on biomolecular amplification of the mass-sensitive SPR biosensing signal with SON complex (Scheme 1). The assay was performed in two simple steps (sandwich technique). First, the chip surface was functionalized with covalently attached thiolated DNA probe, which had the sequence complementary to the part sequence of target miRNA. This step could capture the miRNA to the chip surface. Then, a solution containing SON complex was injected onto the sensor surface to specifically bind another part of the target miRNA. The corresponding sensorgram was shown in Fig. 1a. The SON complex significantly increased the sensitivity of the assay due to the enhanced mass. After the detection, the surface could be regenerated with an appropriate solution.

Optimization of streptavidin concentration

Signal amplification plays a key role in obtaining high sensitivity for the sandwich-type SPR biosensing. The

Fig. 1 **a** Typical sensorgram for the streptavidin-enhanced assay and **b** effect of streptavidin concentration on SPR signal in presence (*gray*) or absence (*white*) of 1 nM target miRNA. Error bar is the standard deviation of three independent measurements



stoichiometric ratio of the streptavidin to biotinylated probe directly affected the composition of the SON complex. Thus, the concentration of streptavidin was optimized to improve the efficiency of the miRNA assay when the concentration of biotinylated probe was fixed. The biotinylated probe concentration adequate for target hybridization was set to 50 nM, while the concentration of streptavidin was varied. At the streptavidin concentrations less than $5 \mu\text{g mL}^{-1}$, the SON complex with single streptavidin and multiple biotinylated probes could bind more than one target molecule (Fig. 1b). As a result, the amount of streptavidin anchored on the chip surface was reduced, and thus the lower signal response was observed. At a streptavidin concentration higher than $5 \mu\text{g mL}^{-1}$, the hybridization site of biotinylated probe might be covered by streptavidin molecules and the steric hindrance for target recognition was increased, thus the response was also low. The maximum signal response was obtained with a concentration of $5 \mu\text{g mL}^{-1}$, which was used in the later sensing process.

Signal amplification performance of MiRNA assay

The amplification strategy was based on the high-affinity interaction of biotin and streptavidin [35]. This interaction allows the construction of a molecular complex between streptavidin and biotinylated molecules, so that only a few binding events of the analyte at the sensing surface may lead to a detectable surface mass due to the large molecular weight of the complex. In order to further clarify the signal amplification of the SON complex on miRNA assay, a comparing experiment was carried out and the result was shown in Fig. 2. The response change with SON complex amplification was approximately 5 times that without enhancement (direct detection), demonstrating that the SON complex greatly amplified the response signal. To confirm the function of streptavidin in the present system, another experiment using biotinylated probe as a substitute of SON complex was performed. The signal increase using SON complex was much greater than that obtained using biotinylated probe (Fig. 2). This result could be attributed to the larger mass of the complex with streptavidin than separated oligonucleotide. In other words, the considerable response change was mainly caused by the large mass of streptavidin.

The nonspecific binding of the SON complex to the sensor surface was also investigated by control experiment. The direct treatment of the thiolated DNA immobilized surface with SON complex produced only very small change in the SPR response, indicating that the amplified SPR assay was not affected by the nonspecific adsorption of the SON complex.

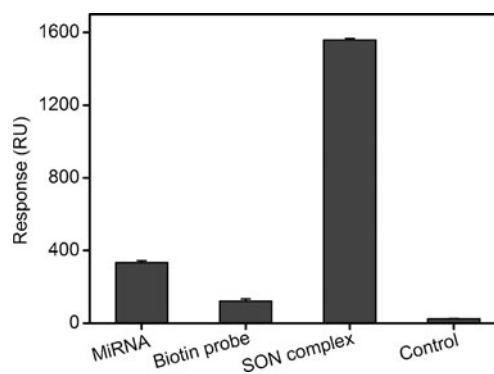


Fig. 2 SPR responses for target miRNA (10 nM) hybridization and signal amplification by biotinylated probe and SON complex. Control is obtained with hybridization buffer in absence of miRNA using the streptavidin-enhanced assay. Error bars represent the standard deviations of three independent measurements

Specificity of MiRNA assay

The specificity of the method for miRNA assay was evaluated using different combinations of four probes and non-complementary sequence, i.e., P1, P2, P3, P4 and miR-29. As shown in Fig. 3, single nucleotide mismatch could be clearly distinguished with the method. It was interesting that the mismatches on the capture probe were consistently much more perceivable than mismatches of detection probe on the SON complex. This phenomenon was likely attributed to the easier dissociation of the miRNA with SON complex than the SON complex itself. When the method was applied to detect miR-29, the response was as small as the background. These results demonstrated that this method displayed excellent selectivity.

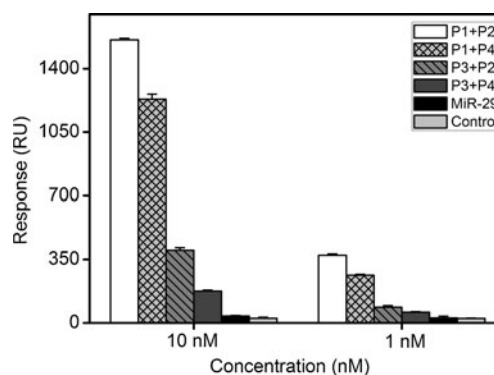
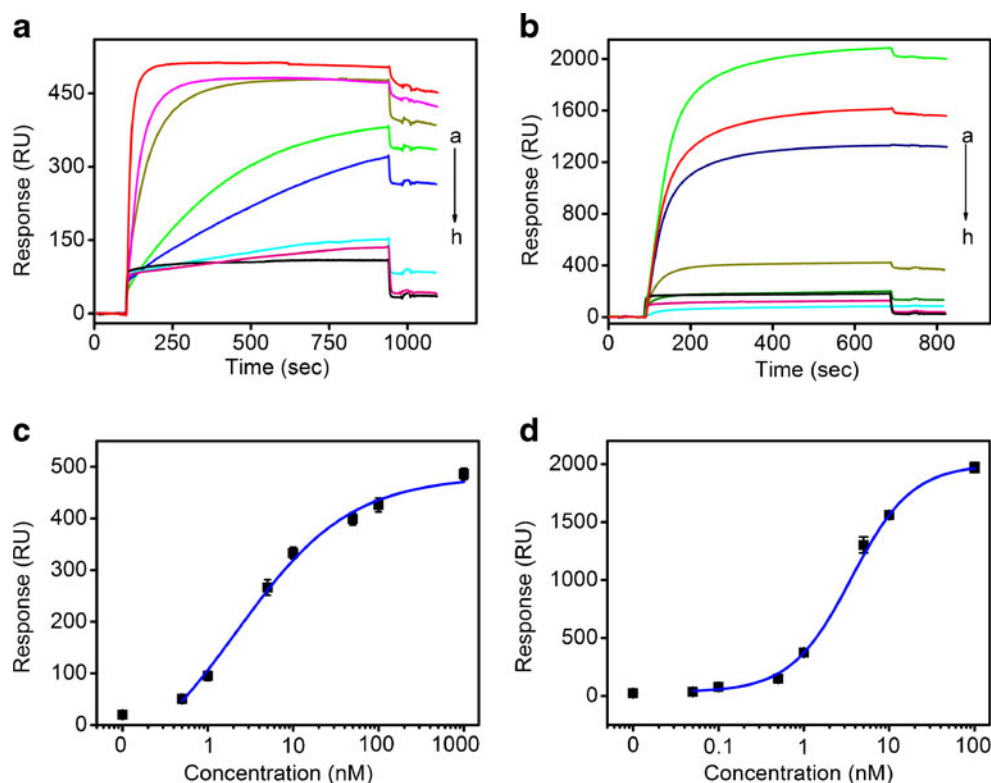


Fig. 3 Detection of miR-122 with four sets of probes, i.e., P1+P2 (complete complementary probes), P1+P4 (biotin labeled P4 has single nucleotide mismatch to miR-122), P3+P2 (thiol labeled P3 has single nucleotide mismatch to miR-122) and P3+P4, and miR-29 (a non-complementary sequence) with P1+P2. Control is obtained with hybridization buffer. Error bars represent the standard deviations of three independent measurements

Fig. 4 **a** SPR sensorgrams for unamplified detection of target miR-122 at 1000, 100, 50, 10, 5.0, 1.0, 0.5 and 0 nM (from **a** to **h**), **b** SPR sensorgrams for amplified detection of target miR-122 at 100, 10, 5.0, 1.0, 0.5, 0.1, 0.05 and 0 nM (from **a** to **h**), and calibration curves for the unamplified (**c**) and amplified (**d**) detection ($n=3$)



Analytical performance

Figure 4a and b show the sensorgrams obtained with direct detection and amplification detection, respectively. The introduction of target miR-122 at different concentrations to the sensor surface induced proportional increases in the response signal. The calibration curves for synthetic miR-122 could be simulated with a 4-parameter logistic equation: $f(c) = (A_1 - A_2)/(1 - (c/c_0)^p) + A_2$. The calibration curve for direct detection of miRNA (Fig. 4c) showed the values of $-(123.43 \pm 91.63)$ RU for A_1 , (481.63 ± 11.86) RU for A_2 , (2.13 ± 1.09) nM for c_0 and 0.65 ± 0.12 for p , while the amplification detection using SON complex showed the values of (33.19 ± 4.19) RU for A_1 , (1997.63 ± 48.91) RU for A_2 , (3.60 ± 0.22) nM for c_0 and 1.22 ± 0.04 for p (Fig. 4d). The limits of detection (LODs) for miRNA were calculated based on a 3σ rule to be 400 pM and 17 pM, respectively. Considering the required sample volume of $\sim 100 \mu\text{L}$ per analysis, the latter corresponded to the smallest detectable absolute amount of less than 1.7 femtomoles. Compared to Northern blot [6], the established SPR biosensor showed a much lower limit of detection due to low blank signal and signal amplification of streptavidin. The detection limit was also comparable to the electrochemical sensor using enzymatic catalysis [36], SPR imaging [37], and antibody enhanced SPR biosensor [32]. Although more sensitive detection of miRNA has been achieved by the fluorescence sensor using cyclic enzymatic signal amplification strategy [38], hairpin probe-based circular

exponential amplification assay [39], and nanoparticle-based biosensors [14, 15], the sensor developed here was simpler and faster (only 30 min, including measurement and regeneration). Typically, the measurement time of electrochemical and fluorescence sensor was about 1–2 h. Moreover, the binding molecules could be removed completely from the sensor chip surface without irreversible loss of its activity. Up to 30 cycles of binding/regeneration processes on the same chip, the SPR response decreased $<15\%$. These results indicated that a well-immobilized Au chip can be regenerated more than 30 times. Therefore, the sensor chip can be reused by simply washing the analyte off from the surface, resulting in a reduced detection cost and a rapid detection process. The average relative standard deviation (RSD) was 5.7 %, which guaranteed the precision of the method.

Table 1 The recoveries determined using the proposed miRNA assay via spiking miR-122 into human total RNA samples

Sample No.	Spiking value (nM)	Assayed value (nM)	Recovery (%)
1	20.00	19.00	95.0
2	2.00	1.60	80.0
3	1.50	1.38	92.0
4	0.5	0.45	90.0
5	0.1	0.09	90.0

Analytical application of MiRNA assay

The practical applicability of the established miRNA assay was evaluated by a standard addition method owing to the fact that RNA samples from breast tumors do not contain miR-122 [40]. Total RNA samples extracted from human breast tumor cells were obtained from Molecular Oncology and Epigenetics Laboratory of the First Affiliated Hospital of Chongqing Medical University. Various amounts of synthetic miR-122 were spiked into human total RNA samples. Then, the method was used to detect target miRNA in these RNA samples. The results were listed in Table 1. The recoveries were between 80 % and 95 % from 0.1 nM to 20 nM in 157.8 ngmL^{-1} total RNA, indicating that the analytical performance of the biosensor was not compromised in complex mixtures. These results demonstrated that the established miRNA biosensing assay provided a potential analytical tool in real biological samples.

Conclusions

A simple, rapid, highly sensitive and selective streptavidin-enhanced SPR biosensor has successfully been developed for miRNA expression profiling. The SPR analytical process for miRNA is PCR-free and ligand-free. With the introduction of streptavidin amplification, as low as 17 pM miRNA could be detected with 30 min. The established miRNA assay also exhibited excellent specificity, good reproducibility, and good precision. This method could be expanded readily to other miRNA detection and provided a promising tool for the basic research and clinical application of miRNA expression profiling.

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