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Surface plasmon resonance biosensor for highly sensitive detection of microRNA based on DNA super-sandwich assemblies and streptavidin signal amplification

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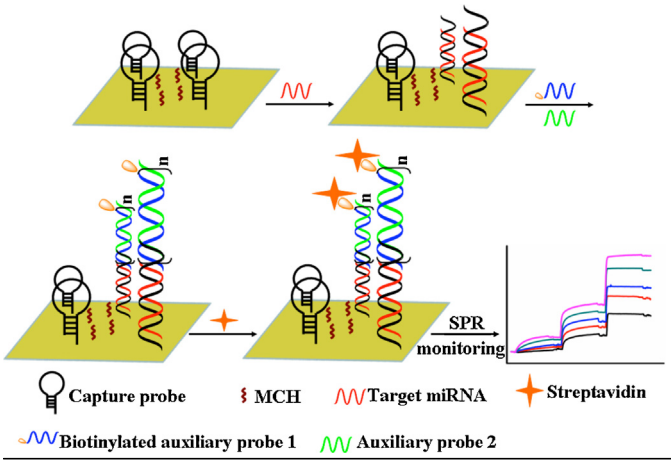
HIGHLIGHTS

- An enzyme-free and rapid SPR sensing strategy has been developed for miRNA detection.
- DNA super-sandwich assembly and streptavidin were used for dual signal amplification.
- The developed method showed high sensitivity and specificity for detection of miRNA.
- The biosensor performed well in analyzing extractions from MCF-7 cells.

GRAPHICAL ABSTRACT



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ABSTRACT

MicroRNAs (miRNAs) play an important regulatory role in cells and dysregulation of miRNA has been associated with a variety of diseases, making them a promising biomarker. In this work, a novel biosensing strategy has been developed for label-free detection of miRNA using surface plasmon resonance (SPR) coupled with DNA super-sandwich assemblies and biotin–strepavidin based amplification. The target miRNA is selectively captured by surface-bound DNA probes. After hybridization, streptavidin is employed for signal amplification via binding with biotin on the long DNA super-sandwich assemblies, resulting in a large increase of the SPR signal. The method shows very high sensitivity, capable of detecting miRNA at the concentration down to 9 pM with a wide dynamic range of 6 orders of magnitude (from 1×10^{-11} M to 1×10^{-6} M) in 30 min, and excellent specificity with discriminating a single base mismatched miRNA sequence. This biosensor exhibits good reproducibility and precision, and has been successfully applied to the detection of miRNA in total RNA samples extracted

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from human breast adenocarcinoma MCF-7 cells. It, therefore, offers a highly effective alternative approach for miRNA detection in biomedical research and clinical diagnosis.

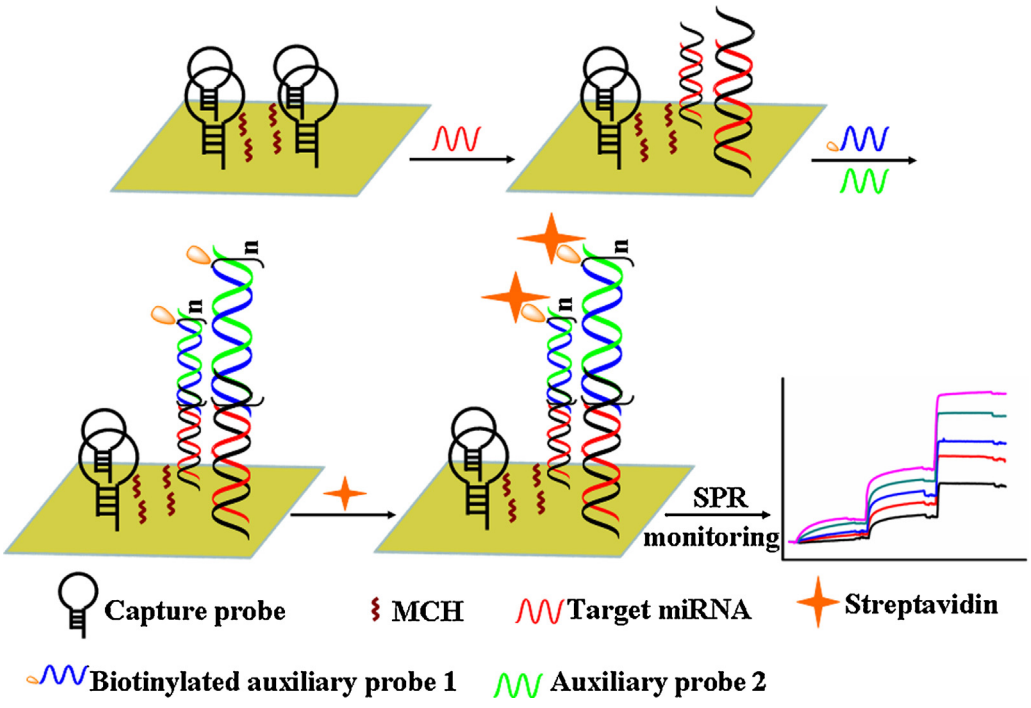
1. Introduction

MicroRNAs (miRNAs), a class of short, endogenous, non-protein coding RNAs, play important roles in gene expression regulation by formation of RNA-induced silencing complex (RISC) with target mRNAs [1,2]. Recently, more evidences have emerged that abnormal expressions of miRNAs are associated with many diseases, including human cancers [3,4], cardiovascular [5], and neurological diseases [6]. Hence, miRNAs have become an emerging class of important biomarkers for clinical diagnosis, and it is of great significance to develop rapid and accurate methods for miRNA detection.

The detection of miRNA can be challenging due to their characteristics of small size, low abundance, and sequence homology. Conventional methods, including Northern blotting [7,8], microarray [9,10], and quantitative real-time PCR (qRT-PCR) [11], have been well established for miRNA detection. However, these methods require tedious procedures and rigorous experimental conditions, and have high operation expense. Therefore, it is necessary to explore novel alternative methods, i.e., biosensing approaches, for miRNA detection.

Recently, various methods for miRNA detection have been proposed, including electrochemical [12,13], bioluminescence [14], electrochemiluminescence [15], colorimetry [16], Raman spectroscopy [17], and surface plasmon resonance (SPR) [18,19]. Among these methods, SPR biosensor, measuring the change in refractive index near the surface that occurs during complex formation or dissociation [20], is an advanced technology for fast, label-free, real-time and in-situ detection of biomolecular targets [18]. SPR biosensing platform has been applied to detect a wide variety of biomolecules (proteins, peptides, nucleic acids, etc.) [18]. However, the sensitivity of SPR for direct detection is not satisfying in certain cases, which has limited the further application of SPR for clinical diagnosis, especially for detection of trace substance in complex biological samples. Thus, a number of amplification strategies, including PCR [21,22], hybridization chain reaction (HCR) [23], enzymes [24,25], protein [26,27] and nanoparticle enhancement [28–31], have been developed to improve the sensitivity of SPR biosensors. Nevertheless, these methods are time-consuming, and have the issues of high nonspecific binding [19]. Recently, a DNA super-sandwich assemblies-based amplification technique for the detection of nucleic acid has been reported [32–34]. In the super-sandwich assay, two single stranded DNAs that have partially complementary segments can hybridize and form long DNA nanostructures, which enables sensitive nucleic acid analyses without the assistance of enzymes and can be implemented at room-temperature [32–34].

In this work, we report the development of a highly sensitive and versatile SPR biosensing platform that is based on DNA super-sandwich assembly and streptavidin signal enhancement for detection of miRNA. Biotin–avidin system has been used as amplification strategy in SPR assays, including the detection of DNA and miRNA [35,36]. However, combining DNA super-sandwich assembly with biotin–avidin system in SPR platform is new, especially for miRNA detection that targets miR-21, a potential cancer biomarker with elevated expression levels in numerous tumor tissues. In this work, we have designed a thiolated hairpin capture DNA probe, CP, and two auxiliary probes, AP1 and AP2, respectively. One terminal of the AP1 is labeled with biotin. The hairpin CP specifically hybridizes with the target miRNA and undergoes a conformational change, resulting in the opening of the terminus site for AP1 hybridization (Scheme 1). Addition of AP1 and AP2 leads to partial hybridization with each other and formation of DNA super-sandwich assemblies on the chip surface.



Scheme 1. Schematic representation of miRNA detection assay using SPR biosensor based on DNA super-sandwich assemblies and streptavidin amplification.

Further signal enhancement is obtained by using streptavidin that attaches to the chip surface via binding to biotinylated AP1 on the long DNA super-sandwich assemblies, resulting in cascading amplification of the SPR signal. This strategy demonstrates highly sensitive and selective detection of miRNA, and can be applied to the determination of miRNA in real samples.

2. Materials and methods

2.1. Reagents

HPLC-purified miRNAs were synthesized by TaKaRa Biotech. (Dalian, China). 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 μM and diluted to the desired concentration with appropriate buffer prior to use. Total RNA extracted from human breast adenocarcinoma MCF-7 cells was obtained from Ambion (TX, USA). 6-Mercapto-1-hexanol (MCH) and streptavidin from *Streptomyces avidinii* were purchased from Sigma–Aldrich (St. Louis, MO, USA). Diethylpyrocarbonate (DEPC) was from Solarbio (Beijing, China). Premix Taq Version 2.0, DL500 DNA Marker and agarose were from TaKaRa Biotech. (Dalian, China). GoldView was purchased from SBS Genetech (Beijing, China). All other reagents were of analytical grade. All aqueous solutions used in the experiments were prepared with Milli-Q water (≥18 MΩ, Milli-Q, Millipore). To avoid miRNA degradation, all buffer solutions were treated with DEPC by mixing 0.1% of DEPC with the solution, and the mixed solutions were kept overnight before autoclaving.

2.2. SPR experiment

Biacore X^{TM} analytical system (Biacore AB, Uppsala, Sweden) and bare gold sensor chips (SIA kit Au) were used in the SPR measurement. All experiments were carried out at an operating temperature of 25 °C ± 1 °C. The Biacore X^{TM} displays results as a time course of resonance units (RU). All sensorgrams were evaluated by BIA evaluation 4.1 software (Biacore, Sweden).

2.3. Probe immobilization

The thiolated capture probe was immobilized on the gold sensor chip by the interaction of Au–thiol. The gold chip surface was first cleaned with fresh Piranha solution (70% H_2SO_4, 30% H_2O_2) for 10 min to remove adsorbed organic impurities, then rinsed with Milli-Q water thoroughly and dried at room temperature. The chip was then docked into the Biacore X^{TM} instrument socket, and Milli-Q water was used as running solution. When a steady state baseline was reached, 1 μM thiolated capture probe in immobilization solution (1 M KH_2PO_4, pH 3.8) was injected into the flow-cell and a flow at a constant rate of 2 μL min^{-1} was maintained for 30 min. After immobilization, the sensor chip was removed from the Biacore X^{TM} instrument, washed with Milli-Q water, and further blocked with 1 mM MCH solution for 4 h at room temperature in the dark. After washing with Milli-Q water, the chip was allowed to dry and re-docked into the instrument for experiment.

2.4. SPR biosensor real-time monitor

The sensor chip functionalized with thiolated capture probe/MCH was used for miRNA detection. Hybridization solution was used as the running buffer in the experiment. Prior to the detection, the regeneration solution (50 mM NaOH) was injected into the flow-cell for 1 min, followed with the running buffer. After the sensorgram reached a steady state, the solution of miRNA diluted with hybridization buffer was injected into the flow cell and flowed for 8 min. Then the chip surface was rinsed with the running buffer. After hybridization, the mixture of AP1 and AP2 was injected and flowed for 8 min. The mixed solution of AP1 (1 μM) and AP2 (1 μM) was prepared freshly. The chip was then equilibrated with the running buffer to remove the unbound materials. Finally, the solution of 200 nM streptavidin in hybridization buffer was injected and flowed for 8 min.

The sensor surface could be regenerated with the regeneration solution to remove the binding analytes on the gold film for the next measurement. Three replicate tests were carried out to the same sample and the average value was used for calculation. All the experiments were carried out at a flow rate of 5 μL min^{-1}. The binding/regeneration processes on the same chip was repeated 20 times to assess the reusability of the sensor chip.

2.5. Gel electrophoresis

AP1 (1 μM) was incubated with AP2 (1 μM) for 8 min in the hybridization buffer (30 mM sodium phosphate, 450 mM NaCl, 3 mM EDTA, 0.25% Triton X-100, pH 7.4) at room temperature before gel electrophoresis analysis. The DNA super-sandwich assemblies were verified by running 10 μL mixed solution in 2% agarose gel for 25 min and detected by GoldView staining under UV light. Gel images were recorded by an imaging system (Bio-Rad Laboratories, USA).

3. Results and discussion

3.1. Principle of the SPR assay

The basic principle of the SPR-based miRNA biosensor is shown in Scheme 1. The assay was performed with simple steps. 5′-Thiolated hairpin CP, containing the sequence complementary to the target miRNA, was immobilized onto the sensor surface by Au–S interaction, and MCH was utilized to block non-specific binding sites. In the presence of target miRNA, the hairpin CP

Table 1
DNA and miRNA sequences employed in this work.

Oligonucleotide	Sequence [5′–3′]
Capture probe	SH-(CH2)6-GGCCG*TCAACATCAGTCTGATAAGCTA*AAACATGATGACGGCC
Auxiliary probe 1	Biotin-TTTTGCACCTGGGGGAGGCCGTCATCAT
Auxiliary probe 2	TCCCCCAGGTGCATGATGACGGCC
miR-21	UAGCUUAUCAGACUGAUGUUGA
Single-base mismatch target	UAGCUUAUCAGACUGAUGUUUA
Double-base mismatch target	UAGCUUAUCAGACUGAUUUUU̲U̲A
Non-complementary sequence	AUUGAAUAUUCUUAUUAU̲U̲AAU̲U̲U
miR-222	AGCUACAUCUGGCUACUGGGUCUC

The italic portion of capture probe represents complementary sequences to miR-21. The underline portion represents mutation bases in target miRNA.

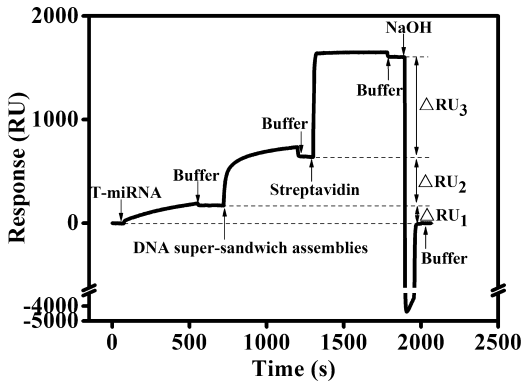


Fig. 1. Typical SPR sensorgram of target miRNA hybridization, DNA super-sandwich assemblies and streptavidin signal amplification.

specifically hybridized with the target miRNA and underwent a conformational change, resulting in the opening of the terminus site for AP1 hybridization. With the addition of AP1 and AP2, a large quantity of DNA was assembled to the chip surface and produced an amplified SPR signal. Furthermore, the added streptavidin attached to the chip surface via binding with biotinylated AP1 on the long DNA super-sandwich assemblies, resulting in a cascading amplification of the SPR signal. This strategy significantly increased the sensitivity of the assay due to combined amplification modes of DNA super-sandwich assemblies and streptavidin. After the detection, the surface could be completely regenerated with the regeneration solution. A typical sensorgram is shown in Fig. 1.

3.2. Characteristics of the proposed assay

The amplification characteristics were further evaluated by SPR measurements. As shown in Fig. 2A (curve c), the introduction of target miRNA led to an increase of SPR signal, which could be attributed to the hybridization process. The SPR signal increased clearly with the addition of AP1 and AP2 mixture, because a large quantity of DNA assembled to the sensor surface. With the addition of streptavidin, there was a great increment of the SPR signal, which could be associated with the biotin binding sites on the DNA super-sandwich structures. For comparison, the traditional sandwich assay, with only AP1 introduced, produced a much smaller SPR signal on the same experimental conditions (Fig. 2A (curve b)). This is because in the traditional sandwich assay, the ratio of target miRNA to AP1 was approximate 1:1, while in the DNA super-sandwich assay, there was much more AP1 available for the subsequent streptavidin binding. In the absence of target miRNA, although AP1 and AP2 can self-assemble to form long DNA nanostructures, the DNA nanostructures cannot be linked to the chip surface, giving no ground for the binding of streptavidin. Therefore only a small SPR signal was observed in Fig. 2A (curve a), largely due to a small scale of nonspecific adsorption. To further verify the formation of the DNA super-sandwich nanostructures, gel electrophoresis was employed. As seen in Fig. 2B, the mixture of AP1 and AP2 could hybridize with each other and form longer DNA fragments (lanes 2 and 3). The gel electrophoresis results confirmed the formation of the DNA super-sandwich nanostructures in the process.

3.3. Optimization of detection conditions

Signal amplification plays a key role in obtaining high sensitivity for the SPR biosensor developed in this study. To achieve higher detection sensitivity, experimental parameters, such as the volume ratio of AP1 to AP2, concentration of AP1 and AP2 and concentration of streptavidin, were optimized. 100 nM target miRNA was used for the optimization study. We first examined the effect of volume ratios of AP1 to AP2, from 3:1 to 1:3, on the SPR signal. The results were shown in Fig. 3A. The highest SPR signal was obtained when the ratio was 1:1. Therefore, the ratio of 1:1 was used throughout the experiment.

Then the optimum concentration of AP1 and AP2 was investigated at a fixed rate of 1:1. As shown in Fig. 3B, the SPR response increased rapidly with the increasing concentration of AP1 and AP2 before reaching a steady value at the concentration of 1 μM. Therefore, 1 μM was chosen as the optimal concentration of AP1 and AP2, and used in the following experiments.

The concentration of streptavidin was also optimized. In Fig. 3C, the concentration of streptavidin was examined from 100 to 300 nM. After 200 nM, the signal reached a plateau. Thus, 200 nM was selected as the appropriate concentration of streptavidin.

3.4. Analytical performance

Under optimal experimental conditions, to evaluate the analytical performance of the biosensor, different concentrations of target miRNA were investigated. The measurements of the target miRNA were repeated three times on the same chip, and the error bars represent the standard deviation among the results of the parallel measurements. The sensorgrams obtained with SPR direct detection and amplification detection are shown in Fig. 4A and B, respectively. The introduction of target miRNA at different concentrations led to proportional increases in the SPR signals.

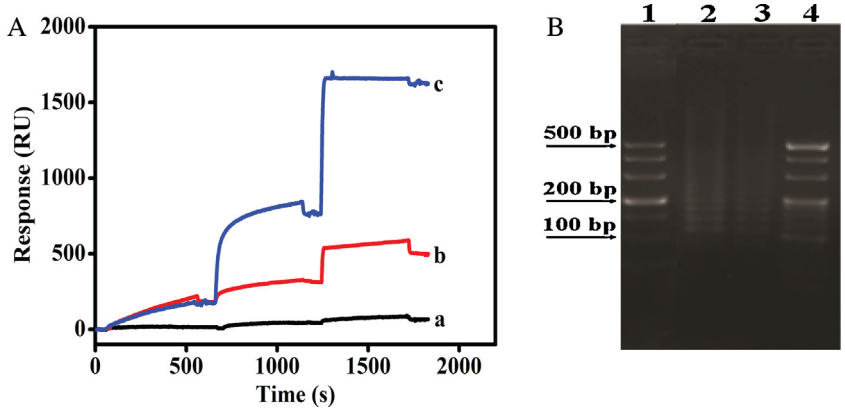


Fig. 2. (A) SPR sensorgrams corresponding to blank (curve a), traditional DNA sandwich (curve b) and DNA super-sandwich assemblies (curve c). (B) The gel electrophoresis results of DNA super-sandwich assemblies. Lanes 1 and 4, DNA ladder markers. Lanes 2 and 3, two ladders of DNA super-sandwich assemblies.

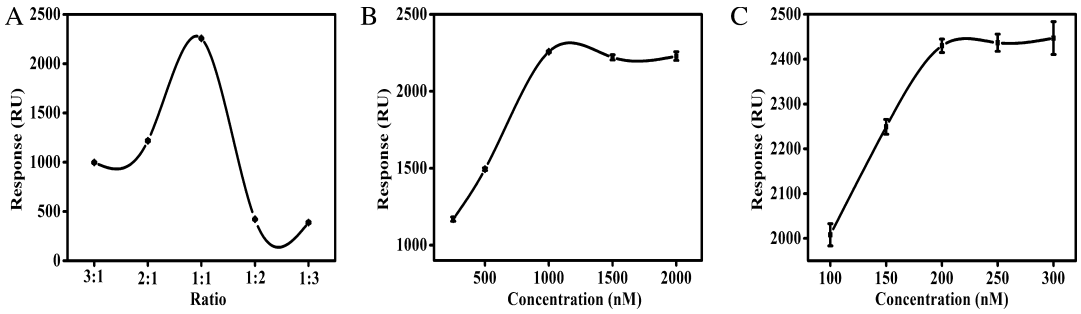


Fig. 3. Dependence of SPR signal on (A) ratio of AP1 to AP2, (B) concentrations of AP1 and AP2, and (C) concentration of streptavidin.

The calibration curves could be obtained with a 4-parameter logistic equation:

$$f(c) = \frac{(A_1 - A_2)}{(1 + (c/c_0)^p) + A_2} \quad (1)$$

where f is the SPR response signal, c is the concentration of target miRNA. The calibration curve for direct detection of target miRNA (Fig. 4C) showed the values of 16.18 ± 6.08 RU for A_1, 421.69 ± 6.55 RU for A_2, 15.98 ± 1.01 nM for c_0 and 0.79 ± 0.06 for p, while the amplification detection using DNA super-sandwich assemblies and streptavidin (Fig. 4D) showed the values of -69.95 ± 7.34 RU for A_1, 2890.50 ± 18.50 RU for A_2, 5.27 ± 0.16 nM for c_0 and 0.47 ± 0.01 for p. Based on the 3σ rule, the limits of detection (LODs) for target miRNA were estimated to be 470 pM for the direct detection and 9 pM for the amplification measurement, respectively. Since the sample volume was 100 μL per analysis, the smallest detectable amount was less than 0.9 fmol. Compared with Northern blotting [8], the SPR biosensor shows a much lower detection limit. The sensitivity of the current assay is also comparable to other works relating to nucleic acid detection by SPR biosensor technique (Table 2) [21,27,36–38].

3.5. Specificity, reproducibility and reusability of the strategy

The specificity of the SPR biosensor reported here was evaluated by detecting five different miRNA sequences, including the perfect complementary target miRNA, single-base mismatch target (SM), double-base mismatch target (DM), non-complementary sequence (NC), and miR-222 at the concentrations of 10 nM and 1 nM, respectively. As shown in Fig. 5A, it is clear that the SPR signal for target miR-21 gives a remarkably distinguished increase as compared with other targets, and the signals for NC and miR-222 are as small as that of the blank. These results indicated that the biosensor displayed excellent specificity for the determination of target miR-21. The high sequence specificity could be attributed to the high affinity and unique specificity of

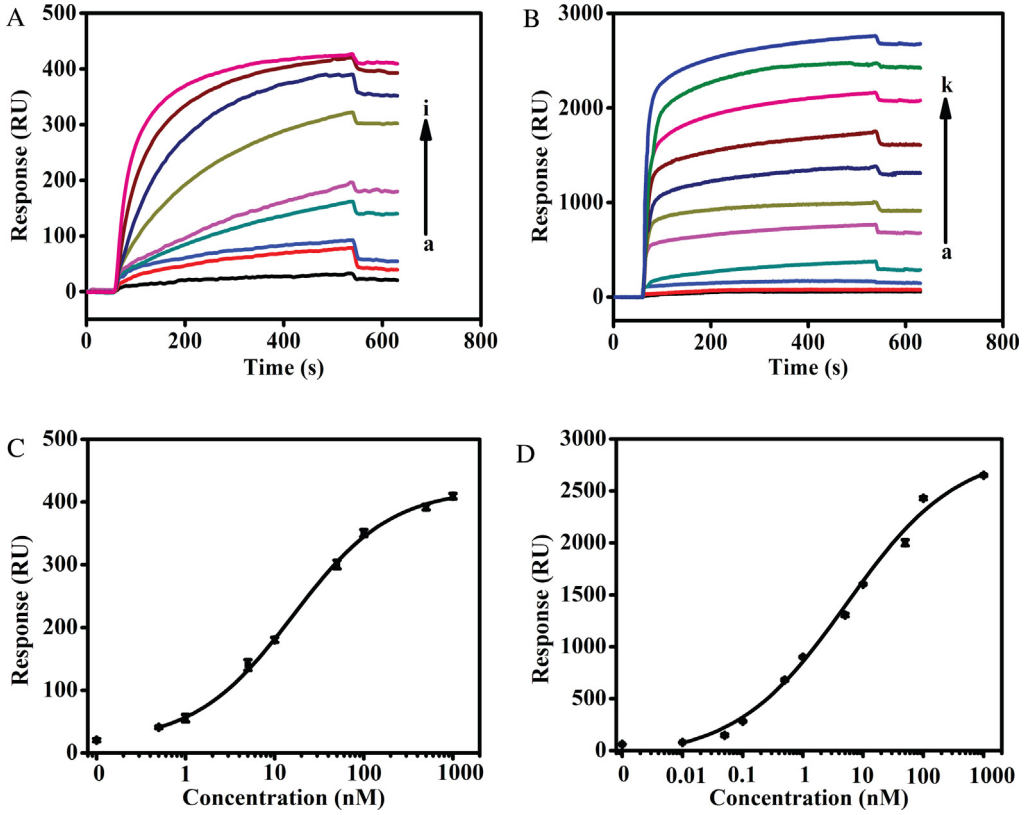


Fig. 4. (A) SPR sensorgrams for unamplified detection of target miR-21 at 0, 0.5, 1.0, 5.0, 10, 50, 100, 500 and 1000 nM (from a to i). (B) SPR sensorgrams for amplified detection of target miR-21 at 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 and 1000 nM (from a to k). (C) The calibration curve for the unamplified direct miRNA detection. (D) The calibration curve for the amplified miRNA detection.

Table 2
Comparisons of different amplification strategies of nucleic acid SPR biosensors.

SPR platform	Target	Amplification strategy	Dynamic range	LOD	Reference
Biacore X	DNA	PCR	0–500 nM	2.5 nM	[21]
SPR imaging	DNA	Exonuclease III	–	10 pM	[37]
laboratory SPR	DNA	Rolling circle amplification and Au nanoparticles	1 pM–10 nM	5 pM	[38]
Biacore X	miRNA	Streptavidin complex	50 pM–100 nM	17 pM	[36]
SPRimager II	miRNA	Protein p19	1 fmol–1 pmol	1 fmol	[27]
Biacore X	miRNA	DNA super-sandwich assemblies and streptavidin	10 pM–1 μM	9 pM	This work

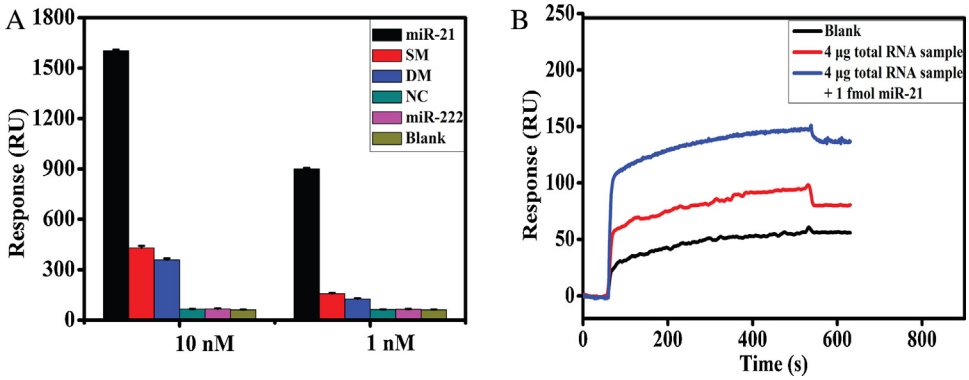


Fig. 5. (A) The SPR response signal for miR-21, single-base mismatch target (SM), double-base mismatch target (DM), non-complementary sequence (NC) and miR-222 at the concentrations of 10 nM and 1 nM, respectively. (B) SPR sensorgrams obtained from blank (black line), 4 μg total RNA sample (red line), 4 μg total RNA sample spiked with 1 fmol miR-21 (blue line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the hairpin structure of CP [39]. In addition, the reproducibility of the established biosensing system was also investigated. Five replicate measurements of miR-21 at the concentrations of 0.1 nM and 1 nM were performed, and the relative standard deviations were 6.3% and 6.1%, respectively, which demonstrated good reproducibility.

In order to study the reusability of the biosensor, repeated regenerations were operated on the same chip. After 20 regenerations, the change of SPR signal was less than 10%, suggesting the same sensor chip could be reused 20 times at least.

3.6. Application of miRNA assay

The SPR biosensor reported here was then employed to detect real miRNA samples. Total RNA extracted from human breast adenocarcinoma MCF-7 cells was used for the assay. The total RNA sample was diluted to 0.4 μg μL^{-1} with DEPC-treated water, and 10 μL total RNA sample diluted to 100 μL with hybridization buffer (4 μg in total) was used for measurement. The SPR response produced by 4 μg total RNA can be clearly distinguished (Fig. 5B). Using the calibration curve established above (Fig. 4D), the amount of miR-21 in the total RNA sample was estimated to be 1.2 fmol in the 4 μg sample. To further evaluate the sensor's performance in real sample analysis, 1 fmol miR-21 was spiked into 4 μg MCF-7 total RNA, and the amount of miR-21 in the spiked sample was estimated to be 2.3 fmol with the recovery rate of 108.3%. These results clearly demonstrated that the analysis was not compromised by the complex components. Therefore, the established miRNA biosensing assay can provide a potentially very useful platform for the detection of miRNA in real biological samples.

4. Conclusion

In summary, we have successfully developed a simple, rapid, and enzyme-free SPR biosensor for miRNA detection. With the cascade signal amplification based on DNA super-sandwich assemblies and biotin–strepavidin system, as low as 9 pM target miRNA could be detected in 30 min. The established miRNA assay exhibits excellent specificity, reproducibility and precision. This novel SPR biosensor offers a highly effective alternative platform for miRNA analysis and clinical diagnostics, and could be further extended to detect and quantify a wider range of DNA and RNA analytes.

Acknowledgments

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