Clinica Chimica Acta 502 (2020) 9–14

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Multi-marker diagnosis method for early Hepatocellular Carcinoma based on surface plasmon resonance

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ARTICLE INFO

Keywords:
SPR
AFP
miRNA-125b
Early Hepatocellular Carcinoma diagnosis
Multi-marker combined detection

ABSTRACT

Early diagnosis of Hepatocellular Carcinoma (HCC) is an important means to raise the survival rate of patients. Multi-marker combined detection is a powerful tool of early HCC diagnosis. Traditional detection methods are not effective and accurate because it is difficult to achieve combined detection of multiple markers. In this paper, we selected Alpha Fetoprotein (AFP) and miRNA-125b as the combined detection markers to improve the simultaneously diagnostic sensitivity and specificity. The anti-AFP monoclonal antibody and the DNA probes paired with the miRNA-125b were modified on the surface of surface plasmon resonance (SPR) sensor respectively to specifically recognize AFP and miRNA-125b in serum. In order to enhance the SPR response signal and detection sensitivity, Double Antibody Sandwich Method (DASM) and S9.6 antibody enhanced method were applied to achieve low detection limit of the two markers. Experimental results showed that AFP (25–400 ng/mL) was accurately detected by DASM and the detection limit of miRNA-125b by S9.6 antibody enhanced method reached 123.044 pM. These results verified the feasibility of the multi-marker detection method in early diagnosis of HCC.

1. Introduction

HCC is the second leading cause of cancer death in China and the overall mortality and morbidity are on the rise [1–2]. Early diagnosis can improve the therapeutic effects and the prognosis of HCC patients [3]. Therefore, it is an important means to improve the survival rate. Although the noninvasive imaging has the high detection rate and accuracy in the diagnosis of HCC, it is often used for advanced diagnosis [4–5]. Studies have shown that, compared with other diagnostic methods, tumor can be detected 3–5 months in advance or tumor recurrence and metastasis can be diagnosed by HCC markers detection in serum. Therefore, the study of tumor markers in serum is the breakthrough point of early tumor diagnosis [6]. The serum AFP is a widely used maker of HCC. However, it remains insensitive and nonspecific in certain circumstances and is therefore imperfect, particularly in the detection of early HCC [7]. The HCC markers other than AFP mainly includes AFP heterogeneity, Des-gamma carboxyprothrombin (DCP), heat shock protein 70 (HSP70), and MicroRNAs. The recognition rate of AFP heterogeneity is low, and it is difficult to detect quantitatively. The significant variation of DCP and HSP70 concentration are related to various physiological changes in human body. Therefore, DCP and HSP70 are less specific as HCC markers [8–10]. MicroRNAs (miRNAs) are a class of non-coding RNA molecules. Through a large number of studies, the mechanism of miRNAs in serum on tumors is more and more clear, and miRNA expression profiles can be drawn for tumors in different parts of the body [11]. Therefore, the use of miRNAs as early tumor markers has become a research hotspot in recent years. In 2008, Li et al. found the miRMA-125b inhibits cell growth and phosporylation of Akt in hepatoma cells and demonstrated the diagnostic miRNA profile for HCC, and for the first time, identified the miRMA-125b with predictive significance for HCC prognosis [12]. In 2012, Liang et al. found miRNA-125b exerts tumor-suppressive effects in HCC through the suppression of oncogene LIN28B expression. The miRNA-125b is underexpressed in most cases of HCC and is inversely related to cell proliferation index in HCC [13]. Comparing the above HCC makers, AFP and miRNA-125b are preferred for the early diagnosis of HCC.

The methods for quantitative detection of AFP in serum, include one-step sandwich immunoassay [14], radioimmunoassay [15–16], fluorescence immunoassay [17–18], immunochromatography test strip [19], and immunosensor [20–22]. The surface plasmon resonance (SPR) based immunoassay gradually replaces the traditional detection method by virtue of its high specificity, good repeatability, simple sample preparation, relatively low cost and short response time. In 2007, Teramura et al. developed an SPR-based AFP assay. However,

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https://doi.org/10.1016/j.cca.2019.12.007
Received 26 August 2019; Received in revised form 21 November 2019; Accepted 8 December 2019
Available online 11 December 2019
0009-8981/

low-concentration AFP could not be detected using monoclonal antibody due to surface noise of the self-made chip. The sandwich-type immunoassay using antibody, monoclonal and polyclonal, reaches the detection range of 0–500 ng/mL [23]. In 2015, Liang et al. used U-bend fiber-optic LSPR biosensor for AFP detection with a detection limit of 5–200 ng/mL [24]. For miRNAs detection, a number of methods have been developed, such as northern blot analysis [25], microarray analysis [26], multiplex ligation-dependent probe amplification (MLPA) [27], SPR biosensor [28], electrochemical sensing platform [29], and the like. As the SPR technology can detect not only the interaction between antigens and antibodies, but also that between DNA and protein, DNA and DNA, the SPR-based immunoassay has become a research point in miRNA detection [30–32]. In 2015, Ding et al. adopted DNA super-sandwich assemblies for label-free detection of miRNA. The detection limit was as low as 9 pM, but another two additional auxiliary probes and signal enhancement protein were added and the process was more complicated [33]. In 2016, Wang at al. developed a novel simple GO-AuNPs-based SPR biosensor (GOAu-SPR) for miRNA detection, in which GO-AuNPs hybrids were used as signal enhancement markers. The detection limit was as low as 1 fM. But the preparation of GO-AuNPs hybrids and the reaction of miRNAs with hybrids took too long to clinical use [34]. In 2017, Liu developed a universal biosensor by using multiple signal amplification strategy to enhance SPR signal for miRNA detection. It can detect miRNA-21 as low as 0.6 fM. However, it needs two more probes and silver nanoparticles for signal enhancement. Also the detection process was too complicated to practical application [28]. Although multi-marker detection could enhance the specificity and sensitivity of HCC diagnosis, there are no reports of joint detection of AFP and miRNA based on SPR up to present.

In this paper, a multi-marker detection method was proposed to measure the concentration of AFP and miRNA-125b simultaneously in one sample using one single SPR chip through one simple step measurement. Due to the problems of complex serum component and the low concentration of AFP and circulating miRNA-125b, the probe molecules of AFP and miRNA-125b were modified on two different areas of the SPR surface to achieve specific detection respectively in a three-channel reference measurement. Furthermore, Double Antibody Sandwich Method (DASM) and S9.6 antibody enhanced method were proposed to improve the specificity and sensitivity of multi-marker detection. This method makes it possible to use one single instrument to detect multiple markers of HCC in clinic.

2. Material and methods

2.1. Method for detecting AFP based on SPR

The CM5 chip in SPR detection system (Biacore 3000, GE, America) was selected for AFP and miRMA-125b detection. The gold surface of the chip is modified with a layer of carboxymethyl dextran matrix. The carboxyl groups need to be activated before binding antibodies to the chip by amine coupling. Then the antibody is bound to the chip by the reaction between the active group and the amino group on the monoclonal antibody (as shown in Fig. 1a).

In the process of pretreating the CM5 chip, the four channels of the CM5 chip were first washed successively with NaOH solution (50 mM), HCl solution (10 mM), SDS solution (0.1%), and H_3PO_4 solution (0.085%). Then, a mixture (100 μL) of EDC (0.4 M) and NHS (0.1 M) with a volume ratio of 1:1 was introduced into the four channels to carry out an activation reaction, and the PBS buffer was injected to remove the residual EDC/NHS mixture. In the subsequent experiment, the first channel was selected as the reference channel, and the second and third channels were used for AFP and miRNA detection respectively. The negative effects caused by non-specific adsorption and equipment noise can be reduced by the differential measurement between the reference and detection channels. AFP and miRNA detection channels were set in series to realize the joint detection of AFP and miRNA.

To achieve specific detection of AFP, the highly homogeneous antibodies produced by a single B cell clone were selected, diluted (150 ug/mL, pH 5.0, 30 μL), and injected into the second channel of the chip with the optimized flow rate of 5 uL/min. After sufficient reaction between protein molecule and the chip, the residual monoclonal antibody was removed by the PBS buffer. 100 μL of ethanolamine-hydrochloric acid solution was introduced into the binding channel to block the excess active site, and then the PBS buffer was added to remove the residual ethanolamine-hydrochloric acid solution. The SPR response curve of the binding process is shown in Fig. 1b. At the same time, ethanolamine was used to block the reference channel without binding antibodies. In the direct measurement of AFP, the AFP solutions with concentrations of 25 ng/mL, 50 ng/mL, 100 ng/mL, 150 ng/mL, 200 ng/mL, 250 ng/mL, 300 ng/mL, 350 ng/mL, and 400 ng/mL were injected. And then the specific combination of AFP with monoclonal antibodies on the golden surface causes the change of SPR signal, which is used to detect AFP concentrations in the sample flowing through the chip surface.

In order to improve the detection sensitivity of AFP, the polyclonal antibody (20 μg/mL) that can specifically recognize AFP was introduced after AFP specifically bound to the monoclonal antibody on the chip surface. One AFP molecule has multiple sites that can bind multiple polyclonal antibody molecules. A sandwich structure of monoclonal antibody, AFP, and polyclonal antibody was formed to amplify the SPR response signal, and the method was known as DASM. The principle of APF measurement is shown in Fig. 1c. Then 300 s of PBS washing was set to dissociate the AFP molecule and the polyclonal antibody molecule. If the residual SPR response ΔRU was more than 20 after the rinsing, 5 mM NaOH solution was used for chip regeneration. It is worth noting that the regeneration process should be performed by multiple injections of NaOH solution in small volume to avoid excessive regeneration, which may wash off the anti-AFP monoclonal antibody bound on the chip surface and shorten the sensor life. Except for special instruction, the temperature of all experiments was set at 25° C and the flow rate was 10 μL/min.

2.2. Method for detecting miRNA-125b based on SPR

Nucleic acid molecules cannot be modified on the surface of CM5 chip by covalent bond coupling, therefore taking the biotin-avidin system as medium to bind the DNA probe to the chip surface. The principle of DNA probe binding is shown in Fig. 2a.

For miRNA-125b detection, the chip cleaning, activation and reference channel processing were the same as mentioned in Section 2.1. The third channel of the CM5 chip was selected as miRNA-125b detection channel. After the chip was activated, 30 μL of streptavidin dilution (200 μg/mL, pH5.0) was applied to the chip surface at a flow rate of 5 μL/min, and then PBS buffer was added to remove residual streptavidin in the channel. The excess active site was then blocked with an ethanolamine-hydrochloric acid solution. 10 μM biotinylated DNA probe solution was introduced into the measurement channel at a flow rate of 5 μL/min to ensure the DNA probe fully react with streptavidin. The SPR response curve of the DNA probe binding process is shown in the Fig. 2b.

For direct detection, samples with different miRNA-125b concentrations were introduced into the SPR sensor chip to form DNA-RNA hybrid by complementary base binding between the DNA probe and miRNA-125b. The formation of hybrids caused changes of the refractive index on the chip surface, thus affecting the SPR response signal.

To enhance the SPR response signal, the S9.6 antibody of 10 μg/mL was introduced to specifically recognize and bind with the DNA-RNA hybrid. The S9.6 antibody enhanced method can reduce the detection limit and improve the detection sensitivity of miRNA-125b. The principle of miRNA-125b measurement is shown in Fig. 3. After S9.6 antibody enhanced measurement, the chip needs to be washed with PBS for

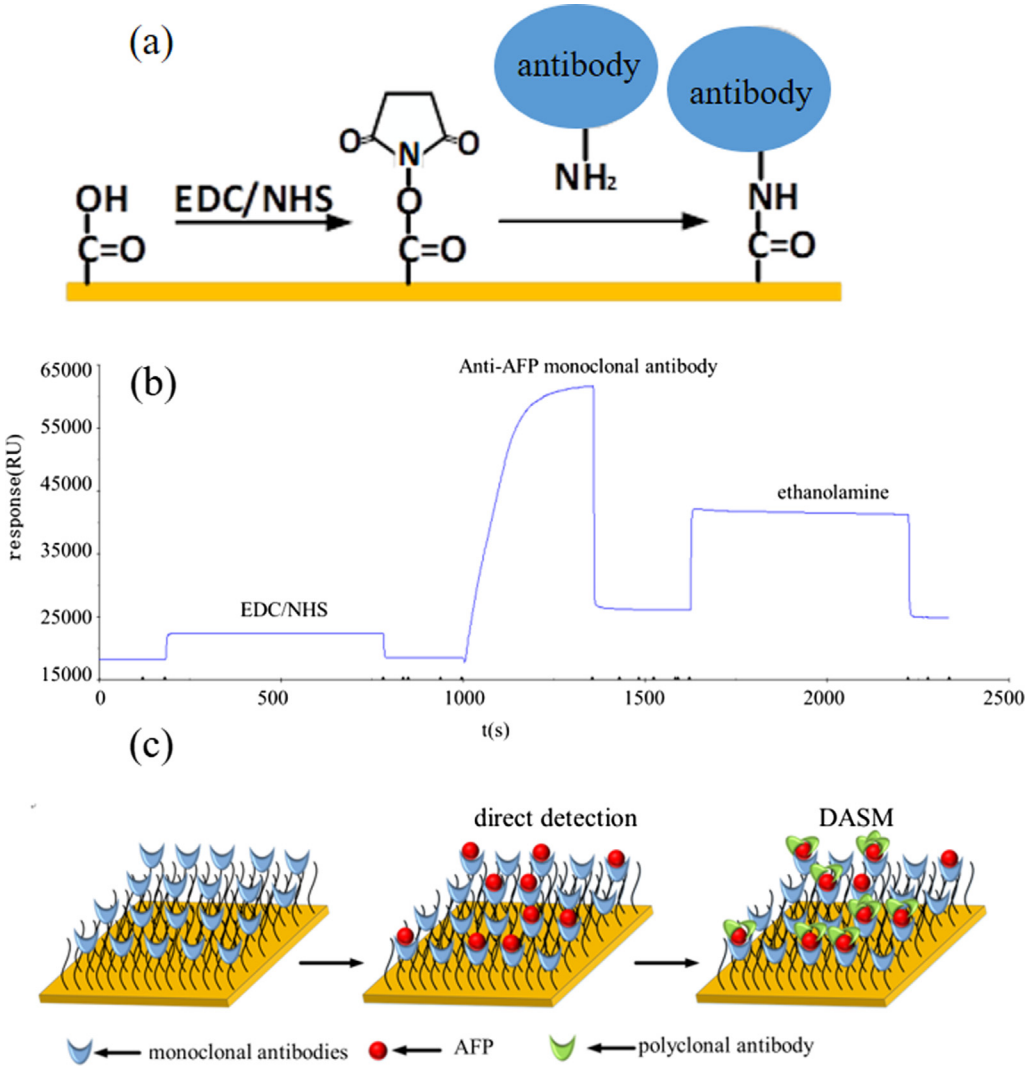

Fig. 1. (a) Schematic diagram of binding AFP monoclonal antibody to the surface of sensor chip, (b) Response curve of anti-AFP monoclonal antibody binding process, (c) Schematic diagram of AFP measurement principle.

5 min first to complete the dissociation of DNA-RNA hybrid with S9.6 antibody, and then rinsed with 10 mM NaOH solution to wash away the miRNA-125b that bound to the DNA probe and achieve chip regeneration. While for direct measurement, only NaOH solution was injected for chip regeneration.

2.3. Combined detection method of AFP and miRNA-125b

The combined detection has the same principle and detection process with the separate detection. In the combined detection, AFP antibody and DNA probe were modified in parallel in the second and third channel of the SPR sensor chip. To detect HCC markers, the sample containing AFP and miRNA-125b was injected into the sample port and serially passed through the first, second, and third channels. Then, the mixture of AFP polyclonal antibody and S9.6 antibody were also serially flowed through the first, second, and third channels. The AFP antigen–antibody complex formed in the second channel was specifically bound to the anti-AFP polyclonal antibody, while the DNA-RNA hybrid formed in the third channel was specifically bound to the S9.6 antibody, thus affecting the SPR response signal of the two channels. The combined detection was realized through these signal variations.

3. Results

3.1. Experimental results of AFP detection based on SPR

The clinical detection range of AFP in serum is 0–400 ng/mL. The AFP concentrations higher than 100 ng/mL and 400 ng/mL indicate low concentration positive and high concentration positive respectively, and both of them need further examination of HCC. In the experiment, we measured AFP with concentrations in the range of 25–400 ng/mL by direct measurement and DASM.

The AFP measurement results are shown in Fig. 4. Both the direct measurement method and DASM got good linearity, but DASM showed better linear correlation and higher sensitivity in the detection of AFP concentration. Under the amplification effect of DASM, the SPR technology is suitable for AFP detection in the clinical range.

3.2. Experimental results of miRNA-125b detection based on SPR

Direct measurement and S9.6 antibody enhanced methods were used to detect miRNA-125b concentrations in three different ranges, 6.25–100 nM, 0–8 nM, and 0–1000 pM.

The measurement results of miRNA-125b in different concentration ranges are shown in Fig. 5. Comparing the measurement results of miRNA-125b in the range of 6.25–100 nM (as shown in Fig. 5a and

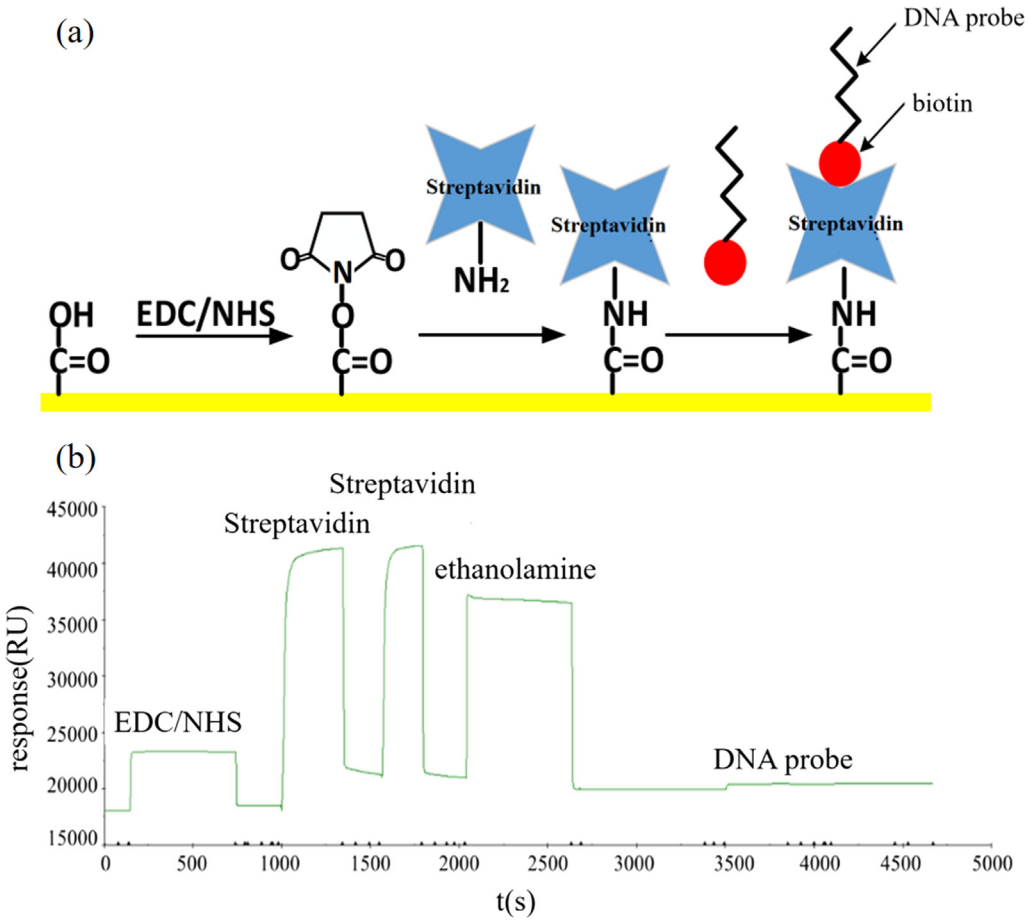

Fig. 2. (a) Schematic diagram of binding DNA probe to the surface of sensor chip, (b) Response curve of DNA probe binding process.

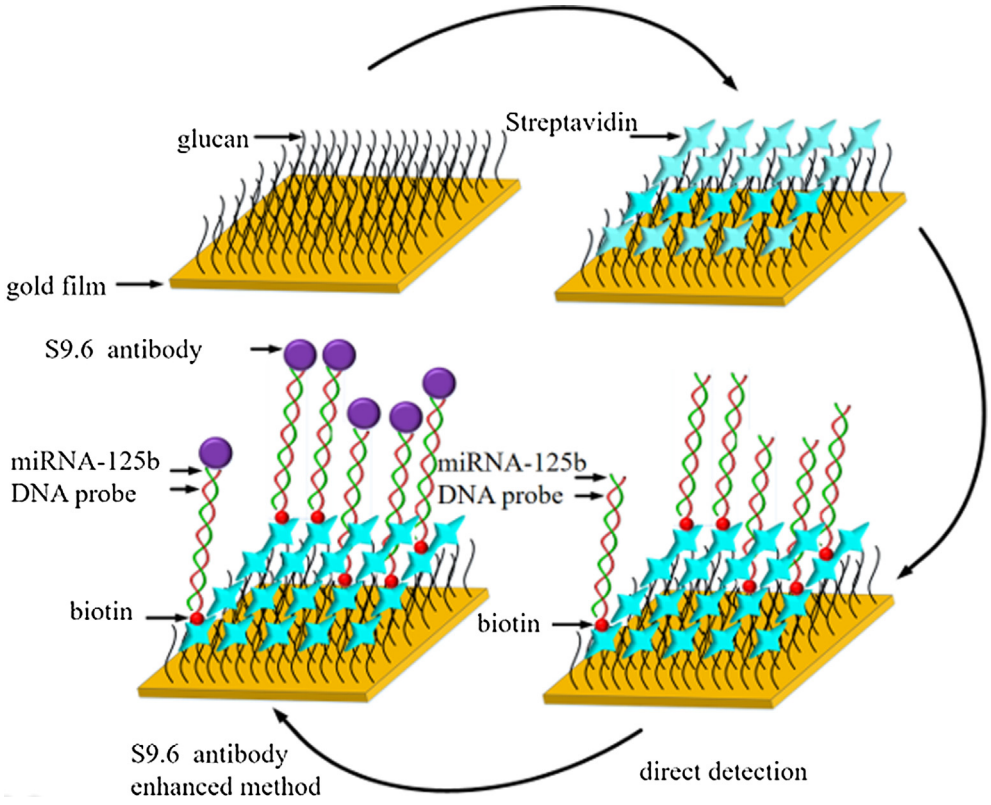

Fig. 3. Schematic diagram of miRNA-125b measurement principle.

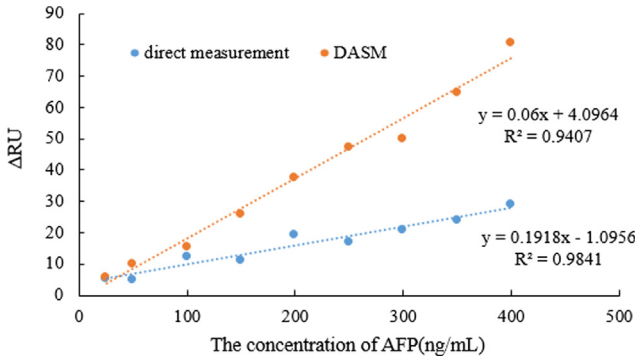

Fig. 4. Comparison of AFP measurement results between direct measurement and DASM.

Fig. 5b), the S9.6 antibody enhanced method obtained higher SPR response signal in the higher concentration range, however the corresponding signal fluctuation becomes more serious. Therefore, the direct measurement got a better linearity than S9.6 antibody enhanced method. The reason for signal fluctuation of enhanced method may be that S9.6 antibodies are not bound to DNA-RNA hybrids one-to-one when the concentration of miRNA-125b is high. Fig. 5c and Fig. 5d show the measurement results of miRNA-125b in two different concentration ranges of 0–8 nM and 0–1000 pM. In the lower concentration ranges (0–8 nM, 0–1000 pM), the direct measurement method was not sensitive enough for miRNA-125b detection, while the S9.6 antibody enhanced method with higher sensitivity can fulfill the requirement of lower concentration detection. The limit of detection (LOD) [24] of miRNA-125b by S9.6 antibody enhanced method reached 123.044 pM. LOD = 3 (SD/S), where SD is the standard deviation of the signal values obtained in seven blank tests and S is the sensitivity. From the above, the S9.6 antibody enhanced method was selected for the detection of the lower concentration range of miRNA-125b, and the direct measurement method was suitable for the higher concentration range.

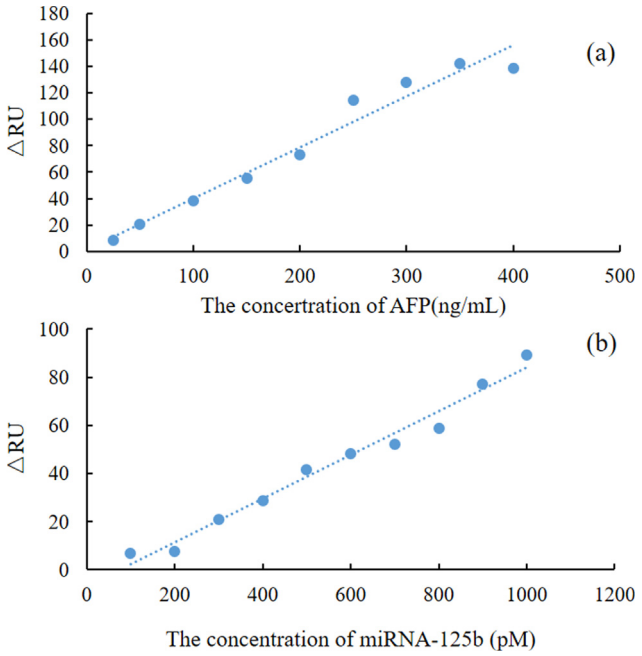

Fig. 6. (a) Fit curve of AFP concentration and SPR response signal in combined detection. (b) Fit curve of miRNA-125b concentration and SPR response signal in combined detection.

3.3. Combined detection results of AFP and miRNA-125b based on SPR

In view of the clinical need for AFP detection and the low level of circulating microRNAs in serum, the combined detection of AFP (concentration range 25–400 ng/mL) and miRNA-125b (concentration

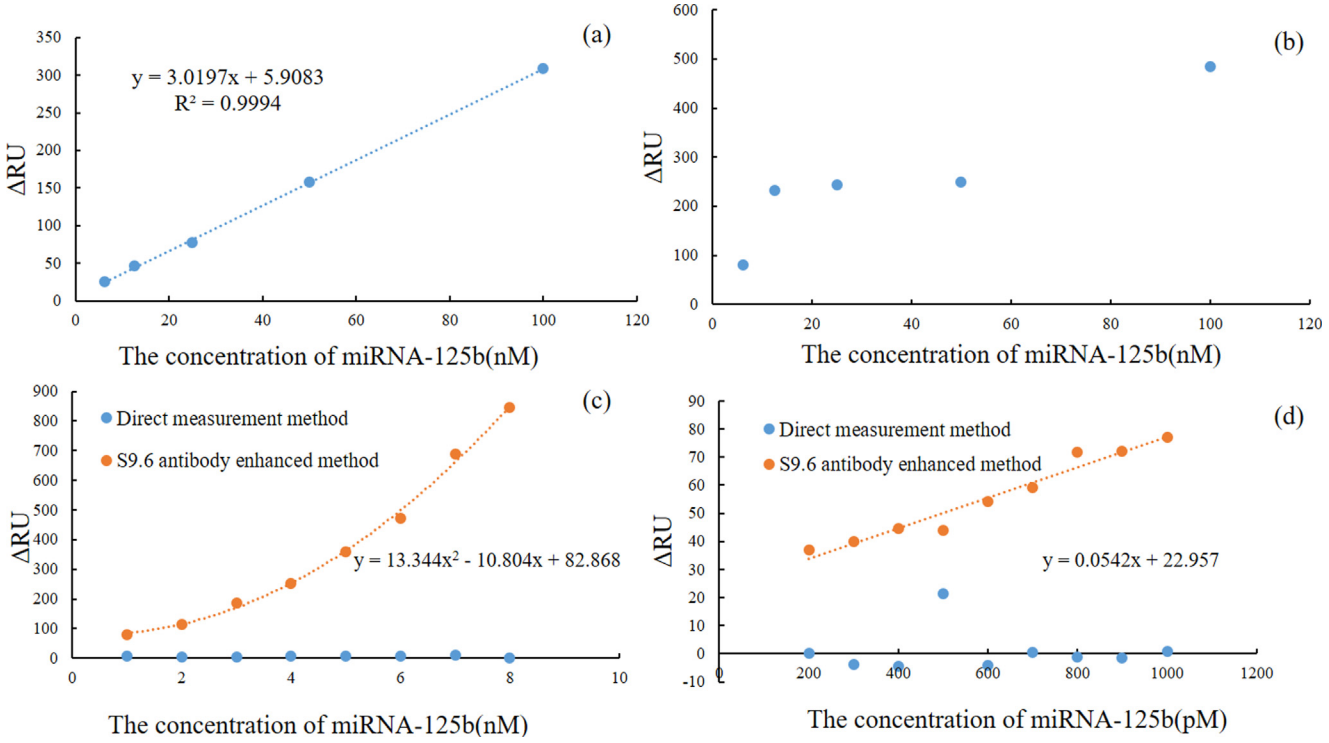

Fig. 5. (a) The fit curve of direct measurement results for miRNA-125b (6.25–100 nM), (b) The measurement results of miRNA-125b (6.25–100 nM) using S9.6 antibody enhanced method, (c) The fit curve of measurement results for miRNA-125b (0–8 nM), (d) The fit curve of measurement results for miRNA-125b (0–1000 pM).

range 0–1000 pM) in mixed sample solution was carried out by antibody enhanced method.

The results of combined detection of AFP and miRNA-125b are shown in Fig. 6. In combined detection, both of AFP in the clinical detection range and miRNA-125b with concentrations lower than 200 pM were specifically recognized and sensitively measured. It shows the feasibility of joint detection of HCC markers, and is expected to achieve early diagnosis of HCC.

4. Conclusions

In this paper, we proposed a method for early detection of HCC based on SPR sensor by using AFP and miRNA-125b as combined HCC markers. AFP monoclonal antibody was selected to bind on the chip surface by amine coupling, realizing the specific detection of AFP, and DASM was applied to enhance the detection sensitivity of AFP. In addition, the DNA probe was bound to the surface by the biotin-avidin system, and the S9.6 antibody enhanced method was used to improve the detection sensitivity of miRNA-125b. Differential measurement of AFP and miRNA-125b was performed with two parallel channels modified by AFP antibodies and DNA probes respectively and one reference channel for background signal subtraction. Test results show that the AFP detection can reach the clinical detection range (25–400 ng/mL), and the low concentration of miRNA-125b can be detected accurately.

CRediT authorship contribution statement

Haixia Yu: Conceptualization, Methodology, Data curation, Funding acquisition, Writing - original draft, Writing - review & editing. **Ruixue Han:** Conceptualization, Methodology, Data curation, Writing - original draft, Writing - review & editing. **Jie Su:** Conceptualization, Methodology, Data curation, Writing - original draft, Writing - review & editing. **Hailong Chen:** Writing - original draft, Writing - review & editing. **Dachao Li:** Conceptualization, Funding acquisition, Writing - review & editing.

Funding

This work is supported by the National Key R&D Program of China (No. 2018YFE0205000, No. 2017YFA0205103), the National Natural Science Foundation of China (No.)81571766, the Natural Science Foundation of Tianjin City (No. 17JCYBJC24400), and the 111 Project of China (No. B07014).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.12.007.

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