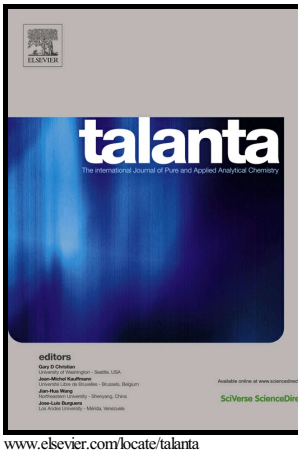

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An Enzyme-Amplified Lateral Flow Strip Biosensor for Visual Detection of MicroRNA-224

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Abstract

An enzyme-based dual-labeled nanoprobe is designed to fabricate a sensitive enzyme-amplified lateral flow biosensor for visual detection of mircoRNA-224 (miRNA-224). The recognition DNA probe (detection probe) and signal amplification enzyme (Horseradish peroxidase, HRP) are immobilized on gold nanoparticle (GNPs) surface, simultaneously. The capture DNA probes are immobilized on the test zone of the lateral flow biosensor. When miRNA-224 is present, the enzyme-based dual-labeled nanoprobes will be captured by forming the “sandwich structure” on the test zone of the lateral flow biosensor, enabling the visual detection for miRNA-224. Sensitivity is amplified by applying the 3,3,5,5-tetramethylbenzidine enzymatic substrate (TMB/H_2O_2 enzymatic substrate) onto the test zone. The enzymatic reactions between the HRP and the TMB/H_2O_2 enzymatic substrate will produce blue products, which deposit on the nanoprobe surface to enhance the visual effect and the corresponding response intensities of the test zone. This enzyme-amplified lateral flow biosensor shows a low limit of detection (LOD) (7.5 pM) toward miRNA-224 in the buffer solution, which is improved by 10-fold than that of the single-labeled lateral flow biosensor. This biosensor has been successfully used for the detection of

the target miRNA-224 detection in A549 cell lysate.

Keywords: MicroRNA; Lateral flow biosensor; Visual detection; Signal amplification; Horseradish peroxidase

1. Introduction

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs of 18–25 nucleotides that can regulate gene expression by interacting with messenger RNAs at sequence-specific sites to catalyze the cleavage of the mRNAs or inhibit translation [1, 2]. MiRNAs are involved in essential biological processes including development [3, 4], differentiation [5, 6], apoptosis [7], metabolism [8]and cell proliferation [9, 10]. It has been suggested that miRNAs expressions are differentially expressed in normal and tumor tissues [11, 12]. Thus, miRNAs expression alterations are relevant to some malignant diseases, making them strong candidates, as well as potential diagnostic markers specific to corresponding tissues or diseases, especially cancers [13, 14]. However, the detection of miRNAs has significant difficulties, due to their short length, trace-amounts and sequence homology among family members [15, 16]. The traditional strategies for detection of miRNA, such as Northern Blot Technique [17, 18], microarrays [19-21], and real-time polymerase chain reaction (RT-PCR) [22, 23], offer ideal platforms for multiplex miRNA expression analysis. However, the requirement for intensive sample, laborious and time-consuming processes impede their effectiveness of miRNA detection [24-26]

Recently, various nucleic acid biosensors have been proposed to detect miRNAs [27, 28]. We have also reported a simple gold nanoparticles (GNPs)-DNA based lateral flow nucleic acid sensor for miRNA assay [29]. The captured GNPs on the test zone enabled the visual and quantitative detection of miRNA. Compared with other nucleic acid biosensors, the lateral flow device based nucleic acid sensors showed the advantages of simplicity, rapid analysis, low cost, and specificity in the miRNA detection, indicating the potential for clinical application and biomedical diagnosis in the malignant diseases.

In this study, an enzyme-amplified lateral flow strip biosensor (E-LFSB) is presented. The detection DNA probe is conjugated simultaneously with amplification enzyme (HRP) on GNPs surface. The capture DNA probe is immobilized on the test zone of the E-LFSB. When the target miRNA-224 is present, the target analyte is sandwiched between the capture DNA probe (immobilized on the test zone of the E-LFSB) and the detection DNA probe (conjugated simultaneously with HRP on GNPs surface). The accumulation of the GNPs enables the visual detection of miRNA-224. The sensitivity is improved by introducing the enzymatic substrate (TMB/H_2O_2 enzymatic substrate) onto the test zone where the HRP is accumulated. The products from catalytic reactions between the HRP and the TMB/H_2O_2 enzymatic substrate exhibit blue color [30, 31] and deposit on the GNPs surface to enhance the visual effect and the intensities of red bands on the test zone of the E-LFSB. The E-LFSB shows 10-fold improvement in the sensitivity compared with single-labeled LFSB. The test for miRNA-224 on E-LFSB can be accomplished within 30 min. A549 cell lysate is selected as the real sample, since miRNA-224 plays a crucial role in A549 cell cycle progression through coordinately regulating the expression of key cell cycle transcripts. After the experimental optimizations, the miRNA-224 can be successfully detected in A549 cell lysate without complex sample pretreatment. This simple and fast approach is practical for sensitive detection of miRNAs in real samples with simple processes.

2. Experimental

2.1 Apparatus and Reagents

The Airjet AJQ 3000 dispenser, Biojet BJQ 3000 dispenser, Clamshell Laminator, and the Guillotine cutting module CM 4000 were purchased from Biodot LTD (Irvine, CA). The DT1032 portable strip reader was purchased from Shanghai Goldbio Tech. Co., LTD (Shanghai, China).

Streptavidin was purchased from Calbiochem (Germany). Horseradish peroxidase (HRP), 3,3,5,5-tetramethylbenzidine (TMB) TMB/H_2O_2 enzymatic substrate (T8665), $HAuCl_4$, sucrose, hydroxylamine, $Na_3PO_4 \cdot 12H_2O$, Tween 20, Triton X-100, trisodium citrate, bovine serum albumin (BSA), sodium chloride-sodium citrate (SSC) buffer (pH = 7.0), and phosphate buffered saline (PBS, pH = 7.4) buffer were purchased from Sigma-Aldrich and used without further purification. Glass fibers (GF-06), cellulose fiber sample pads (JN8025), and laminated cards (MT101B) were purchased from Jiening Biological Technology & Co.Ltd. (Shanghai, China). Nitrocellulose membrane (HFB18002) was purchased from Millipore (Billerica, MA).

Target miRNAs were purchased from Suzhou Gene-Pharma Co., Ltd. (Suzhou, China) and purified using high-performance liquid chromatography:

Target miRNA-224: 5'-AAA AUG GUG CCC UAG UGA CUA CA-3'

Interference miRNA: 5'-GGA UUA UUG UUA AAU AUU GAU AAG GAU-3'

The oligonucleotides were purchased from Sangon Biological Engineering Technology & Co.Ltd. (Shanghai, China) and purified using high-performance liquid chromatography. The sequences were as follows:

Detection probe (probe 1): 5'-/ThioMC6-D/-TGT AGT CAC-3'

Capture probe (probe 2): 5'-GGC ACC ATT TT-/Biotin/-3'

Control probe (probe 3): 5'-/Biotin/-GTG ACT ACA-3'

2.2 Preparation of Dual-Labeled Bioconjugates

The dual-labeled bioconjugates HRP-GNPs-DNA were prepared according to the reported method with slight modifications [32]. 20 μL of HRP solution (1 %, w/v) was added to 1 mL of GNPs (The GNPs with average diameter of 15 nm were synthesized by the procedures reported previously [29]). The pH of this mixture solution was adjusted to 9.0 by adding diluted NaOH solution (0.1 M). The mixed solution was incubated for 30 min at 37 °C with gentle shaking, and then this solution was centrifuged under 4 °C with a rate of 13000 rpm for 30 min. The supernatant clear solution was discarded and the remaining nanoparticles in the bottom of the tube

were re-suspended in 200 μL of water. Subsequently, 20 μL of 2 M of NaCl/10 mM of $Na_3PO_4 \cdot 12H_2O$ buffer solution (pH 7.4) was dropped into the mixture at a rate of 1 μL/30 min. The low speed for adding the salt solution is to decrease the aggregation of nanoparticles at the greatest extent, as well as enabling more DNA probes and HRP to be immobilized on the surface of GNPs, which will lead to the high sensitivity for detection. The thiolated DNA detection probe was added into the solution to a final concentration of 1.5 μM, and then incubated at 37 °C for 3 h. 20 μL of 10 % BSA solution was added to the passivate the GNPs for 30 min. The mixture was centrifuged (13000 rpm, 30 min, 4 °C) and the supernatant was discarded. After being washed with 200 μL of PBS for 3 times to remove the unbound HRP and DNA, the as-obtained ruby sediments were re-suspended in 200 μL of stock solution (20 nM of $Na_3PO_4 \cdot 12H_2O$ containing 5% BSA, 0.25% Tween-20 and 10% sucrose) and stored at 4 °C for further use.

2.3. Preparation of Streptavidin-Biotinylated DNA Conjugates

The preparation of streptavidin-biotinylated DNA conjugates followed the previously reported methods [29]. 200 μL of 2.5 mg/ml of streptavidin was mixed with 50 nmol biotinylated DNA probe (capture probe/control probe). The mixture was incubated on a shaker for 1 h. After adding 500 μL PBS into the mixture, the solution was centrifuged in dialysis tube for 20 minutes at 6000 rpm under 4°C. The above steps were repeated for 3 times to remove the unbound DNA. The remaining solution in filter was diluted to 600 μL with PBS.

2.4. Preparation of E-LFSB

The E-LFSB consisted of four components: sample application pad, conjugate pad, nitrocellulose membrane, and absorption pad. All components were laminated into a sheet of plastic orderly using the Clamshell Laminator (Biodot, Irvin, CA). The sample application pad was made from cellulose fiber (JN8025, Jiening) and saturated

with a Tris-HCl buffer (pH 8.0) containing 0.23% of Triton X-100, 0.05 M of Tris-HCl and 0.15 M of NaCl. Then, the pad was dried at 37°C for 2 h and stored in desiccators at room temperature (RT). The nitrocellulose (NC) membrane was pretreated and blocked by BSA solution [33]. The test zone and control zone on the NC membrane were prepared by dispensing streptavidin-biotinylated capture probe and streptavidin-biotinylated control probe solutions, respectively. The distance between the test and control zones was 3 mm. The membrane was then dried at 37°C for 1 h and stored at 4°C in a dry state. Finally, the sample pad, conjugate pad, nitrocellulose membrane, and absorption pad were assembled on a plastic adhesive backing (60 mm × 30 cm) using the clamshell laminator. Each part overlapped 2 mm to ensure that the solution could migrate through the E-LFSB during the assay. E-LFSBs with a 3-mm width were cut using the Guillotin CM 4000 cutting module [34-36]. The DNA-GNPs-HRP conjugate was dropped on the conjugate pad prior to the test.

2.5. Assay Procedure

A typical miRNA test on E-LFSB contains two steps of reactions: 1) hybridization reaction and 2) signal enhancement. In the first step, 5 μL of DNA/GNPs/HRP conjugate was initially loaded on the conjugate pad. Then 100 μL of running buffer (25% SSC buffer containing 4% BSA) with the desired amount of target miRNA-224 was applied to the sample pad. During the assay process, the solution migrated up by capillary force. After a complete hybridization assay, the accumulation of HRP-GNPs-DNA bioconjugates on the test zone and the control zone of the E-LFSB was visualized as a characteristic red band within 20 min. In the final step, 0.7 μL of "signal amplification solution" (TMB/H_2O_2 enzymatic substrate) was applied on the test zone and control zone, respectively. The enzymatic reactions between the captured HRP on the E-LFSB and TMB/H_2O_2 enzymatic substrate produced insoluble blue products, which deposited on the GNP surface to enhance the visual effect and the intensities of red bands within 9 min. The whole process required only 30 min.

For quantitative measurements, the optical intensity of the blue band was recorded using the portable "strip reader" instrument combined with "GoldBio strip reader" software.

3. Results and Discussion

3.1. Principle of E-LFSB

Scheme 1

The principle of E-LFSB based on HRP-GNP-DNA dual-labeled bioconjugate for miRNA-224 detection is illustrated in Scheme 1 and enzyme modification was introduced into a lateral flow device. As shown in Scheme 1a, a model enzyme, HRP, is firstly immobilized on the surface of the GNP through an absorption process, and then the thiolated detection DNA probe is introduced to the leftover surface of the GNP through a self-assembling method [32]. The two biotinylated DNA probes (capture probe and control probe) are pre-immobilized on the nitrocellulose membrane through biotin–streptavidin interaction to form test zone and control zone respectively. In a typical hybridization assay, the sample solution containing target miRNA is applied on the sample pad. The solution migrates by capillary action and passes the conjugate pad, and then rehydrates HRP-GNP-DNA bioconjugates. The target miRNA hybridizes with the thiolated detection probe of the HRP-GNP-DNA bioconjugates. The as-formed hybrids continue to migrate along the strip and are captured on the test zone through the reaction between the target miRNA and the capture probe. The accumulation of HRP-GNP-DNA bioconjugates on the test zone produces a characteristic red band. The excess HRP-GNP-DNA bioconjugates continue to migrate through the control zone and are captured by the control probes, thus forming the second red band on the control zone (Scheme 1b). The formation of this "sandwich type" structure was reported previously [29]. Meanwhile, the HRP is captured on the test and control zones. Followed by applying TMB/H_2O_2 enzymatic substrate to the test zone and control zone, the enzymatic reactions between the HRP and TMB/H_2O_2 enzymatic substrate will produce blue products, which deposit on the GNP surface to enhance the visual effect and the intensities of red bands (Scheme 1c).

In the absence of miRNAs, only one blue band can be observed on the control zone.

Fig. 1

Fig. 1 presents the typical photo images of the performances of a) GNPs-DNA single-label-based nucleic acid sensor, b) HRP-GNPs-DNA dual-label-based nucleic acid sensor before and c) after treated with TMB/H_2O_2 enzymatic substrate by testing 0 nM and 0.25 nM of target miRNA-224. As described in Fig. 1a and 1b, relative weak red bands can be observed from the test zones of the single-label-based nucleic acid sensor and the HRP-GNPs-DNA dual-label-based nucleic acid sensor before treated with TMB/H_2O_2 enzymatic substrate. Meanwhile, an obvious increase of the visual effect on the test zone is shown from HRP-GNPs-DNA dual-label-based nucleic acid sensor after treated with TMB/H_2O_2 enzymatic substrate (Fig. 1c), as a result of the deposition of blue products. No obvious enhancement can be observed from control sample. Therefore, this signal enhancement strategy is suitable for sensitive miRNA detection.

3.2. Optimization of the Preparation of E-LFSB

Fig. 2

One of the most important factors affecting the E-LFSB sensitivity and reproducibility is the use of various buffers. Appropriate buffers would minimize the nonspecific adsorption, as well as increasing the sensitivity of the E-LFSB. Four kinds of running buffers, including PBS, Tris-HCl + $MgCl_2$, NaCl + Na_3PO_4 and 1/4 SSC + 4% BSA were investigated by comparing the analytical performances of the E-LFSB on detecting 0.25 nM of miRNA-224 (Fig. 2a). It was found that the best S/N ratio was obtained with the SSC + BSA buffer. The amounts of SSC and BSA in buffer were further optimized, and the optimal amount was 25% of SSC and 4% of BSA in buffer (data not shown).

The amount of HRP/GNPs/DNA conjugate loaded on the conjugate pad has a great effect on the intensities of HRP captured on both test and control zones. Therefore, the effect of HRP/GNPs/DNA conjugate on the E-LFSB response by loading different volumes of HRP/GNPs/DNA conjugate on the conjugate pad was studied (Fig. 2b).

The S/N ratio initially rises with the increase of the conjugate volume, while further increasing the conjugate amount reduces the S/N ratio, because of the increased background signal. Therefore, 3 μL of DNA/GNPs conjugate on conjugate pad was the optimal amount and used in the following study.

The sensitivity is improved by introducing the enzyme modification into the E-LFSB. The blue products produced by the enzymatic reactions will deposit on the GNP surface to enhance the visual effect and the intensities of red bands, as well as increase the E-LFSB sensitivity. Therefore, two factors affecting enzymatic catalytic activity were investigated for optimizing the analytical performances of the E-LFSBs, including the enzymatic reaction time and the amount of TMB/H_2O_2 enzymatic substrate introduced in the enzymatic reactions. The S/N ratios on E-LFSBs for testing of 0.25 nM miRNA-224 are compared with different TMB/H_2O_2 substrate volume ranging from 0.3 to 1.1 μL at different enzymatic reaction times (6 min, 7min, 8 min, 9 min, 10 min, 11 min and 12 min). From Fig. 2c we can see that with the enzymatic reaction time increase, the S/N ratio increases because more blue products are deposited on the test zone. When the reaction time exceeds 9 min, the S/N ratio decreases dramatically as the blue products are shaded into yellow color. Therefore, 9 min of the enzymatic reaction is optimal. Enzyme catalytic activity is dependent on the substrate volume. In the current study, 0.7 μL of TMB/H_2O_2 enzymatic substrate was found to be the optimal and used in the following experiment (Fig. 2d).

3.3. Analytical Characteristics

To evaluate the performances of E-LFSBs, sample solutions containing different concentrations of miRNA-224 (ranging from 0 to 75 nM) were measured by the HRP-GNPs-DNA dual-label-based E-LFSBs under the optimal experimental conditions. Fig. 3 presents the typical photo images and the resulting calibration curve of the E-LFSBs in the presence of miRNA-224 with different concentrations. The visual detection for miRNA-224 can be achieved by observing the blue bands on the test zones of the E-LFSBs. The intensities of the blue bands were recorded by a portable strip reader and the obtained data was applied for the quantitative analysis of

miRNA-224. The detection limit of 7.5 pM for miRNA-224 was estimated based on the 3 times of signal to noise ratio. The sensitivity was improved by 10-fold by applying the E-LFSB for target miRNA analysis compared with the single-label-based lateral flow nucleic acid sensor [29]. The resulting plot of response vs. miRNA concentration was linear over the 7.5 pM to 75 nM range and was suitable for quantitative work. As shown in Fig.3, no obvious response can be observed from the test zone of the E-LFSB in the interference miRNA in absence of target miRNA-224, indicating the nonspecific adsorption has no significant influence on the reaction system for target miRNA detection under the optimized experimental conditions.

Fig. 3

Reproducibility is of significant importance to evaluate the E-LFSBs. The reproducibility of the E-LFSB was examined by testing the sample solutions at different concentration levels (0 nM, 0.25 nM, and 2.5 nM) of miRNA-224 (Fig. S1). Each concentration was tested six times on six E-LFSBs and the corresponding RSD values of optical responses were 8.45%, 8.50% and 3.55% respectively, indicating fine analytical reproducibility.

Another important parameter for evaluating the biosensors is the selectivity. To evaluate the selectivity of the E-LFSB, the performances were examined from the interference miRNA. As shown in Fig. 4, even when the interference sample of miRNA is applied at concentration as high as 25 nM, which is 100-fold in excess as compared to that of target miRNA-224, the response from the interference miRNA remains as low as the background signal. The high hybridization selectivity of the E-LFSB is achieved by taking the advantages of the binding affinity and specificity between the target miRNA-224 and the DNA probes. For the stability (life time) study, the performances of the E-LFSBs were tested after 9 weeks' storage at 4°C. As shown in Fig. S2, compared with one week, the responses of E-LFSBs with 0.25 nM of miRNA-224 remained almost the same after 9 weeks indicating the E-LFSBs have the desirable stability.

Fig. 4

3.4. Detection of MiRNA-224 in A549 Cell lysate with the E-LFSB

The above-mentioned results demonstrate that the E-LFSBs show the intrinsic advantages including the stability, high hybridization specificity, and meanwhile the enzyme modification can offer the signal amplification strategy for miRNA-224 analysis. The intrinsic advantages can merit and meet the demands of miRNA detection in aqueous solutions and real samples. To further evaluate the performances of the E-LFSBs, these biosensors were applied to detect miRNA-224 in A549 cell lysates. The A549 cell lysate samples were prepared by spiking the lysed A549 cells with various amounts into running buffers. Fig. 5 shows the photo images of E-LFSBs for the detection of miRNA-224 in the presence of various amounts of A549 cells. The intensities of the test zone rise with an increase in the A549 cell amount. The visual detection limit for miRNA-224 in A549 cell lysates is 0.1 million. The resulting calibration curve indicates that the E-LFSBs are capable of detecting miRNA-224 detection in A549 cell lysates. Furthermore, in the present of the interference components such as the unrelated nucleic acids or proteins existing in the A549 cell lysate, the E-LFSB exhibits the anti-interference ability and high hybridization specificity towards target miRNA. The E-LFSBs provide significant advantages for miRNA-224 detection, such as short detection time (within 30 min), simple procedure for real sample pretreatment (lysed cells). Most importantly, compared with the previously reported single-labeled lateral flow biosensor, the E-LFSBs show the improvement in the sensitivity.

Fig. 5

4. Conclusion

In summary, a sensitive E-LFSB was developed for miRNA-224 detection. The nanoprobes dual-conjugated with the enzyme and detection DNA probe were coupled with a capture DNA probe immobilized lateral flow biosensor via the target miRNA-224. The sensitivity was improved by introducing the enzyme modification into the lateral flow biosensor, and the E-LFSB was capable of detecting of 7.5 pM

target miRNA-224 within 30 min under optimal conditions. Although the E-LFSB does not endow the sensitivity as the official devices (~fm) [37], the proposed E-LFSB shows advances in simplicity, low-cost and high speed. In addition, the present E-LFSBs were capable of analyzing miRNA-224 in A549 cell lysate by visual and quantitative detection with simple sample pretreatment. Furthermore, the E-LFSB can be extended to detect other types of miRNA. Further study will involve the detection of mismatched miRNA and multiplex miRNA on the E-LFSB in aqueous solutions and human fluids, e.g. human plasma.

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References

[1] B. John, A.J. Enright, A. Aravin, T. Tuschl, C. Sander, D.S. Marks, PLoS Biol., 2 (2004) e363.
[2] J. Krol, I. Loedige, W. Filipowicz, Nat. Rev. Genet., 11 (2010) 597-610.
[3] J. Brennecke, D.R. Hipfner, A. Stark, R.B. Russell, S.M. Cohen, Cell, 113 (2003) 25-36.
[4] X. Karp, V. Ambros, Science, 310 (2005) 1288-1289.
[5] C.-Z. Chen, L. Li, H.F. Lodish, D.P. Bartel, science, 303 (2004) 83-86.
[6] J. Dostie, Z. Mourelatos, M. Yang, A. Sharma, G. Dreyfuss, Rna, 9 (2003) 180-186.
[7] P. Xu, M. Guo, B.A. Hay, Trends Genet., 20 (2004) 617-624.
[8] M.N. Poy, L. Eliasson, J. Krutzfeldt, S. Kuwajima, X. Ma, P.E. MacDonald, S. Pfeffer, T. Tuschl, N. Rajewsky, P. Rorsman, Nature, 432 (2004) 226-230.
[9] A.M. Cheng, M.W. Byrom, J. Shelton, L.P. Ford, Nucleic Acids Res., 33 (2005) 1290-1297.
[10] Y. Wang, D.N. Keys, J.K. Au‐Young, C. Chen, J. Cell. Physiol., 218 (2009) 251-255.
[11] G.A. Calin, C.M. Croce, Cancer Res., 66 (2006) 7390-7394.
[12] G.A. Calin, M. Ferracin, A. Cimmino, G. Di Leva, M. Shimizu, S.E. Wojcik, M.V. Iorio, R. Visone, N.I. Sever, M. Fabbri, N. Engl. J. Med., 353 (2005) 1793-1801.
[13] I. Alvarez-Garcia, E.A. Miska, Dev., 132 (2005) 4653-4662.
[14] G.A. Calin, C.M. Croce, Nat. Rev. Cancer, 6 (2006) 857-866.
[15] H. Dong, J. Lei, L. Ding, Y. Wen, H. Ju, X. Zhang, Chem. Rev., 113 (2013) 6207-6233.
[16] A.W. Wark, H.J. Lee, R.M. Corn, Angew. Chem., Int. Ed., 47 (2008) 644-652.

[17] M. Lagos-Quintana, R. Rauhut, W. Lendeckel, T. Tuschl, Science, 294 (2001) 853-858.

[18] A. Válóczi, C. Hornyik, N. Varga, J. Burgyán, S. Kauppinen, Z. Havelda, Nucleic Acids Res., 32 (2004) e175-e175.

[19] O. Barad, E. Meiri, A. Avniel, R. Aharonov, A. Barzilai, I. Bentwich, U. Einav, S. Gilad, P. Hurban, Y. Karov, Genome Res., 14 (2004) 2486-2494.

[20] Y.H. Yang, S. Dudoit, P. Luu, D.M. Lin, V. Peng, J. Ngai, T.P. Speed, Nucleic Acids Res., 30 (2002) e15-e15.

[21] J.Q. Yin, R.C. Zhao, K.V. Morris, Trends Biotechnol., 26 (2008) 70-76.

[22] C. Chen, D.A. Ridzon, A.J. Broomer, Z. Zhou, D.H. Lee, J.T. Nguyen, M. Barbisin, N.L. Xu, V.R. Mahuvakar, M.R. Andersen, Nucleic Acids Res., 33 (2005) e179-e179.

[23] T.D. Schmittgen, K.J. Livak, Nat. Protoc., 3 (2008) 1101-1108.

[24] S.C. Chapin, D.C. Appleyard, D.C. Pregibon, P.S. Doyle, Angew. Chem., 123 (2011) 2337-2341.

[25] Y. Cheng, X. Zhang, Z. Li, X. Jiao, Y. Wang, Y. Zhang, Angew. Chem., 121 (2009) 3318-3322.

[26] I. Lee, S.S. Ajay, H. Chen, A. Maruyama, N. Wang, M.G. McInnis, B.D. Athey, Nucleic Acids Res., 36 (2008) e27.

[27] Y. Peng, Z. Gao, Anal. Chem., 83 (2011) 820-827.

[28] Y. Zhang, Z. Li, Y. Cheng, X. Lv, Chem. Commun., (2009) 3172-3174.

[29] X. Gao, H. Xu, M. Baloda, A.S. Gurung, L.-P. Xu, T. Wang, X. Zhang, G. Liu, Biosens. Bioelectron., 54 (2014) 578-584.

[30] M.J. Linman, K. Sugerman, Q. Cheng, Sens. Actuators, B, 145 (2010) 613-619.

[31] X. Liu, Q. Wang, Y. Zhang, L. Zhang, Y. Su, Y. Lv, New J. Chem., 37 (2013) 2174-2178.

[32] J. Li, S. Song, X. Liu, L. Wang, D. Pan, Q. Huang, Y. Zhao, C. Fan, Adv. Mater., 20 (2008) 497-500.

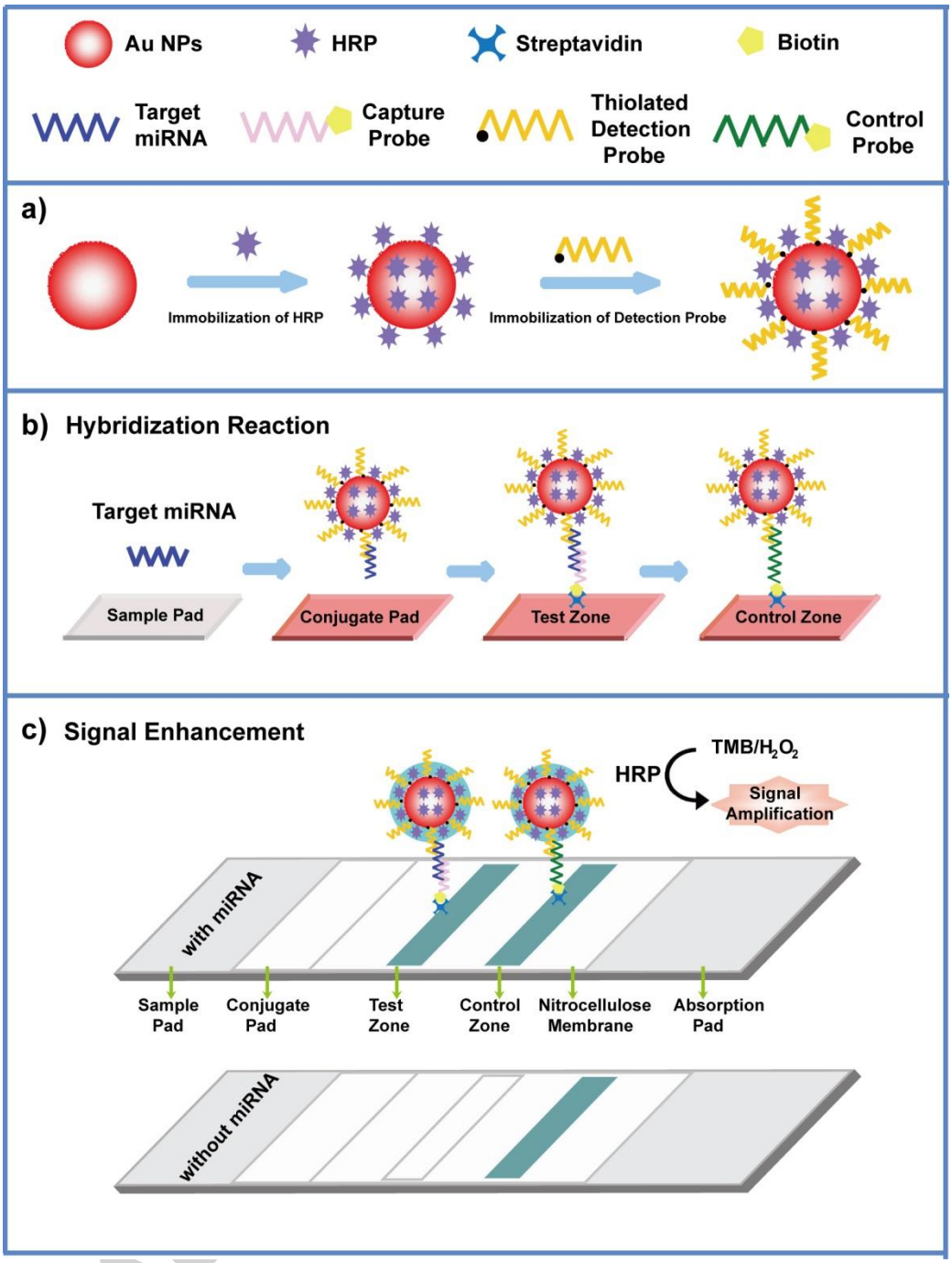
[33] G. Liu, Y.-Y. Lin, J. Wang, H. Wu, C.M. Wai, Y. Lin, Anal. Chem., 79 (2007) 7644-7653.

[34] G. Liu, X. Mao, J.A. Phillips, H. Xu, W. Tan, L. Zeng, Anal. Chem., 81 (2009) 10013-10018.

[35] X. Mao, Y. Ma, A. Zhang, L. Zhang, L. Zeng, G. Liu, Anal. Chem., 81 (2009) 1660-1668.

[36] H. Xu, X. Mao, Q. Zeng, S. Wang, A.-N. Kawde, G. Liu, Anal. Chem., 81 (2008) 669-675.

[37] S. Catuogno, C. L. Esposito, C. Quintavalle, L. Cerchia, G. Condorelli, V. Franciscis, Cancers 3 (2011) 1877-1898.



Scheme 1. a) Schematic diagram of two-step preparations of dual labeled HRP-GNPs-DNA bioconjugates; b) schematic diagram of the hybridization reaction step on the E-LFSB; c) the signal enhancement step on E-LFSB.

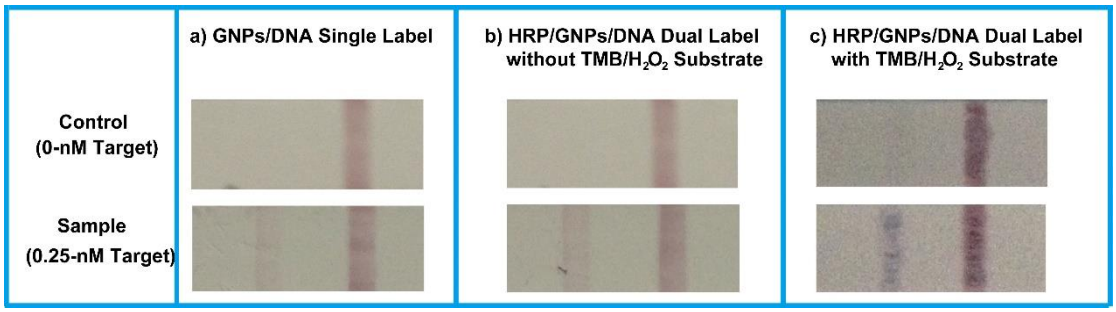


Fig. 1. Typical photo images of 0 nM (control solution) and 0.25 nM (sample solution) of target miRNA-224 on a) GNPs/DNA single-label-based nucleic acid sensor, HRP/GNPs/DNA dual-label-based nucleic acid sensor b) before and c) after treated with TMB/H_2O_2 enzymatic substrate. Running buffer: 25 % SSC + 4% BSA; dispensing time of test zone: 3; HRP/GNPs/DNA bioconjugate volume loaded on the conjugate pad: 3 μL; enzymatic substrate: TMB/H_2O_2; enzymatic reaction time: 9 min; volume of enzyme substrate: 0.7 μL; total assay time: 30 min.

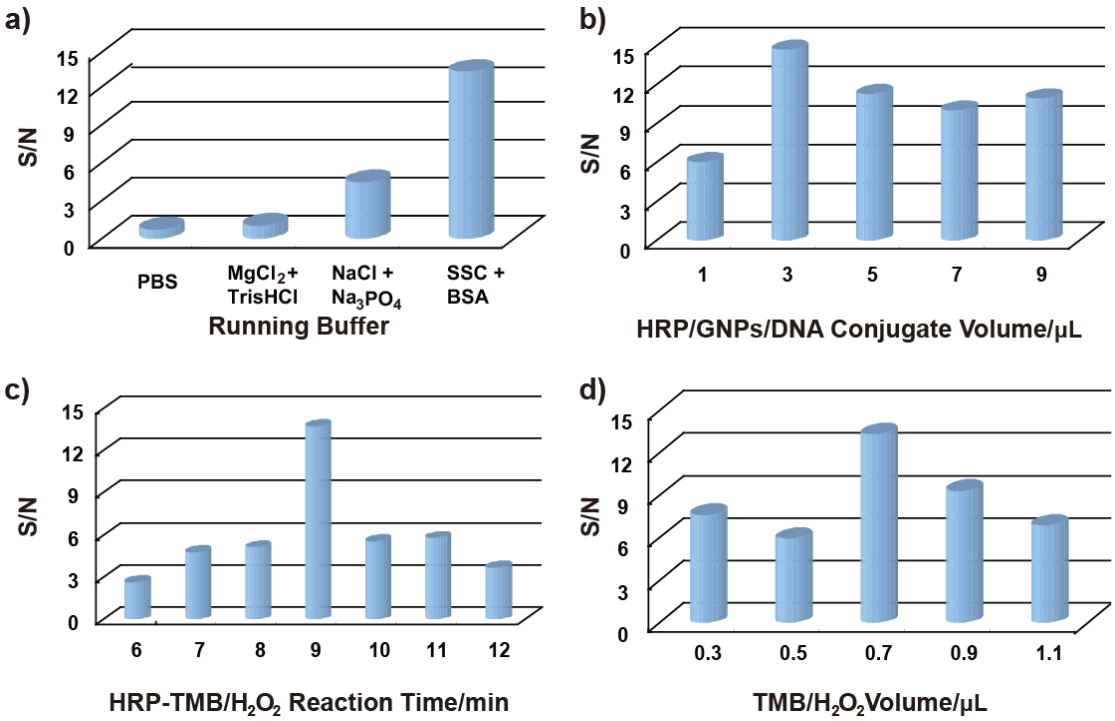


Fig. 2. a) Effect of running buffer component on the S/N ratio of the E-LFSB; b) effect of HRP-GNPs-DNA volume on the S/N ratio of the E-LFSB; c) effect of enzymatic reaction time between HRP and TMB/H_2O_2 substrate on the S/N ratio of the E-LFSB; d) effect of TMB/H_2O_2 substrate volume on the S/N ratio of the E-LFSB. MiRNA-224 concentration: 0.25 nM.

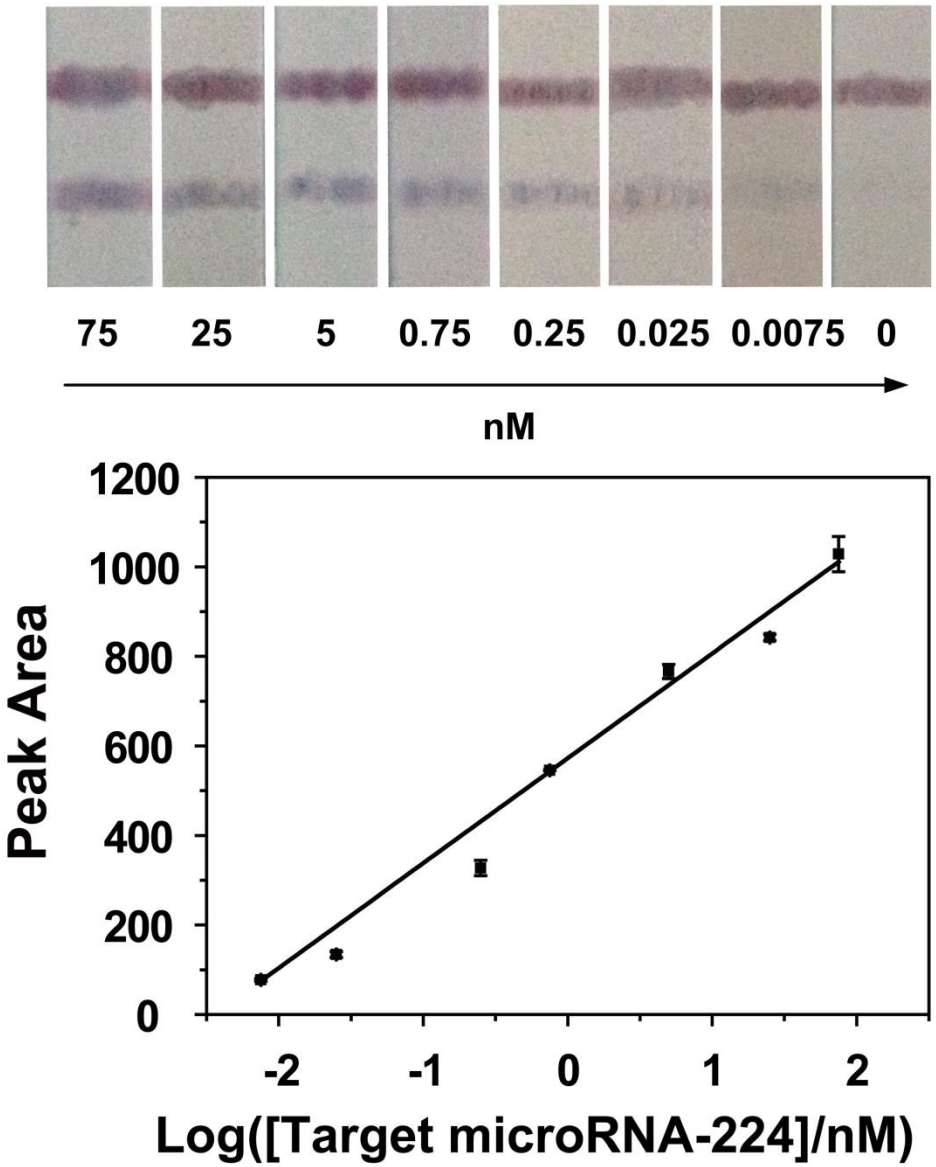


Fig. 3. The photo images and the resulting calibration curves of the E-LFSBs with different concentrations of miRNA-224. Experimental conditions were the same as in Fig. 1.

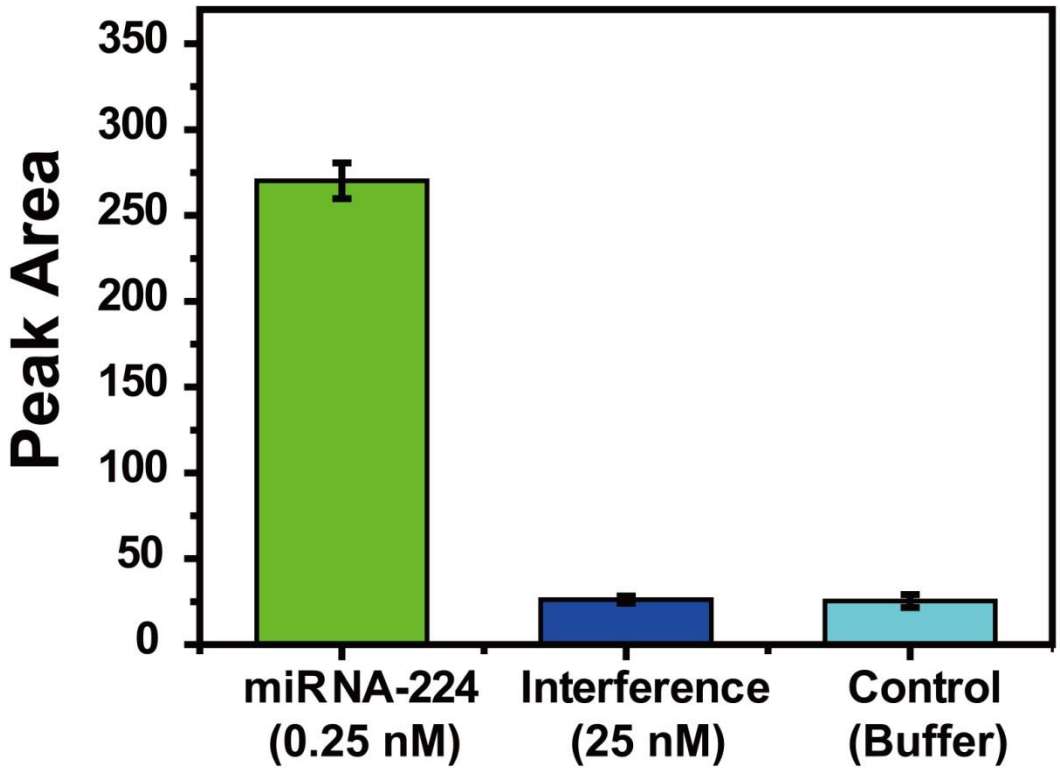


Fig. 4. The optical intensities (peak areas) of the E-LFSB test zones in the presence of 0.25 nM miRNA-224 (a), 25 nM interference miRNA (b), 0 nM miRNA-224. Experimental conditions were the same as in Fig. 1.

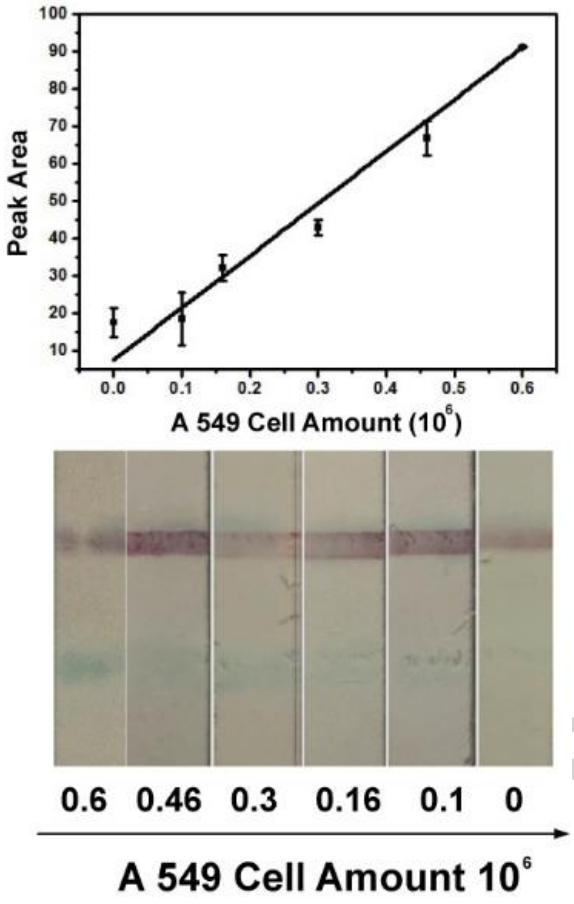
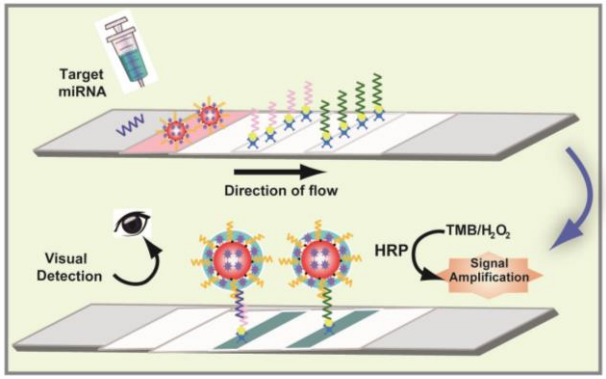


Fig. 5. The photo images and the resulting calibration curves of the E-LFSBs in the presence of various amounts of A549 cells: 0.6 million, 0.46 million, 0.3 million, 0.16 million, 0.1 million and 0 million (from left to right). Experimental conditions were the same as in Fig. 1.

Graphical abstract



Research Highlights:

1. An enzyme-amplified biosensor was developed for microRNA detection.
2. The biosensor enables the visual detection of target microRNA.
3. The biosensor exhibits the improvement in the sensitivity.
4. MicroRNA-224 can be detected in A549 cell lysate within 30 min.