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ORIGINAL PAPER

Catalytic hairpin assembly-assisted lateral flow assay for visual determination of microRNA-21 using gold nanoparticles

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Abstract

The authors describe an improved lateral flow assay based on (a) the use of catalytic hairpin assembly (CHA), and (b) on signal amplification performed at the interface of gold nanoparticles (AuNPs). The combination of the amplification capability of the CHA reaction and the unique optical properties of AuNPs results in an assay that has a sensitivity that is improved by more than two orders of magnitude. MicroRNA-21 was employed as a model analyte to prove the concept. The presence of microRNA-21 triggers the self-assembly of two hairpin DNAs into double stranded DNA and exposing biotin molecules on the surface of AuNPs. Hence, the target becomes recycled and the signal is strongly amplified. The AuNPs carrying biotin are captured on the test line of the strip to display a red zone. This enables the visual recognition of microRNA without the need for any instrumentation. The fast quantitation of microRNA via the red band intensity is accomplished with the help of software, and the limit of detection is 0.89 pM. The enhanced lateral flow assay was employed to the determination of microRNA-21 in cell extracts and spiked serum samples.

Keywords Signal amplification · Cancer cells · Human serum · Quantification · Hairpin probes · DNA nanotechnology

Introduction

Lateral flow assay (LFA), as a point-of-care (POC) detection strategy, has received extensive attention from a wide range of research fields, especially for the detection of disease and cancer related biomarkers [1–4]. Due to the need of high concentration AuNPs for visual detection, the sensitivity of traditional LFA is inferior to instrumentation-based methods [5, 6]. Therefore, its further generalization for early biomarker diagnosis was hindered. Considering the low concentration of biomarker, it is urgent to develop new method to improve the LFA's performance.

The need has driven several attempts including: (i) the development of alternative signal transduction technologies, such as fluorescence signal [7, 8], surface enhanced resonance scattering signal [9, 10], or thermal contrast signal [11]; (ii) modifications of the strip itself [12, 13] and (iii) sample pre-amplification. Among them, sample pre-amplification is of particularly useful for trace-level nucleic acids analysis. Polymerase chain reaction (PCR) is the most widely used and effective signal amplification method. The integration of PCR with LFA has realized the detection of bacteriophage λ-DNA down to 30 copies [14]. Whilst sensitive, the process involves the participation of the thermal cycler to precisely control temperature, which constrains the portability for POC detection. Meeting this challenge, several isothermal amplification methods based on protein enzyme have been employed to couple with LFA [15, 16]. These enzymes operate at constant temperature with high efficiency, therefore, these methods achieved excellent amplified performance. However, the high cost, easily deactivation properties, stringent store conditions of protein enzymes limit the real application in LFA detection.

Wenjing Wang and Axiu Nie contributed equally to this work.

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Alternatively, isothermal amplification based on the use of hairpin DNA probe alleviates the involvement of protein enzyme and performs at room temperature. Thus, it can be potentially employed to improve the performance of LFA. Recently, Ying et al. have developed a series of sensitive LFAs based on hybridization chain reaction (HCR) using two hairpin DNA probes [17, 18]. However, these methods require multiple incubation steps due to the "sandwich-type" detection model. First, the initiator is incubated with two hairpin DNA probes to form reporter probe-HCR complexes. Subsequently, the complexes need to be further incubated with target and capture probe to realize detection. These multiple incubation steps make the detection process time-consuming. In addition to HCR, catalytic hairpin assembly (CHA) is another commonly used hairpin-based amplification method [19]. Two hairpin DNA probes are designed to coexist in the solution. In the presence of a trigger, the first hairpin is opened with the help of toehold. The other hairpin DNA is subsequently opened to replace the trigger as a catalyst through the branch migration reaction. This leads to numerous hairpin assembly events. The reaction has been reported to couple with a variety of signal readouts to amplify detection signals both in vivo and in vitro with low background and high turnover rates [20, 21].

We try to overcome these obstacles in LFA for nucleic acids detection by integrating with on-particle CHA amplification. As a proof of concept, we choose microRNA-21 as the detection model and two hairpin DNA probes are rationally designed accordingly. One of the DNA Hairpin 1 (H1) is anchored on the AuNPs using Au-S chemistry at 5′ end, while 3′ end of H1 is modified with biotin (H1-bio). Due to the formation of the hairpin structure, biotin molecule on H1 probe is close to AuNPs. The probe is not able to be captured by streptavidin (SA) on the cellulose membrane of LFA strip because of the steric hindrance effect. In the presence of microRNA-21 and the other Hairpin 2 (H2), CHA amplification is initiated on AuNPs to reuse the target, generating numerous double stranded DNA exposing biotin molecules outside. With the help of CHA pre-amplification, the detection limit of LFA nucleic acids analysis has been improved by more than two orders of magnitude. The detection process is one step incubation, cost effective and easy to generalize to other targets, providing great potential in improved LFA.

Materials and methods

Materials

40% acrylamide mix solution, ammonium persulfate (APS), 1, 2-bis(dimethylamino)-ethane (TEMED), diethylpyrocarbonate (DEPC)-treated water, 6× loading buffer and DNA ladder were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China https://www.sangon.com/). Streptavidin (SA) and Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) were obtained from Sigma-Aldrich (USA https://www.sigmaaldrich.com/). Tween-20, chloroauric acid ($HAuCl_4$) and trisodium citrate were provided by Sinopharm Chemistry Reagent Co., Ltd. (Shanghai, China http://www.reagent.com.cn/). rRNasin RNase Inhibitor was obtained from Wuhan Tianyuan Huida Biotechnology Co., Ltd. (Wuhan, China http://www.tybio.com.cn/). Phosphate buffered solution (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 137 mM NaCl, pH = 7.4) was employed as catalytic hairpin assembly reaction buffer and running buffer for lateral flow assay. Dulbecco's modified Eagle's medium (DMEM), RPMI Medium Modified medium (RPMI-1640), trypsin, fetal bovine serum (FBS) were provided by Thermo Fisher Scientific *Corp.* (USA https://www.thermofisher.com/cn/zh/home/brands/gibco.html). Total RNApure Kit was bought from Beijing Zoman Biotechnolgy Co., Ltd. (Beijing, China http://www.zomanbio.com/). YeaRed nucleic acid gel staining dye (10,000 × in water) was provided by Yisheng Biotechnolgy Co., Ltd. (Shanghai, China https://www.yeasen.com/). Nitrocellulose (NC) membrane (Sartorius CN140), adhesive bakcing (DB-6), absorption pad (H5072), conjugate pad (Ahlstrom 8964), as well as sample pad (GL-b02) were purchased from Shanghai Jieyi Biotechnology Co., Ltd. (Shanghai, China http://www.joey-bio.com/index.html).

All the chemicals were of analytical grade and used without further purification and modification. RNase-free water was used throughout the whole RNA experiment. All the oligonucleotides were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China https://www.sangon.com/) with HPLC purification for modified sequences and with UltraPAGE purification for others. Table S1 shows all the oligonucleotide sequences in detail. Huam serum sample was obtained with the permission from the Huazhong Agricultural University Hospital and acquired with consent from volunteer who was informed about the research purposes. This consent procedure (HZAUHU-2018-009) was approved by The Scientific Ethic Committee of Huazhong Agricultural University.

Apparatus

Lateral flow strips were prepared using a CM4000 cutting system and a ZX1000 dispense platform supplied by Biodot (USA https://www.biodot.com/). Transmission electron microscopy (TEM) images were acquired on a JEM-2010 transmission electron microscope. UV − vis absorption spectra were obtained with a LAMBDA 25 UV − vis spectrometer (PerkinElmer, United Kingdom http://www.thermofisher.com/). Hydrodynamic size was measured by dynamic light scattering (DLS) using a Malvern Zetasizer (Nano-ZS) system.

Preparation of DNA hairpin-modified gold nanoparticles (AuNPs)

AuNPs were modified with thiol and biotin-labeled H1 following a salt-aging method [22]. 13 nm AuNPs with the concentration of approximately 12 nM were synthesized using the classical citrate-reduction method [23]. Please refer to Electronic Supporting Material for more details.

Assembly of lateral flow strips

Figure 1b provides a clear illustration for the assembly of the test strips. The LFA test strips are composed of sample pad, conjugate pad, NC membrane, absorption pad and adhesive backing. According to the size of AuNPs (13 nm), 8 μm NC membrane is selected. Specifically, all components are sequentially assembled on the adhesive backing at first and they are cut into 3.6 mm width with the cutter. Each of the adjacent parts have a 2 mm overlap to ensure the continuous flow of running buffer. SA (0.4 mg·mL^{-1}) is dispensed on the NC membrane as T line and dried at room temperature for 3 h before use. The test strips were kept in dark and dry conditions at 4 °C.

Native polyacrylamide gel electrophoresis (PAGE)

The on-particle CHA was verified using 12% PAGE. Different samples with total volume of 10 μL, final concentrations of 100 nM containing 1 × loading buffer, were loaded onto the 1 mm gel. The electrophoresis was carried out in 1 × TBE buffer at a constant potential of 100 V for 1 h. The gels were stained with YeaRed dye solution for 15 min. After staining and washing, the gels were imaged with a Bio-rad gel imaging system.

Cell culture and total RNA extraction

Human embryonic kidney cells (HEK293T), human lung adenocarcinoma cells (A549) and cervical cancer cells (HeLa) were obtained from American Type Culture Collection (ATCC). HEK293T cells were seeded in RPMI-1640 culture medium containing 10% FBS and 1% antibiotic (penicillin–streptomycin, 10000 U mL^{-1}) in a constant environment with 5% CO_2 at 37 °C. A549 cells and HeLa cells were cultured in DMEM medium with 10% FBS and 1% antibiotic under the same condition as HEK293T cells. All the cells were collected in the period of logarithmic phase. Total RNAs containing microRNA-21 were extracted from cells according to instruction manual provided along with the Total RNApure Kit after cell counting. The final RNA extracts were dissolved in RNase-free water and stored at −80 °C refrigerator for later use.

Improved lateral flow assay procedures

3 μL 12 nM AuNPs-H1 probes and H2 probes were mixed and incubated with different concentrations of target DNA

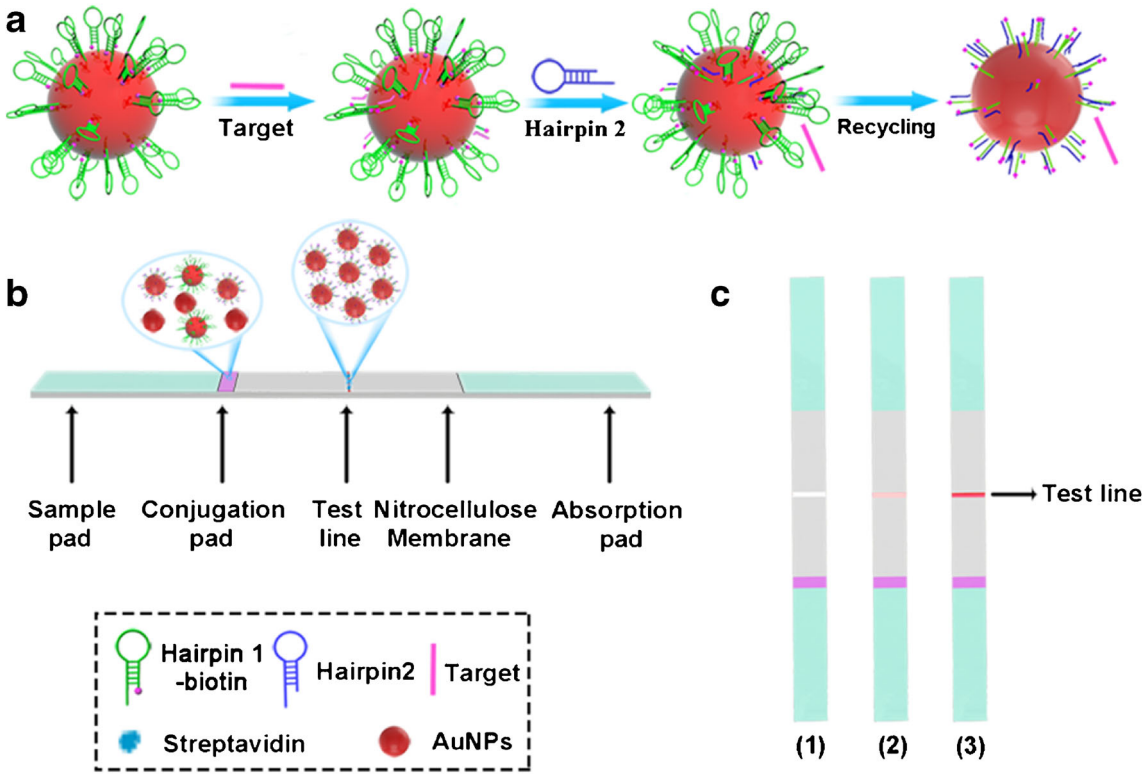

Fig. 1 Schematic presentation of microRNA-21 detection using on-particle CHA assisted lateral flow assay

(analogue of microRNA-21) for 50 min at room temperature. Equal stoichiometry of H1 and H2 were used for the CHA reaction. Finally, 6 μL of sample was pipetted onto the conjugation pad and the strip was moved to a 1.5 mL EP tube containing phosphate buffered solution for running about 2 min. Each measurement was repeated at least three times. For comparison, non-amplification involved strategy for analyzing DNA target (analogue of microRNA-21) was carried out without addition of H2 probe. All the other conditions were the same as before. MicroRNA-21 was detected in the same way, except for using RNase-free water and rRNasin RNase Inhibitor throughout the experiment. The results of LFA were recorded using a Canon camera, the intensity of T line bands was used to quantify the target concentrations with the help of ImageJ software. The brightness of the surroundings was tuned and a proper calibration of the color intensity was carried out at exactly the same conditions.

Real sample analysis

The real sample used include total RNA extracts and human serum. For the detection of total RNA extracts, cells were counted during the preparation of extracts. 3 μL 12 nM AuNPs-H1 probes, equal stoichiometry H2 probes, cell extracts containing 10^6 cells were mixed in phosphate buffered solution. The final volume of the mixture was adjusted into 6 μL with DEPC water. After incubation for 50 min at room temperature, the sample was pipetted onto the strip and underwent LFA measurement.

For human serum detection, the blood was centrifuged to get serum, and was diluted by a factor of 100 with water. Different amount of microRNA-21 were added into diluted serum to carry out recovery experiments. Similarly, 3 μL 12 nM AuNPs-H1 probes and equal stoichiometry H2 probes were mixed and incubated with different concentrations of microRNA-21 in diluted serum for 50 min at room temperature. The final concentration of microRNA-21 were 0.1 nM, 10 nM and 1 μM. DEPC water was added to ensure the final volume was 6 μL. The sample was pipetted onto the strip and underwent LFA measurement.

Results and discussion

Design principle of on-particle CHA assisted LFA

The principle of on-particle CHA assisted LFA for microRNA-21 detection is depicted in Fig. 1a. It is realized by using microRNA-21 as an initiator and two hairpin DNA probes as assistant strands. Two stable hairpin probes with 8 nt toehold overhang are designed according to the specific sequence of microRNA-21. The secondary structures are provided by IDT Oligo Analyzer. As shown in Fig. S1, the melting temperatures of H1 and H2 are all designed to be above 60 °C. This design can increase the stability and decrease the non-specific leakage reaction possibility in the absence of target. The mechanism of the reactions is depicted in Fig. S2. First, domain 1* on strand T serves as a toehold to bind domain 1 on H1 and open the hairpin structure. Domain 3* on H1 then serves as a second toehold to initiate another strand displacement to open the H2, and the intermediate of T-H1-H2 is formed. Finally, T dissociates from H1 and catalyzes another hairpin assembly reaction, thus amplifying the signal.

Notably, similar to those proof-of-concept studies reported previously [24–26], we do not incorporate control line in our experiment for simplicity. However, it can be easily realized by modifying another single stranded DNA on AuNPs and its complementary strand on NC membrane. In our study, the capture of H1 modified AuNPs (AuNPs-H1) on T line is realized through biotin and SA interaction. The T line is predispended with SA. H1 as a target capture probe is anchored on AuNPs through Au–S bond at 5′-end. Biotin modified at 3′-end is close to AuNPs due to the formation of the stable hairpin structure. The binding between AuNPs-H1 and SA on the strip is prevented because of the steric hindrance effect, displaying negligible red signal on LFA strip (Fig. 1c. (1)). In the presence of microRNA-21, H1 is opened via toehold mediated strand displacement reaction to expose biotin towards outside, exhibiting a pale red line on the strip because of the capture of AuNPs-H1 (Fig. 1c. (2)). Furthermore, the exposed part incorporating in the H1 is designed to hybridize with H2 through another toehold-mediated strand displacement reaction to form H1-H2 duplex. The target microRNA-21 is displaced simultaneously and can be used for initiating next reaction between H1 and H2. This process generates numerous H1-H2 duplex exposing biotin molecules outside, thus the signal is amplified significantly. As the reaction goes on, a strong red line can be ovserved (Fig. 1c. (3)). By monitoring the different shade of red bands on the strip, qualitative and quantitative analysis of target can be easily realized by either visualization or reading the T line intensities with ImageJ software.

Choice of materials

During recent years, AuNPs, quantum dots, upconversional nanoparticles and superparamagnetic nanobeads [3, 6, 7, 27] are frequently used in LFA as the signal readout. Among them, AuNPs own the intrinsic merits including easy to synthesize and modify, long-time stability, good biocompatibility and tunable plasmonic properties. Therefore, they have been the most widely used signal labels in LFA, especially in commercialization. For these reasons, we select AuNPs, and modify them with hairpin DNA probes. Immobilizing DNA strands on AuNPs not only improves the stability of DNA in complex sample, but also accelerates the reaction kinetics of

DNA hybridization [28]. AuNPs have two important roles: as the signal output for visual detection and quantification, and as probe carriers to carry out signal amplification at their surfaces. The characterization of AuNPs and AuNPs-H1 probes are shown in Fig. S3.

Feasibility tests

To testify the design, 12% PAGE was conducted. As displayed in Fig. 2a, when mixing H1 and H2 together (lane 5), nearly all of them exhibit bands as they are separately loaded in lane 2 and lane 3 respectively. Negligible H1-H2 hybridization products formes at the top of line 5, implying minimum leaking between H1 and H2 without target addition. Incubation target with H1 triggers the open of hairpin, indicated by the large molecular weight complex formation and disappearance of H1 band in lane 6. Further adding H2 to H1-T, a strong band of H1-H2 complex appears, and the target strand is released (lane 7). This result confirms the feasibility of our signal amplification strategy in aqueous solution. In order to further testify the feasibility of on-particle CHA amplification, H1 probes were anchored on AuNPs and LFA was conducted. As shown in Fig. 2b, the intensity of red band for H2, target together with AuNPs-H1 probe is remarkably stronger than that of AuNP-H1 or AuNPs-H1 and H2 mixtures. This indicates on-particle CHA amplification reaction has taken place as expected successfully. Inset displays the digital photograph images of the strip.

Optimization of experimental conditions for LFA

To achieve optimal detection performance, experimental conditions, including the concentration of SA, the volume of AuNPs-H1 probe, and the reaction buffer were systematically studied. According to the results in Fig. S4 and S5, 0.4 mg·mL^{-1} SA, 3 μL AuNPs-H1 probe and phosphate buffered solution were chose as the optimal conditions in the following experiments.

Comparison of the detection performance

The detection performance of on-particle CHA assisted LFA was confirmed by comparing with non-amplification involved strategy. Under the above optimized conditions, quantitative analysis was carried out. First, we employed DNA target (analogue of microRNA-21) to evaluate the performance of the LFA. Figure 3a displays the digital photograph images of the strip with DNA target concentrations varying from 5 pM to 0.5 μM. It is obvious that the color intensity of the red band on the T line increases with increasing DNA target concentration. The quantitative analysis is further carried out with ImageJ software. The T line signal intensity increases along with the increase of target DNA concentration (Fig. 3b). Corresponding linear relationship is shown in Fig. 3c, there is a good linear relationship between the T line signal intensity and the logarithm of the DNA target concentration. The detection limit for DNA target is calculated to be 0.80 pM ($S/N > 3$).

In addition, we performed traditional LFA without employing amplification strategy for comparison. We incubated the target with AuNPs-H1 probes without adding H2 probe for signal amplification. Similarly, the color intensity of the red band also increases with increasing DNA target concentration (Fig. S6). However, the T line intensities with the aid of on-particle CHA amplification are obviously higher than without amplification at the same target concentrations (Fig. S7). The detection limit of traditional LFA without employing amplification strategy is calculated to be 0.78 nM (Fig. S6), which represents about more than two orders of magnitude higher than amplified lateral flow assay. By comparison, the

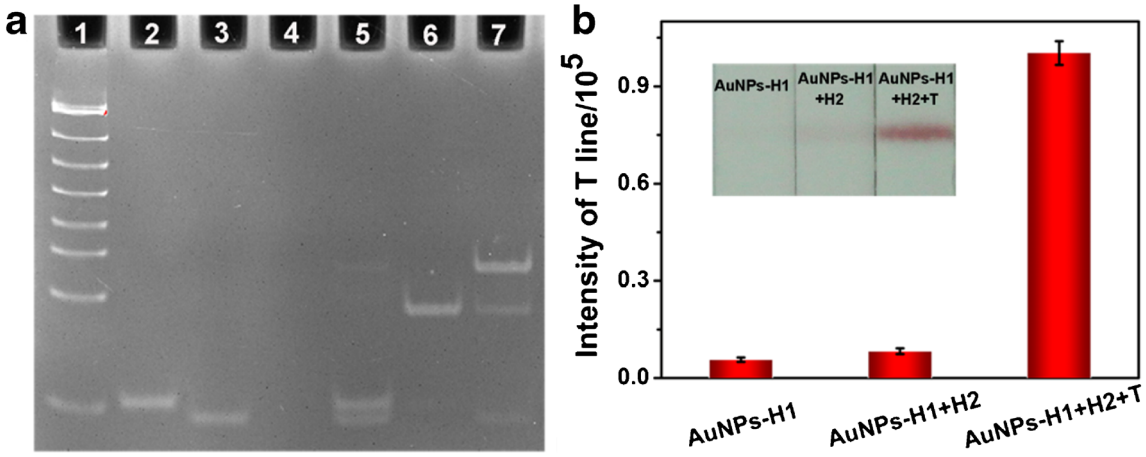

Fig. 2 Feasibility analysis of on-particle CHA assisted LFA for microRNA-21 detection. **a** PAGE gel: lane 1: Marker; lane 2: H1; lane 3: H2; lane 4: T (the short single stranded DNA was not stained); lane 5: H1 + H2; Lane 6: H1 + T; lane 7: H1 + H2 + T. **b** Lateral flow assay: T line intensity in the presence of AuNPs-H1 probe, AuNPs-H1 + H2, AuNPs-H1 + H2 + T. Inset: The corresponding digital photograph images of LFA strips. The quantification data were acquired by Image J software analysis

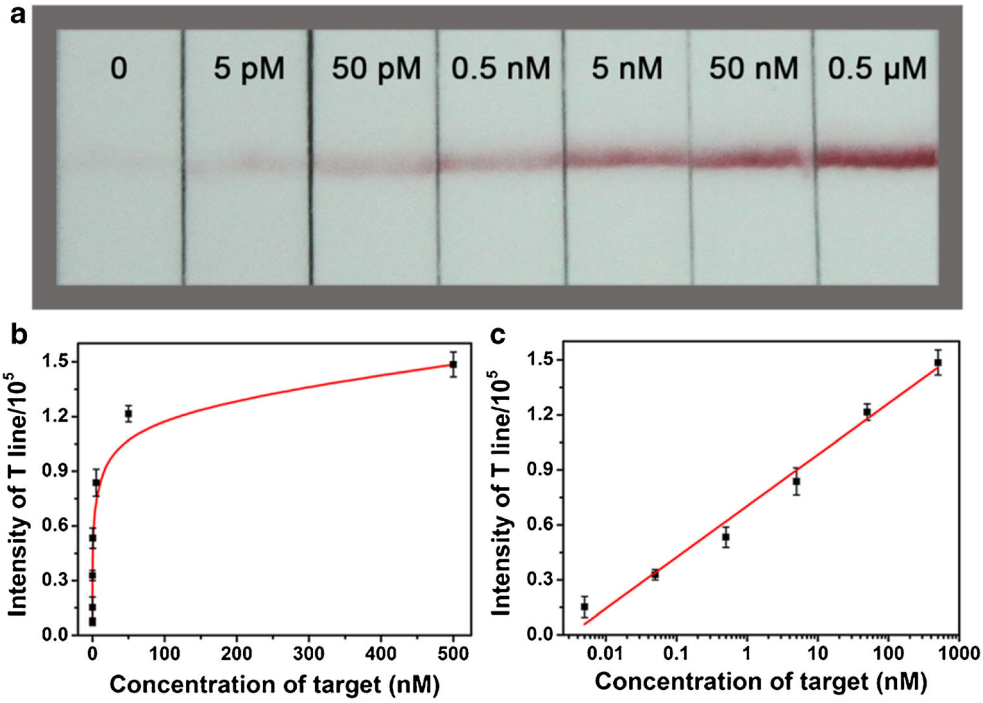

Fig. 3 Performance of the LFA with on-particle CHA amplification in response to various concentrations of DNA target. **a** Digital photograph images of LFA strips for 0, 5 pM, 50 pM, 0.5 nM, 5 nM, 50 nM, 0.5 μM targets. **b** Calibration plot of red band intensity vs target concentration. **c** Linear relationship of red band intensity vs logarithm of DNA concentration. (Error bars indicate the standard errors of three independent experiments of three strips.) The quantification data were acquired by Image J software analysis

method of on-particle CHA based enzyme-free signal amplification strategy is confirmed to significantly improve the detection performance of LFA.

Furthermore, we utilized this detection platform to detect microRNA-21 which is related to cancer. Under the same conditions, the concentration of microRNA-21 from 10 pM to 1 μM was added. We observe the same phenomenon as that to DNA target. The detection limit turns out to be 0.89 pM, which is slightly higher than DNA target (Fig. 4). This result may due to the degradation of microRNA-21 during the whole experiment. We compare our method with the recently reported nanomaterial-based optical methods in Table 1. It is observed that the limit of detection (LOD) and detection linear range of our assay are better than other optical methods

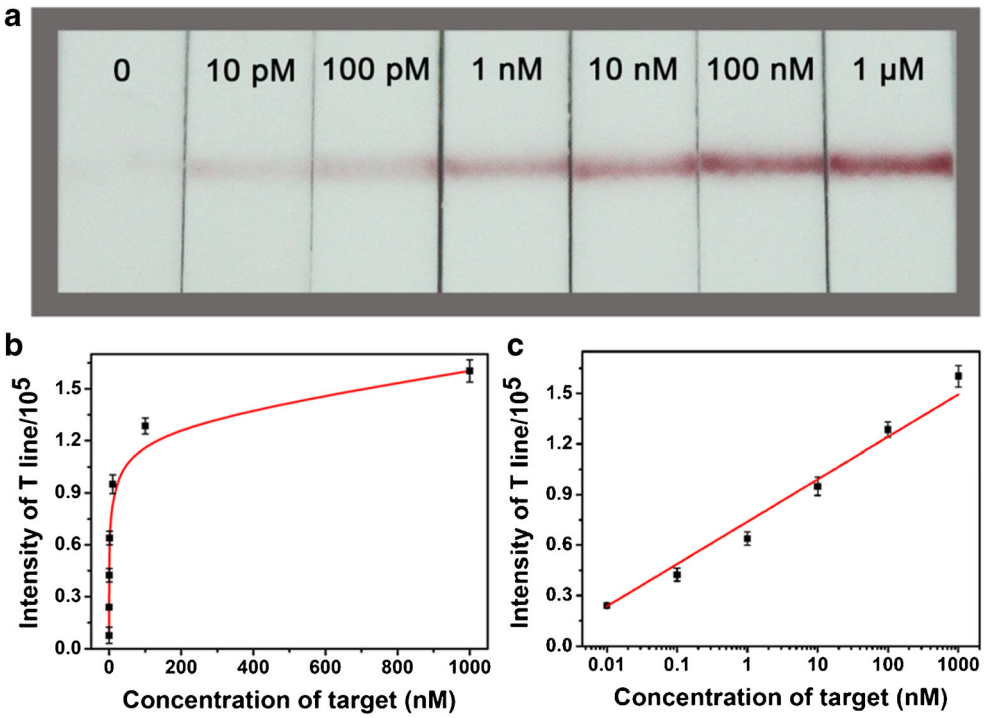

Fig. 4 Performance of the LFA with on-particle CHA amplification in response to various concentrations of microRNA-21. **a** Digital photograph images of LFA strips for 0, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM microRNA-21. **b** Calibration plot of red band intensity vs target concentration. **c** Linear relationship of red band intensity vs logarithm of microRNA-21 concentration. (Error bars indicate the standard errors of three independent experiments of three strips.). The quantification data were acquired by Image J software analysis

Table 1 Comparison of the proposed method with recently reported nanomaterial-based optical methods for determination of microRNA-21

Materials	Detection Method	Amplification strategy	LOD (pM)	Linear range (nM)	Ref.
Gold nanoclusters and carbon nanotubes	Fluorescence	/	36	0.01–100	[29]
Molecular beacons	Fluorescence	/	500	1–50	[30]
AuNPs	Colorimetric	HCR	6.80	0.02–10	[31]
AuNPs	LFA	RCA	40	0.02–200	[32]
AuNPs	LFA	CHA	0.89	0.01–1000	This work

RCA stands for rolling circle amplification

because of CHA signal amplification. In addition, our method is user-friendly and enzyme-free without the involvement expensive instrumentations, suggesting its potential use for early POC cancer diagnosis at home or in rural area with limited medical resources.

Specificity

The specificity of our method was further investigated using different RNA targets including random sequence, one-base mismatch (MT1) sequence, three-bases mismatch (MT3) sequence under the same condition. As displayed in Fig. 5a, compared with the blank soution, the intensity of the red band increases dramatically in the presence of microRNA-21. However, when random, MT1 and MT3 sequences are employed instead of microRNA-21, negligible changes are observed to take place compared with the blank sample. The results reveal that the method exhibits excellent specificity for the target.

Real application of the method in cancer cells and human serum

To assess the utility of the method in discriminating the amounts of microRNA-21 in different cell lines, we extracted total microRNA including microRNA-21 from A549, HeLa and HEK293T cells with an extraction kit. According to the results shown in Fig. 5b, the expression level of microRNA-21 in A549 cells is significantly higher than HeLa cells, which is in a good agreement with the reported literature [33]. Besides, it has been reported that HEK293T cells express very low level of microRNA-21 [34]. Our experimental data also shows that there is negligible signal for HEK293T cell line using our method. The three cell lines expressing different levels of microRNA-21 are clearly differentiated and monitored by observing the signal intensity on the T line. Therefore, the method can be utilized as a POC strategy to analyze microRNA in cancer cells for clinical use.

To further assess the practicability of the method, the recovery experiments were carried out by adding different concentrations of microRNA-21 into normal human serum samples obtained from the Huazhong Agricultural University Hospital (Wuhan, China). Three different concentrations of microRNA-21 were added into the diluted human serum. The results in Table S2 indicates that the recoveries of 3 serum samples at different microRNA-21 content range from 98.59% to 102.5% with RSD values varying from 2.11% to 4.75%. This result suggests the strategy can be used for quantitative detection of microRNA-21 in diluted human serum.

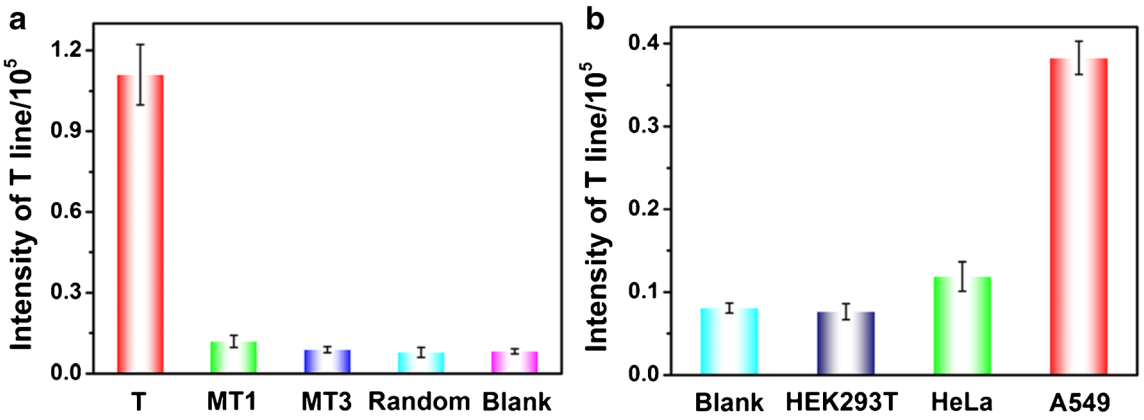

Fig. 5 **a** Specificity investigation of the method. Samples from left to right are: microRNA-21 (T), one-base mismatch (MT1) sequence, three-bases mismatch (MT3) sequence, random sequence and blank. **b** Detection of microRNA-21 in HEK293T, HeLa, and A549 cell lines. Error bars represent the standard deviations of three repetitive measurements. The quantification data were acquired by Image J software analysis

Conclusions

A novel on-particle CHA enzyme-free signal amplification assisted LFA platform for the improved detection of microRNA-21 was successfully demonstrated with one-step incubation. Taking advantage of the signal amplification features of CHA and the strong binding force between biotin and SA, the real application of the method in cancer cells and human serum was realized. The detection limit of LFA for nucleic acids analysis improved about 900-fold compared with non-amplification based LFA. The limitation of the method is that AuNPs modification takes long time and the method is not applicable in blood plasma. We will continue to make efforts to solve these problems in our future work. Furthermore, the detection strategy can be expanded to other biomarkers, which will have wide application prospect in clinical prognosis and drug development.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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