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Universal point-of-care detection of proteins based on proximity hybridization-mediated isothermal exponential amplification†

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We have developed a point-of-care (POC) lateral flow biosensor (LFB) for universal protein detection based on a proximity hybridization-mediated protein-to-DNA signal transducer, isothermal exponential amplification (EXPAR) and catalytic hairpin assembly (CHA) with high sensitivity and specificity. In this assay, a protein signal transducer was employed to convert the input protein to the output DNA signal. Antibody conjugated DNA1 was firstly hybridized with the output DNA (DNA3). The binding of antibody conjugated DNA1 and DNA2 to the same protein was able to increase the local concentrations, resulting in strand displacement between DNA3 and DNA2. DNA3 with nicking endonuclease recognition sequences at the 5′ end then hybridized with hairpin probe 1 to mediate EXPAR in the presence of nicking endonuclease and polymerase. A large number of single strand DNA were produced in the circle of nicking, polymerization and strand displacement. The resulting ssDNA products were further amplified by CHA to generate double-stranded DNA products. The double-stranded DNA products were detected with a lateral flow biosensor within 5 min. This proposed assay has very high sensitivity and selectivity with a dynamic response ranging from 1 fM to 100 nM, and the detection limit was 0.74 fM. This work provides a universal and simple method for protein detection.

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Introduction

Developing point-of-care methods for the detection of protein has a major impact in diverse areas, such as clinical diagnosis, therapy progress monitoring, prognosis estimation, and disease prevention.[1,2]

Until now, different methods have been developed for the detection of proteins. Traditional immuno-methods such as western blot, dot blot, enzyme-linked immunosorbent assay (ELISA),[3,4] immunohistochemistry,[5,6] and immunoaffinity liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay[7,8] are widely used analytical techniques in both clinical diagnosis and research. Although these methods are sensitive, all these approaches are time-consuming, laborious, and expensive. Therefore, continuing efforts have been made to seek ideal methods for the fast, sensitive, cost-effective, and easy-to-use detection of proteins.

Recently, many signal transducers were used to convert the input target molecule into a sequence-specific output DNA signal, and then the output DNA signal was further amplified by DNA signal amplification methods. For example, Ghadiri's group designed a novel nucleic acid detection strategy through converting the target nucleic acid to a predesigned output DNA for signal amplification.[9] Le and his co-workers have developed a molecular translator based on binding-induced DNA strand displacement for the homogeneous detection of protein, including platelet derived growth factor BB (PDGF BB) and prostate specific antigen (PSA).[9–14] These detection methods are effective and sensitive. However, most of them lack portability and require expensive apparatus.

Nowadays, point of care detection assays have attracted a great deal of research interest because of their simplicity, portability, short assay time, and cost-effectiveness. Lateral flow biosensors, as one of the POC methods, are widely applied in the detection of protein, DNA or RNA.[15–20] The results of LFBs can be observed by the naked eye within 5 min, and the quan-

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titative analysis can be realized by scanning the colour intensity of the red band on the test zone with a LFB reader. Catalytic hairpin assembly (CHA) is a commonly used approach for DNA signal amplification due to its robustness and simplicity. LFB combined with CHA has been applied to test nucleic acid, proteins or heavy metals with high specificity.[21–24] Zhou's group has developed a LFB for the detection of nerve growth factor-beta based on a molecular translator and CHA with a detection limit of 10 fM.[24] However, the detection sensitivity of the LFB based on CHA is relatively low due to its limited amplification efficiency.

In this work, we developed a lateral flow biosensor for universal protein detection based on a proximity hybridization-mediated protein-to-DNA signal transducer and isothermal exponential amplification with high sensitivity and specificity. We employed a protein-to-DNA signal transducer to covert the protein signal into DNA signal. The output DNA was firstly amplified by EXPAR to generate a great number of ssDNA. The resulting ssDNA was further amplified by CHA and visually detected with an LFB. Nerve growth factor-beta (NGF-β), a neurotrophin implicated in the modulation of pain perception, was chosen as the detection target.

Experimental

Reagents and chemicals

Gold nanoparticle (AuNP) solutions (15 nm and 20 nm in diameter) were obtained from BBI solution (Crumlin, UK). Nerve growth factor-beta (NGF-β), anti-NGF-β monoclonal antibody, anti-digoxin antibody, goat anti-mouse IgG antibody (secondary antibody), bovine serum albumin (BSA) and streptavidin (SA) were purchased from Sigma-Aldrich (Steinheim, Germany). The Bst DNA polymerase and NbBbvCI nicking endonuclease were purchased from New England Biolabs (New England, USA). Glass fiber, cellulose fiber sample pads, laminated cards and nitrocellulose membranes were purchased from Shanghai Jieyi Biotech (Shanghai, China). All other reagents were purchased from standard commercial sources and were of analytical grade. rCutSmar buffer (20 mM tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 100 μg mL^{-1} recombinant albumin, pH 7.0) was used as the reaction buffer for the protein-to-DNA signal transducer, EXPAR, and CHA. The DNA oligonucleotide probes were obtained from Sangon Biotech (Shanghai, China). The oligonucleotide sequences are listed in Table 1.

A lateral flow dispenser (HM3035), lateral flow paper cutter (ZQ2002), and portable strip reader (DT1030) were purchased from Shanghai Goldbio Tech (Shanghai, China).

Preparation of DNA1/DNA2-anti-NGF-beta antibody conjugates

The amine modified DNA1 and DNA2 were conjugated with anti-NGF-beta monoclonal antibody by the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) activity, respectively. Firstly, 20.6 mg of EDC and 11.5 mg of NHS was mixed in 1 mL of water for 10 min. 10 μL of this mixed solution was added to a 200 μL solution containing 13.5 mg mL^{-1} anti-NGF-beta antibody, 10 μM DNA 1 or 10 μM DNA2 in carbonate buffer (pH 11.0), and reacted for 2 hours. The solution was then centrifuged for 5 min at 4000 rpm and rinsed two times with 200 μL of water for 5 min at 4000 rpm. Finally, the supernatant was abandoned and the precipitate was resuspended to a final volume of 200 μL with rCutSmar buffer. Final protein concentrations were determined with a Pierce BCA protein assay kit.

Table 1 The oligonucleotide sequences

Oligonucleotide	Sequences
DNA1	5′-NH_2-C6-TTTTTTTTTTTTTTTTCGTACGTAGG - 3′
DAN2	5′- CCTACGTACGAATTTTTTTTTTTTTT-C6-NH_2-3′
DNA3	5′-GATACGGC▲TGAGG CCTACGTACGAA-3
Hairpin probe 1 (H1)	5′TTTGATACGGCTGCGTCGTAGGCCTCAGCCGTA TCAAA-3′
Hairpin probe 2 (H2)	5′-TTTGATACGGCTGTGTCGTAGGCCTCAACCA TGTGTAGACGAGTGAGGCCTACGACACAGC-digoxin-3′
Hairpin probe 3 (H3)	5′- TAGGCCTCACTCGTCTACACATGGTTGAGGC CTACGACACAGCACCATGTGTAGACGAG-biotin-3′

The loop portion of each hairpin is underlined. ▲ represents the nicking endonuclease site of Nb·BbvCI.

Construction of the lateral flow biosensor

The LFB consisted of a sample pad, a conjugate pad, a nitrocellulose membrane and an absorbent pad. Fiber glass (16 mm in width) was used as sample pads after being treated with sample pad buffer (4% Triton x-100, 0.1% SDS, 1% BSA, 100 mM boric acid, 2% PEG-4000, 2% sucrose, pH 8.0). Untreated fiber glass was prepared as the conjugate pad (0.8 cm × 30 cm) by dispensing 3 μL cm^{-1} of the AuNP-anti-digoxin antibody complex onto the surface with a dispenser. The conjugate pad was dried and stored under low-humidity conditions at room temperature. Streptavidin (1 mg mL^{-1}) and anti-mouse IgG antibody (1 mg mL^{-1}) were dispensed at 1 μL cm^{-1} onto the nitrocellulose membrane (25 mm × 30 cm, capillary rate: 140 ± 40 s, thickness: 145 ± 20 μm) simultaneously, to form the test zone and control zone, respectively, with a lateral flow dispenser. The gap between the test zone and the control zone was approximately 5 mm. The membrane was dried at room temperature for 12 h and stored under low-humidity conditions. Finally, the four components of the lateral flow DNA biosensor were assembled on plastic adhesive backing (6 cm × 30 cm). Each part overlapped by 2 mm to ensure solution migration through the strip during the assay. Strips were cut into 3 mm width with a lateral flow paper cutter.

Protein detection strategies

The protein-to-DNA signal transducer consisted of anti-NGF-β antibody conjugated DNA 1, anti-NGF-β antibody conjugated DNA2 and signal-output element (DNA3). Briefly, 100 μL of

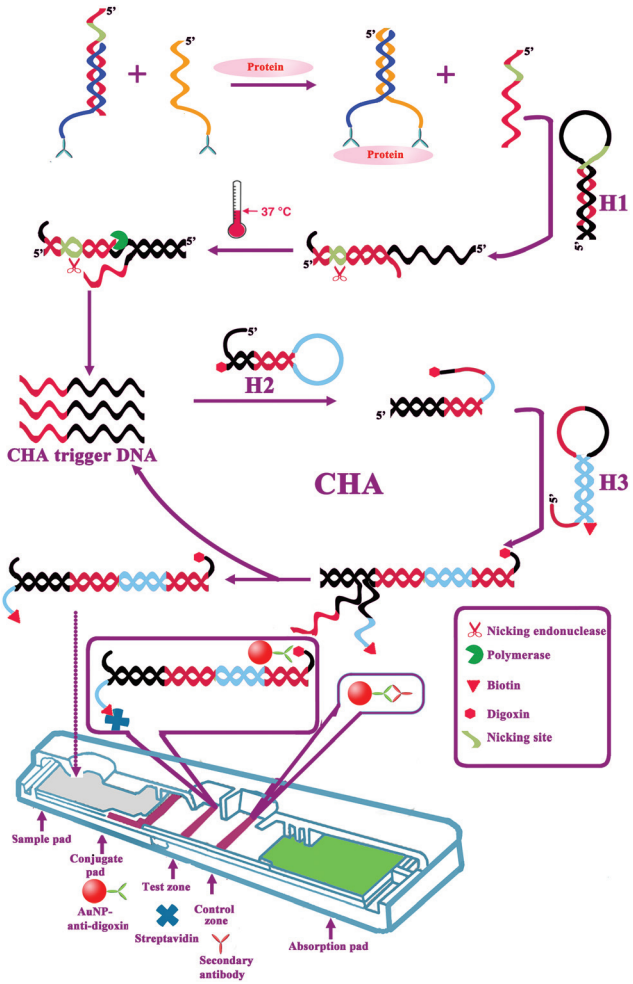
rCutSmar buffer containing 3 μM anti-NGF-β antibody-DNA1 and 2 μM DNA3 was heated to 55 °C for 5 min, and the solution was allowed to cool down to 25 °C slowly in a period of 2 hours to form the anti-NGF-β antibody conjugated DNA1/DNA3 duplex. Proximity hybridization-mediated isothermal exponential amplification and CHA were performed in 100 μL of rCutSmar buffer containing 300 nM anti-NGF-β antibody conjugated DNA1/DNA3 complex, 300 nM anti-NGF-β antibody conjugated DNA2, 200 nM H1, 30 U Bst polymerase large fragment, 20 U Nb·BbvCI nicking endonuclease, 100 μM dNTPs, 300 nM H2, 300 nM H3, and different concentrations of the target protein at 37 °C for 90 min. Finally, the reaction products were directly loaded onto the sample pad for the LFB assay. The red bands were observed within 5 min, and then quantitative detection was performed by recording optical intensities of the test zone and the control zone with a portable lateral flow biosensor reader. The optical intensities measured by the reader mainly included the peak height and the area integral.

Results

Detection principle

The protein signal transducer (Scheme 1) consists of target-recognition elements (antibody conjugated DNA1 and DNA2) and a signal-output element (DNA3). DNA1 conjugated with the NGF-β antibody is firstly hybridized to DNA3 to form a stable DNA1/DNA3 duplex. DNA3 is designed to have a nicking endonuclease recognition site near its 5′ end (reseda region). The DNA2 sequence is designed to be complementary with DNA1. In the absence of the target protein, the strand displacement activity is poor between DNA2 and DNA3 at 37 °C and the releasing of output DNA3 by competing DNA2 is extremely limited. In the presence of the target protein, the binding of the same protein to the two antibodies which are coupled with DNA1/DNA2 assembles DNA1 and DNA2 together, resulting in increasing their local effective concentration. This process raises the melting temperature for the intramolecular hybridization (DNA1 and DNA2), favouring the strand displacement reaction between DNA2 and DNA3. As a result, the output DNA3 is released for further signal amplification.

The releasing output DNA3 was further amplified by EXPAR and CHA with 3 kinds of hairpin probes. DNA3 recognizes and partially hybridizes with H1 to form a partial complementary double strand DNA containing a complete nicking endonuclease recognition site near its 5′ end. EXPAR is initiated in the presence of Nb·BbvCI nicking endonuclease, Bst polymerase and dNTPs to produce a large number of single strand DNA (CHA trigger DNA) in the circle of nicking, polymerization and strand displacement. The resulting ssDNA recognizes and partially hybridizes with 3′-digoxin modified H2 causing the hairpin probe to undergo a conformational change leading to stem separation and forming a H2-ssDNA intermediate. Then the H2-ssDNA intermediate catalyzes the dynamic assembly of 3′-biotin modified H3 to produce H2–H3 duplex accompanying



Scheme 1 Schematic illustration for protein detection based on proximity hybridization-mediated isothermal exponential amplification.

the release of ssDNA. Meanwhile, the released ssDNA can further initiate another CHA reaction for signal amplification. Finally, a great amount of duplex DNA products with biotin and digoxin modification at the 3′ end is synthesized. The hairpin probe retains its original stem–loop structure in the absence of the target protein, because the hairpin probes were designed to be more stable in the hairpin structure than in the heterodimer structure. The amplification products can be detected with a lateral flow biosensor. The LFB consists of four components: sample pad, conjugate pad, nitrocellulose membrane, and absorption pad. The anti-digoxin antibodies conjugated with gold nanoparticles (AuNPs) are pre-dispensed on the conjugate pad. Streptavidin and goat-anti-mouse IgG antibody (secondary body) are dispensed on the nitrocellulose membrane respectively to form the test zone and control zone. The sample solution containing the CHA product and running buffer is applied on the sample pad of the LFBs. The solution migrates along the LFB by capillary action through the conjugate pad, where anti-digoxin antibody–AuNP conjugates have been dispensed. Anti-digoxin antibody coupled with digoxin of the CHA duplex DNA product to form a biotin-duplex DNA-

digoxin–anti-digoxin–AuNP complex. The complexes are captured at the test zone by the specific reaction between pre-immobilized streptavidin and biotin. The accumulation of AuNPs on the test zone is then visualized as a characteristic red band. Excess anti-digoxin–AuNP conjugates continue to migrate and are captured at the control zone by immuno-reactions between the anti-digoxin antibody and the pre-immobilized secondary antibody on the nitrocellulose membrane surface, thus forming a second red band. In the absence of the target protein, no CHA duplex DNA product is produced; therefore, no red band is observed at the test zone. In this case, the red band at the control zone manifests that the LFB is working correctly. Visual detection is realized by observing the colour change caused by the accumulation of gold nanoparticles on the test zone, and quantitative detection can be performed by scanning the colour intensity of the red band on the test zone using a portable lateral flow biosensor reader.

Feasibility study

In this work, EXPAR and CHA were used to amplify the DNA and produce a large number of digoxin- and biotin-coupled duplex DNA for the point of care detection of proteins. We conducted agarose gel electrophoresis for the reaction products obtained from different stages of the isothermal reaction. As shown in Fig. 1(A), the last band at the bottom of lane 3 indicated the DNA3 was displaced by the hybridization of DNA1 and DNA2. The last band at the bottom of lane 4 displayed the products of EXPAR. The bright band in land 7 indicated the products of the H2–H3 duplex. We next investigated the optical response of 100 nM target on the LFB with and without EXPAR and CHA. In the case without EXPAR and CHA, there was no hairpin 1 or hairpin 2 or hairpin3 in the reaction buffer. As shown in Fig. 1B, the optical response of the sample with 100 nM target protein after the EXPAR and CHA reaction was 34 times higher than that of the negative control. It should be noted that the optical response of the sample without EXPAR and CHA (2: without hairpin probe 1; 3: without hairpin probe 2; 4 without hairpin probe 3) is comparable to that of the negative control. These experimental results showed that the EXPAR and CHA did occur as expected for signal amplification.

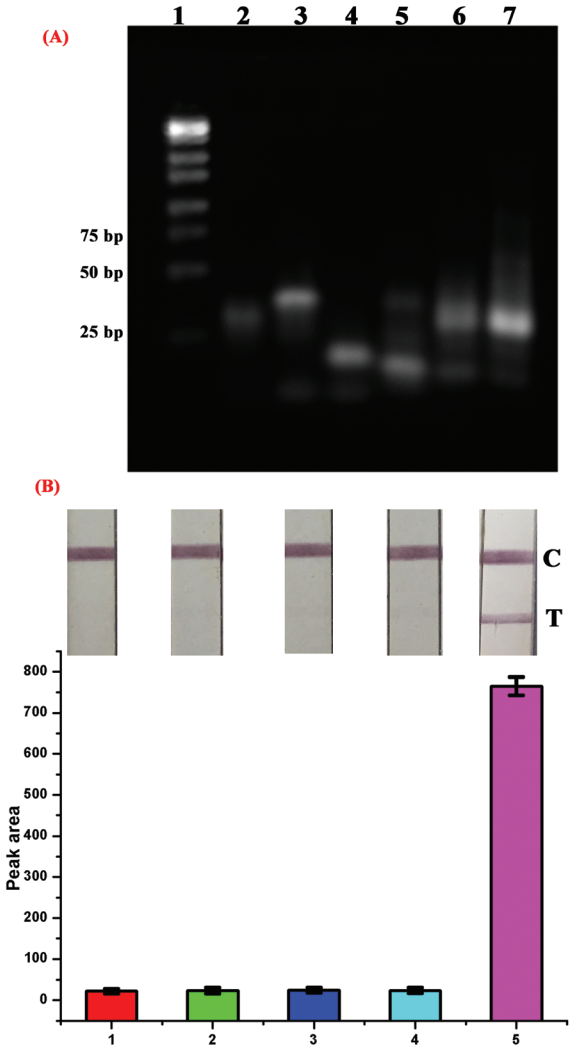


Fig. 1 Verification of the signal amplification of the proposed assay. (A) Gel electrophoresis analysis of different products. 1: DNA marker, 2: Ab-DNA1 + DNA3, 3: Ab-DNA1 + DNA3 + Ab-DNA2 + target for 90 min, 4: Ab-DNA1 + DNA3 + Ab-DNA2 + H1 + target for EXPAR for 90 min, 5: Ab-DNA1 + DNA3 + Ab-DNA2 + H1 + target for EXPAR for 0 min, 6: all reaction components for 30 min 7: all reaction components for 90 min (B) 1: NC, 2: without H1, 3: without H2, 4 without H3, 5, with all reaction components.

Optimization of detection conditions

To detect proteins with high sensitivity and selectivity, the reaction time and reaction temperature were optimized systematically. In this assay, the reaction time is one of the most important factors that affect the analytical performance of the assay. Usually, the products of the H2–H3 duplex increased with the increase of the reaction time due to the increase of the products of DNA3 and CHA trigger ssDNA. As shown in Fig. 2A, the peak area elevated gradually with the increase of reaction time from 0 min to 90 min in the presence of 100 nM target protein. Over 90 min, the optical response increases very slowly but the background signal increases greatly, leading to the sharp decrease of the signal-to-noise (S/N) ratios. Therefore, 90 min was selected as the reaction time in this assay.

The reaction temperature affects the amount of duplex DNA generated in the presence of the target protein. We compared the S/N ratio of 100 nM target protein with four different reaction temperatures (25 °C, 37 °C, 42 °C and 50 °C). As shown in Fig. 2B, the optical responses increase sharply from 25 °C to 37 °C and remain in the plateau in the range from 37 °C to 50 °C. The S/N ratio decreased when the temperature was over 37 °C due to the increase of the background signal, which can be explained by the conformational change of the hairpin probe at a higher temperature, leading to the stem separation and nonspecific binding of the H2 and H3 probe. Therefore, an isothermal amplification temperature of 37 °C was selected in the following experiments.

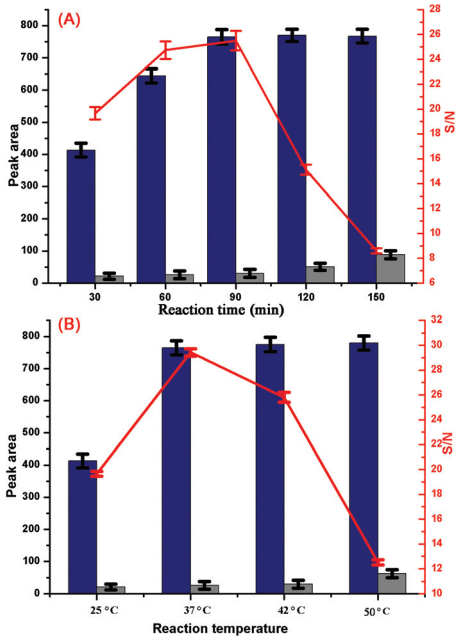


Fig. 2 (A) Effect of the reaction time on the S/N ratio with 100 nM protein target of the proposed assay. (B) Effect of reaction temperature on the S/N ratio with 100 nM protein target of the proposed assay.

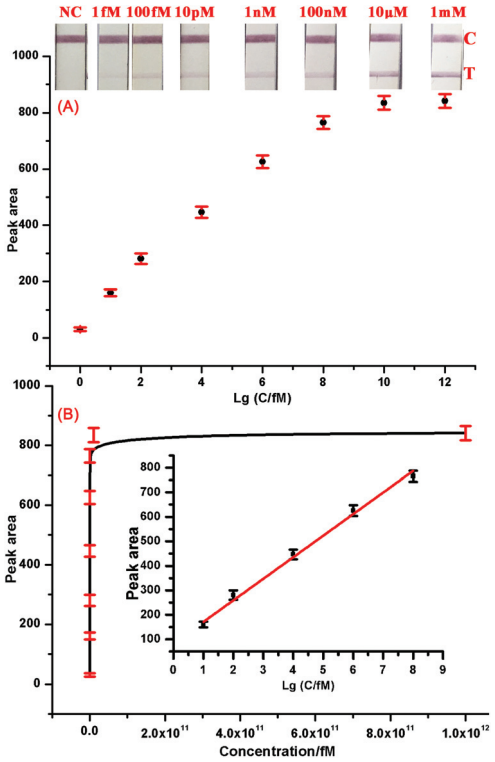


Fig. 3 (A) Photo images and curve plot of detection results of the LFB with different concentrations of the target protein. (B) The calibration curve plotted with the peak areas *vs.* NGF-β concentrations. Inset: The linear relationship between the peak areas and the logarithm of NGF-β concentrations. Error bars represent standard deviation, $n = 3$.

Analytical performances

We have examined the sensitivity and dynamic range of the LFB using different concentrations of the target protein under optimal conditions. As shown in Fig. 3A, the colour intensities of the red bands on the test zone increased with the increase of target protein concentrations. No distinct red band was observed on the test zone in the negative control (NC). For quantitative detection, the optical responses were further confirmed by recording the colour intensities of the red bands with a portable strip reader. The peak areas *vs.* the incremental concentrations of the target have been plotted as shown in Fig. 3B, which followed a sigmoid increase. The resulting calibration curve shows that the peak areas are proportional to the logarithm of target protein concentrations in the range from 1 fM to 100 nM (inset of Fig. 3B). The red band on the test zone was observed with as low as 1 fM. Based on 3 times standard deviation of 3 measurements of negative control samples (NC), the detection limit (LOD) was calculated to be 0.74 fM. Based on the molecular weight of NGF-β (13.5 kDa), the LOD is 9.9 fg mL^{-1}. The detection limit of our proposed approach is 2 orders of magnitude better than the antibody-based immunoassay.[3,4] The LOD is comparable or even superior to those of previously reported protein detection approaches (Table S1, ESI†).[25,26] Generally, human basal serum NGF-β concentrations are between 20 pg mL^{-1} and 30 pg mL^{-1}, and this LFB holds great promise in the monitoring of NGF-β by the naked eye due to its superior detection sensitivity.

The specificity of the LFB for NGF-β was studied by testing the responses of the assay to other kinds of growth factors including PDGF, TGF-α, TGF-β, EGF, IGF-I and aFGF, which are similar to NGF-β. As shown in Fig. 4, 100 nM NGF-β could produce a visible bright red band on the test zone of the LFB, whereas all other growth factors at a concentration of 10 μM did not show a red band on the test zone of the LFB. These

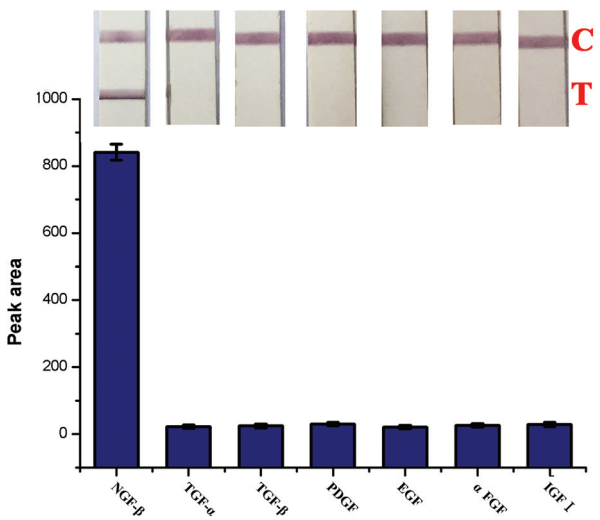


Fig. 4 Photo images and histograms of the LFBs for specificity analysis with NGF-β and other kinds of growth factors which are similar to NGF-β.

results indicated that our constructed LFB exhibited excellent selective responsive to NGF-β.

To demonstrate whether our developed LFB could be applied to real sample analysis, a spiking test was carried out in human serum and saliva samples. Aliquots of the serum and saliva samples were spiked with different concentrations of the target protein. As displayed in Table S2 (ESI†), the recovery values and the relative standard deviations were in the range of 95.3% – 105.5% and 4.3% – 6.2%, respectively. These results indicated that the possible interference from other proteins to NGF-β analysis was negligible.

Conclusions

We have demonstrated a lateral flow biosensor for the amplified detection of the protein target using a protein-to-DNA signal transducer for converting the input protein into output DNA signal, and EXPAR and CHA for signal amplification. Compared to other reported antibody-based immunoassay or protein-to-DNA signal transducer methods, the sensitivity of the LFB developed here is higher due to the higher amplification efficiency by two kinds of isothermal amplification technologies. By using this assay, a target protein concentration as low as 0.74 fM could be detected by the naked eye within 1.5 h. The response of the optimized assay is highly linear over a range of 1 fM to 100 nM. This new detection method is expected to be a great potential platform for protein universal detection.

Author contributions

Zibin Tang: Methodology, formal analysis, investigation, validation and writing – original draft. Wenyong Zhao: Methodology, formal analysis, investigation and validation. Yuling Deng: Methodology, formal analysis, investigation and validation. Yuanzhong Sun: Investigation and validation. Cailing Qiu: Investigation. Binhua Wu: Investigation. Juan Bao: Investigation. Zhangquan Chen: Methodology, writing – original draft and writing – review and editing. Luxin Yu: Conceptualization, methodology, resources, supervision, formal analysis, project administration, writing – original draft and writing – review and editing.

Conflicts of interest

The authors declare no conflicts of interest.

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