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Strip biosensor for amplified detection of nerve growth factor-beta based on a molecular translator and catalytic DNA circuit

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We have demonstrated a new visual detection approach based on a molecular translator and a catalytic DNA circuit for the detection of nerve growth factor-beta (NGF-β). In this assay, a molecular translator based on the binding-induced DNA strand-displacement reaction was employed to convert the input protein to an output DNA signal. The molecular translator is composed of a target recognition element and a signal output element. Target recognition is achieved by the binding of the anti-NGF-β antibody to the target protein. Polyclonal anti-NGF-β antibody is conjugated to DNA1 and DNA2. The antibody conjugated DNA1 is initially hybridized to DNA3 to form a stable DNA1/DNA3 duplex. In the presence of NGF-β, the binding of the same target protein brings DNA1 and DNA2 into close proximity, resulting in an increase in their local effective concentration. This process triggers the strand-displacement reaction between DNA2 and DNA3 and releases the output DNA3. The released DNA3 is further amplified by a catalytic DNA circuit. The product of the catalytic DNA circuit is detected by a strip biosensor. This proposed assay has high sensitivity and selectivity with a dynamic response ranging from 10 fM to 10 pM, and its detection limit is 10 fM of NGF-β. This work provides a sensitive, enzyme-free, and universal strategy for the detection of other proteins.

Introduction

Nerve growth factor (NGF) is a small-molecule protein that is important for the growth, maintenance, and survival of certain target neurons. Nerve growth factor-beta (NGF-β) is the active form of NGF. The detection of NGF-β is of central importance for monitoring the survival and maintenance of sympathetic and sensory neurons.[1] Several methods have thus far been developed for the detection of NGF-β. Conventional antibody-based methods such as Western blot, dot blot, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry are widely used analysis techniques in both clinical diagnosis and research.[2] These methods, however, are time-consuming, laborious, high cost, and low sensitivity. Therefore, continuing efforts have been made to seek ideal tools for the fast, sensitive, cost-effective, and easy-to-use detection of NGF-β.

Molecular translators can convert input target molecules into unique output DNA for signal amplification. For example, Ghadiri's group have developed a novel nucleic acid detection method to convert any target nucleic acid to a predesigned output DNA.[3] Le and co-workers have designed a molecular translator based on binding-induced DNA assembly for the fluorescence detection of proteins including prostate specific antigen (PSA) and platelet derived growth factor (PDGF).[4] Although these approaches are effective, most of them necessitate the use of expensive instruments, require a well-trained operator, and lack portability.

Recently, strip biosensors have attracted a great deal of research interest because of their portability, simplicity, short assay time, and cost-effectiveness. The result of the assay can be observed by the naked eye, and quantitative analysis can be realized by recording the color intensity of the red band on the test zone with a portable strip biosensor reader.[5] To improve the detection sensitivity, various amplification strategies have been developed such as polymerase chain reaction (PCR),[6] strand-displacement amplification (SDA),[7] rolling circle amplification (RCA),[8] and nicking endonuclease signal amplification (NESA).[9] Although these methods have resulted in great progress in sensitivity improvement, all of them require the use of enzymes for signal amplification, which increases the cost and complexity of the analysis. As an alternative, enzyme-free approaches have been developed; catalytic DNA circuit is a commonly used enzyme-free approach for DNA signal amplification due to its robustness, cost-effectiveness, and simplicity.[10] In this work, for the first time, we propose a strip biosensor for detecting NGF-β based on a molecular translator and catalytic DNA circuit.

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Experimental

Reagents and apparatus

Nerve growth factor-beta and polyclonal anti-NGF-β antibody, anti-digoxin antibody, goat anti-mouse IgG antibody (secondary body), bovine serum albumin (BSA), streptavidin (SA), and gold nanoparticles (AuNPs, 20 nm) were obtained from Sigma-Aldrich (Steinheim, Germany). Glass fiber (CFSP001700) and nitrocellulose membrane (HF24002XSS) were obtained from Millipore (Billerica, MA). Other chemicals were purchased from standard commercial sources and were of analytical grade. All buffer solutions used in this study were prepared in our laboratory with ultrapure water (18.2 MΩ cm^{-1}) from a Millipore Milli-Q water purification system (Billerica, MA).

The oligonucleotide sequences were synthesized and purified through HPLC by Shanghai Sangon Biological Engineering Technology (Shanghai, China). The oligonucleotide sequences are listed in Table 1.

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

The anti-NGF-β antibody–DNA1/DNA2 conjugates were synthesized by a zero-length crosslinker (EDC/NHS). Briefly, anti-NGF-β antibody (5 mg mL^{-1}) and 0.1 M EDC were added to PBS (0.1 mM phosphate buffer [pH 7.4] and 100 nM NaCl), and amine-modified DNA was added to the antibody solution at a 10 : 1 ratio. Sulfo-NHS was then added to the mixture to bring its final concentration to 5 mM followed by mixing to dissolve and reaction for 2 h at room temperature. The excess DNA was removed by ultrafiltration (100 000 molecular weight cutoff). Final protein concentrations were determined with a Pierce GCATM protein assay kit (in product no. 23227, Pierce, Rockford, IL).

Table 1 Oligonucleotide sequences[a]

Oligonucleotide	Sequences
DNA1	5'-NH_2-C6-TTTTTTTTTTTTTTTTTGCTAGGTAAAGAGA-3'
DAN2	5'- TTTACCTAGCAATTTTTTTTTTTTTTT-C6-NH_2-3'
DNA3 (output DNA, C7)	3* 2* 5'-ATGGGCGCA CCTCTCTTT 1* ACCTAGC-3'
DNA3 (C9)	3* 2* 5'-ATGGGCGCA CCTCTCTTT 1* ACCTAGCAA-3'
DNA3 (C11)	3* 2* 5'-ATGGGCGCA CCTCTCTTT 1* ACCTAGCAATT-3'
DNA3 (C13)	3* 2* 5'-ATGGGCGCA CCTCTCTTT 1* ACCTAGCAATTGC-3'
Hairpin1 (H1)	1 2 5'- GCAATTGCTAGGT AAAGAGAGG 3 4* TGCGCCCAT CCATGTGTAGA 3* 2* ATGGGCGCA CCTCTCTTT 5* 6* CCTTGTCA TAGAGCAC-digoxin-3'
Hairpin2 (H2)	3 4 5'-TGCGCCCAT TCTACACATGG 3* 2* ATGGGCGCA CCTCTCTTT 4* CCATGTGTAGA-biotin-3'

[a] Domains are named by numbers, and their complementary domain is denoted by asterisks.

Preparation of anti-digoxin antibody–AuNP conjugates

Conjugation reactions were performed by adding 20 μL of 1 mg mL^{-1} anti-digoxin antibody to 2 mL of a tenfold-concentrated AuNP solution (pH 8.4). The solution was then incubated at room temperature with gentle shaking for 1 h. Subsequently, a 10% wt/vol BSA stock solution (pH 7.0) was added to the AuNP solution to a final concentration of 1% wt/vol. After incubation at room temperature for one hour, the resulting AuNP solution was concentrated by centrifugation for 30 min at 13 400 × *g*. The supernatant was discarded without disturbing the pellet, and the pellet was resuspended with 100 μL of suspension solution comprising 20 nM Na_3PO_4, 10% BSA, and 0.05% NaN_3. The resuspended solution was stored at 4 °C.

Preparation of strip biosensor

The strip biosensor was constructed as shown in Fig. 1C. The biosensor consists of a sample pad, a conjugate pad, a nitrocellulose membrane and an absorbent pad. The sample pad (glass fiber; 17 mm × 30 cm) was prepared by soaking it in the sample pad buffer (pH 8.0; 1% triton, 3% BSA, 2% glucose, and 50 mM boric acid) for 5 min. The prepared sample pad was then dried and stored at room temperature. The conjugate pad (8 mm × 30 cm) was prepared by dispensing a certain volume of AuNP–anti-digoxin conjugate onto the glass fiber using a dispenser (Shanghai Kinbio, Shanghai, China). The amount of dispensed AuNPs on the conjugate pad was 4 μL cm^{-1}. The prepared conjugate pad was dried at room temperature for 12 h and stored at 4 °C. Streptavidin (30 μL, 1 mg mL^{-1}) and secondary antibody (30 μL, 7.4 mg mL^{-1}) were dispensed onto the nitrocellulose membrane at the speed of 1 μL cm^{-1} to form the test and control zones, respectively. The distance between the test zone and the control zone was about 6 mm. The membrane was then dried at room temperature for 12 h and stored at 4 °C. Finally, the four elements of the strip biosensor were assembled on plastic adhesive backing (60 mm × 30 cm). Each part overlapped by 2 mm to ensure solution migration through the strip during the assay. Strips were cut into 4 mm widths with a paper cutter (programmed high speed cutter, Shanghai Kinbio, Shanghai, China).

Construction of the molecular translator

A schematic of the molecular translator for the detection of NGF-β is shown in Fig. 1A. The DNA probe for the molecular translator was prepared at a final concentration of 5 μM by mixing 20 μL 50 μM anti-NGF-β antibody conjugated DNA1 with 13.3 μL 50 μM output DNA3 in 166.7 μM TE buffer containing

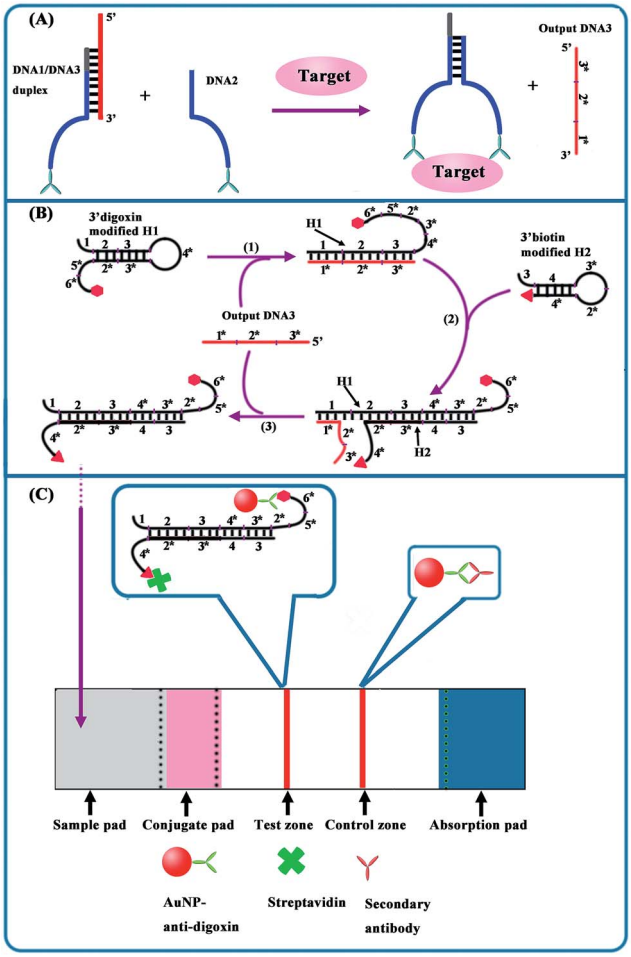

Fig. 1 (A) Schematic of the molecular translator. The input NGF-β is converted into output DNA3 by the binding-induced strand-displacement activity of the molecular translator. (B) The output DNA3 triggers a catalytic DNA circuit for signal amplification by producing a large amount of duplex DNA. (C) Schematic illustration of the strip biosensor for the visual detection of duplex DNA. AuNP–anti-digoxin antibody is dispensed on the conjugate pad. Streptavidin and secondary antibody are dispensed on the test zone and control zone, respectively.

10 mM $MgCl_2$ and 0.05% tween 20, heating to 90 °C for 5 min, and allowing the solution to slowly cool to 25 °C over 3 hours. The molecular translator solution (partially complementary, anti-NGF-β antibody conjugated DNA1/DNA3 complex) was stored at 5 °C.

Detection of NGF-β by the strip biosensor

Strand displacement of the molecular translator was performed in 20 μL TE buffer containing 10 mM $MgCl_2$ and 0.05% tween 20, 10 nM DNA1/DNA3 complex, 10 nM DNA2 and different concentrations of NGF-β. The reaction mixture was incubated at 25 °C for 45 min. Subsequently, 200 nM H1 and 300 nM H2 were added to the reaction solution and incubated for 90 min at 42 °C. Finally, the reaction mixture was loaded onto the sample pad together with 40 μL PBS. The red bands were observed within 5 min, and the biosensor was scanned with a portable strip biosensor reader (Shanghai Kinbio, Shanghai, China) 10 min later. The optical intensities of the test zone and the control zone were recorded simultaneously by the reader, which automatically located the red bands in a fixed reaction area and then measured strip parameters such as peak height and area integral.

Results and discussion

Principle of the strip biosensor

The molecular translator (Fig. 1A) is composed of a target-recognition element (DNA1 and DNA2) and a signal-output element (DNA3). DNA1 conjugated with NGF-β antibody is initially hybridized to DNA3 to form a stable DNA1/DNA3 duplex. DNA2 sequences are designed in such a way that the complementary sequences between DNA1 and DNA2 are 4 nt shorter than the complementary sequences between DNA1 and DNA3. In the absence of NGF-β, the strand displacement activity between DNA2 and DNA3 is poor at 25 °C. The displacement of output DNA3 by competing DNA2 is extremely minimal. However, in the presence of NGF-β, the binding of the same target protein to its antibodies, which are coupled with DNA1 and DNA2, brings DNA1 and DNA2 into close proximity, increasing their local effective concentration. This process triggers the strand-displacement reaction between DNA2 and DNA3. As a result, the output DNA3 is released for the next signal amplification (Fig. 1A).

The released DNA3 containing three domains (domain 1*, 2*, and 3*) recognizes and hybridizes with the complementary domain of haipine1 (H1) by a toehold-mediated strand displacement reaction. This causes H1 to undergo a conformational change, leading to stem separation. The domain 3 of hairpin H2 then hybridizes to the newly opened toehold 3* of H1. This initiates a branch migration to open H2, forming the DNA3–H1–H2 complex. The last step of the catalytic DNA circuit is the spontaneous dissociation of DNA3 from the H1–H2 duplex. During this circular process, the released DNA3 can act as a catalyst to trigger another catalytic DNA circuit, and a great number of H1–H2 duplexes are finally produced. H1 is modified with a digoxin group at the 3′ end, and H2 is modified with a biotin group at the 5′ end (Fig. 1B). The amplification product can be detected by a strip biosensor.

The strip biosensor consists of four components: a sample pad, conjugate pad, nitrocellulose membrane, and absorption pad. The anti-digoxin antibodies conjugated with AuNPs are dispensed on the conjugate pad. Streptavidin and goat anti-mouse IgG antibody (secondary body) are dispensed on the nitrocellulose membrane to form the test zone and control zone, respectively. The sample solution containing the catalytic DNA circuit product and running buffer is applied on the sample pad of the strip biosensor. The solution migrates along the strip biosensor by capillary action through the conjugate pad, where anti-digoxin–AuNP conjugates have been deposited. The anti-digoxin antibody couples with the catalytic DNA circuit 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 in 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 anti-digoxin antibody and the pre-immobilized secondary antibody on the AuNP surface, forming a second red band. In the absence of NGF-β, no duplex DNA product is produced; therefore, no red band is observed in the test zone. In this case, the red band in the control zone indicates that the strip biosensor is working properly. Visual detection is performed by observing the color change caused by the accumulation of AuNPs in the test zone, and quantitative detection can be realized by recording the color intensity of the red band in the test zone with a portable strip biosensor reader (Fig. 1C).

Optimization of experimental parameters

In this assay, the reaction time of the catalytic DNA circuit is one of the most important factors affecting assay sensitivity. Usually, the catalytic DNA circuit can be enhanced by increasing the reaction time. As shown in Fig. 2A, the optical response is gradually elevated as the reaction time is increased from 0 min to 90 min in the presence of NGF-β at 10 pM and 1 pM and then kept at a constant level. However, further increasing the reaction time led to an increase in the background signal. Therefore, 90 min was selected as the optimal reaction time for the experiments.

The reaction rate of the toehold-mediated catalytic DNA circuit depends primarily on the length of the toehold. As shown in Fig. 2B, the peak area of the red band on the test zone of the strip biosensor increases as the toehold lengths are increased from 7 nt to 11 nt. A further increase in toehold length to 13 nt leads to a lower optical response due to the irreversible toehold binding reaction. Therefore, an 11 nt toehold length was chosen for this assay.

Under optimal conditions, we examined the sensitivity and dynamic range of the strip biosensor using different concentrations of NGF-β. As shown in Fig. 3A, the color intensities of the red bands on the test zone increased with increasing NGF-β concentration. No distinct red band was observed on the test zone in the absence of NGF-β. For quantitative detection, the optical responses were further confirmed by recording the color intensities of the red bands using a portable strip reader. A good linear relationship between peak area and the logarithm of the NGF-β concentration was obtained from 10 fM to 10 pM (Fig. 3B, inset). The red band on the test zone was observed for NGF-β concentration as low as 10 fM, which can be considered the limit of detection (LOD). This detection limit is three orders of magnitude better than the antibody-based immunoassay.[2] Generally, human basal serum NGF concentrations are between 1.5 pM and 2.2 pM; therefore, this strip biosensor holds great promise in monitoring NGF-β with the naked eye due to its superior detection sensitivity and large dynamic range.

Due to this assay's potential for clinical application, assay performance in complicated matrices is an important

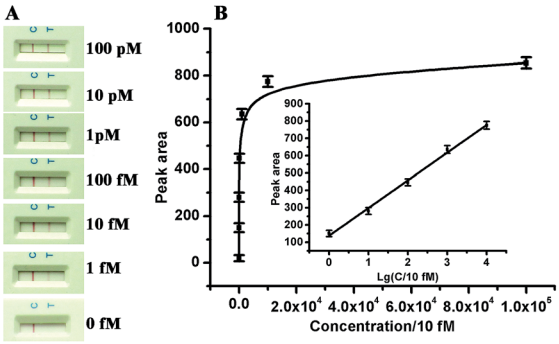

Fig. 3 (A) Photos of strip biosensor detection results for different concentrations of NGF-β. The images were recorded with a digital camera. (B) The calibration curve plotting peak areas *vs.* NGF-β concentrations. Inset: the linear relationship between the peak areas and the logarithm of NGF-β concentrations. Error bars represent standard deviation, $n = 3$.

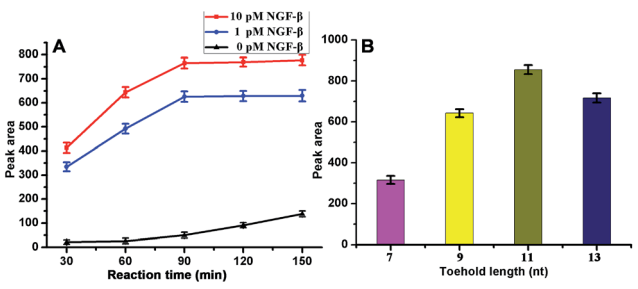

Fig. 2 (A) Effect of the catalytic DNA circuit reaction time on the *S*/*N* ration of 10 pM and 1 pM NGF-β assay. (B) Effect of toehold length on the reaction rate of the catalytic DNA circuit. Four DNA3 strands (named C7, C9, C11, C13) were chosen to trigger the catalytic DNA circuit; their corresponding toehold lengths are 7, 9, 11, and 13 nt. $C = 10$ nM, H1 = 200 nM, H2 = 300 nM, NGF-β = 10 pM. Reaction time = 90 min.

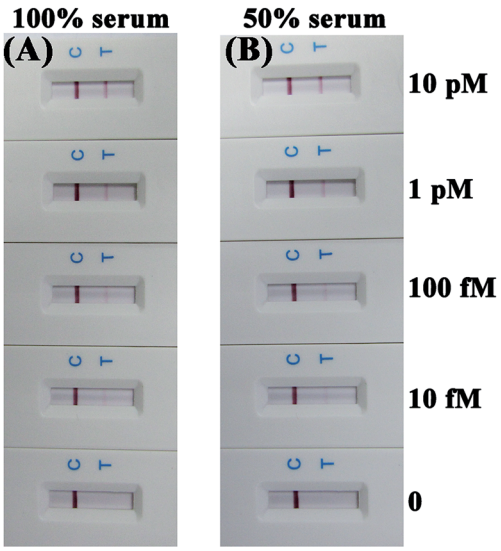

Fig. 4 Photo of the strip biosensor detection results for different concentrations of NGF-β in serum matrices. (A) Different concentrations of NGF-β in pure serum matrix. (B) Different concentrations of NGF-β in 2-fold diluted serum matrix.

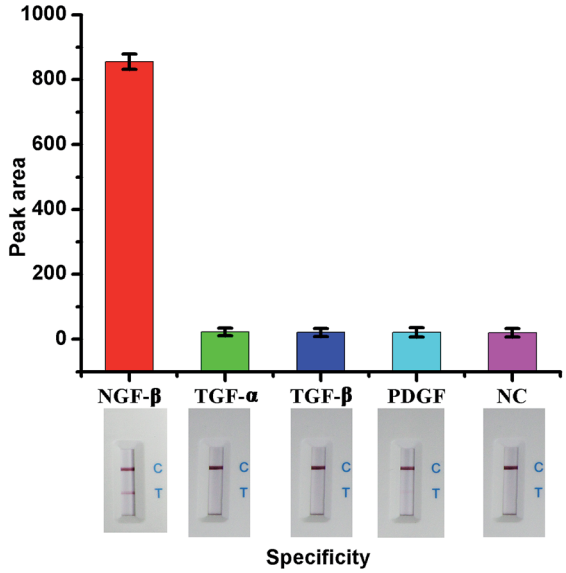

Fig. 5 Plots and photos demonstrating the specificity of the strip biosensors for NGF-β.

parameter to evaluate. We studied the performance of our proposed approach in pure and 2-fold diluted serum with different concentrations of NGF-β ranging from 10 fM to 10 pM. As shown in Fig. 4, the strip biosensor can detect as low as 10 fM NGF-β in both the pure and 2-fold diluted serum.

The specificity of the strip biosensor for NGF-β was studied by testing the assay responses to other kinds of growth factors including PDGF, TGF-α, and TGF-β, which are similar to NGF-β. As shown in Fig. 5, 1 pM NGF-β produced a visible bright red band on the test zone of strip biosensor, whereas all other growth factors at a concentration of 10 pM did not yield a red band on the test zone of the biosensor. These results indicate that our constructed strip biosensor exhibits excellent specificity for NGF-β.

Conclusions

In summary, we have successfully fabricated a strip biosensor for the amplified detection of NGF-β using a molecular translator for converting the input protein to an output DNA signal and a catalytic DNA circuit for signal amplification. Compared to other reported antibody-based immunoassays, the sensitivity of the strip biosensor developed here is higher. This assay allows NGF-β at concentrations as low as 10 fM to be detected by the naked eye. The response of the optimized assay is highly linear over the concentration range of 10 fM to 10 pM. Using other recognition elements such as aptamer or complementary DNA to replace the antibody, this proof of concept approach can further reduce the assay cost. This new NGF-β detection method is expected to be a great potential platform for protein detection.

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