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Terminal protection of a small molecule-linked loop DNA probe for turn-on label-free fluorescence detection of proteins

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ABSTRACT

A novel label-free turn-on fluorescence biosensor for the determination of streptavidin (SA) was proposed. Using terminal protection of small molecule-linked DNA chimeras, which can protect DNA from degradation by various exonucleases when the small molecule moieties are bound to their protein target, we designed a loop probe, where the 3′-end was modified with biotin to resist digestion by exonucleases in the presence of target SA. Coupled with an intercalating dye, SYBR Green I, strong enhancement of the fluorescence signals was obtained compared with that in the absence of SA. A linear correlation equation was obtained for SA from 0 to 200 nM with a limit detection of 0.4 nM. This strategy holds great promise for practical applications with good specificity and sensitivity.

1. Introduction

Investigations of small molecule–protein interaction are of great importance in chemistry, biology (Stockwell, 2004) and medicine (Howitz et al., 2003), which can not only help to reveal the mechanisms of many important physiological processes, but also provide the opportunities for molecular diagnostics, anticancer therapies (Vassilev et al., 2004) and biomedical research. Numerous analytical methods are proposed for the detection of proteins (Wang et al., 2005) and small molecules (Kanoh et al., 2003). Monoclonal antibodies and aptamers are typically used for protein detection (Wu et al., 2010), but generally, aptamers appears to have more advantages such as low molecular weight, simple and reproducible synthesis, thermal and long-term stability, reusability, and signal transduction (Famulok et al., 2007). However, the limited number of aptamers and proteins with RNA aptamers restricts their applications. In addition, RNA aptamers are unstable and easily degraded by ribonucleases. The capability of signal transduction of many aptamer assays is not enormous (Iliuk et al., 2011; Lubin and Plaxco, 2010). Oligonucleotides terminally tethered with protein binding small molecules may represent an ideal option for the detection of these proteins. (Wu et al., 2009) developed a novel method using the binding of small molecules linked to DNA by protein, called terminal protection of small molecule linked DNA (TPSMLD). The small organic molecules that bind to target protein receptors with reasonable affinity can resist degradation by the 3′ single-strand-specific exonuclease I. This interesting finding led to the detection of DNA (Wu et al., 2011) and proteins (Cao et al., 2012; He et al., 2013; Zhao et al., 2014).

Several biosensors are constructed based on TPSMLD, including electrochemical (Cao et al., 2012; Wu et al., 2009), fluorescence (Chen et al., 2014; He et al., 2013; Wu et al., 2011) and colorimetric methods (Yang and Gao, 2014; Zhao et al., 2014). In the fluorescence DNA analysis, some signal amplification strategy such as rolling circle amplification (Wang et al., 2013a) and other signal amplification technology (Wang et al., 2014; Wang et al., 2013a) can achieve a lower detection limit, but they usually need fluorescent reporters, which cause complex procedures, high cost and lower reproducibility. The development of a simple, inexpensive and label-free biosensor should be more attractive.

In this work, with a combination of Exo I and Exo III, we proposed a universal strategy for constructing a label-free fluorescence dsDNA biosensor for the sensitive detection of proteins. Owing to the specific properties of Exo I and Exo III, the DNA probe could be digested completely in the absence of target proteins, thus avoiding the incomplete digestion influence, and the sensor

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generating only a low background signal. Compared to other structures, like DNA labeled with fluorophore and quencher (Ou et al., 2013; Zhou et al., 2013), two single stranded DNA (Yang and Gao, 2014) and reorganized G-quadruplex structure (Wei et al., 2012; Zhao et al., 2014), the single-stranded DNA probe with two stem-loop structures could reduce the difficulty of design about DNA, avoid non-specific hybridization interference and effectively reduce the experiment costs. In the experimental process, this method has simple steps to operate and need less reaction time. In our design, streptavidin (SA) was chosen as a model target to conduct this experiment.

2. Materials and methods

2.1. Reagents and materials

Exonuclease I (Exo I) and exonuclease III (Exo III) were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China.); and SA, and SYGB Green I (SG I) from the Sangon Biotechnology Co., Ltd. (Shanghai, China). The original SG I solution was 10,000-fold concentrated and diluted to 100-fold with ultrapure water before use. All other chemicals were of analytical grade and used without additional purification. All solutions in the experiments were prepared using ultrapure water which was obtained through a Millipore Autopure WR600A system (Millipore, Ltd., U.S.A.) and had an electric resistance $\geq 18\ M\Omega$.

The oligonucleotides used in this work were synthesized by the Sangon Biotechnology Co., Ltd. (Shanghai, China) and the sequences of the synthesized oligonucleotides(5′→3′) are as follows:

CGTCGAACGAGACTTTTTTTTTTGTCTCGTTCGACGCGGCA-CATTGCGTTTTTTTTTTCGCAATGTGCCG-biotin.

2.2. Fluorescence measurement

The fluorescence spectra were recorded at room temperature in a quartz cuvette on an F-4500 spectrophotometer (Hitachi, Japan). The excitation wavelength was 497 nm, and the emission wavelengths were in the range 510–600 nm with both excitation and emission slits of 5 nm.

2.3. Terminal protection based fluorescence assays for the detection of streptavidin

The DNA concentration was estimated on a Nanodrop ND-1000 (Thermo Scientific, USA) with the DNA absorbance at 260 nm. First, a 10 μL aliquot of buffer solution (100 mM Tris–HCl, 10 mM $MgCl_2$, 20 mM DTT, pH 8.0) containing 1 μM biotin-linked loop DNA was denatured at 90 °C for 5 min and cooled to room temperature for further experiments. Then, 10 μL SA (final concentration range from 0 to 800 nM) was added to the DNA solution and the mixture was incubated for 30 min at room temperature to allow the complete interaction between SA and biotin. After that, Exo I and Exo III (final concentration, 0.5 U/μL) were added to the mixture to perform the digestion reaction for 60 min Then, SG I (final concentration, 2.5-fold) was added and well-mixed. Finally, the mixture was diluted to 200 μL and its fluorescence intensity measured.

2.4. The verification of terminal protection based SA-biotin reaction using polyacrylamide gel electrophoresis (PAGE)

An 8% native polyacrylamide gel was prepared for gel electrophoresis analysis using $1 \times TAE/Mg^{2+}$ buffer (tris 40 mM, acetic acid 20 mM, Na_2EDTA 2 mM, pH 8.0, Mg^{2+} 12.5 mM). The sample solutions were prepared as follows: 4 μM DNA and 800 nM SA in 20 μL buffer solution was incubated for 30 min, and then 20 U Exo I and 20 U Exo III were added, followed by another one hour digestion. The blank sample solution was prepared in the same way but without SA, and the control sample was the DNA probe solution (4 μM) without any enzyme treatment. The gel ran at 100 V for 90 min in $1 \times TAE/Mg^{2+}$ buffer. Then, it was stained in stains-all solution (0.1 g stains-all, 450 mL formamide, and 550 mL H_2O) for 30 min After this procedure, the gel was illuminated under sunlight for 5–10 min to obtain the stained bands. Finally, the PAGE results were photographed using a digital camera.

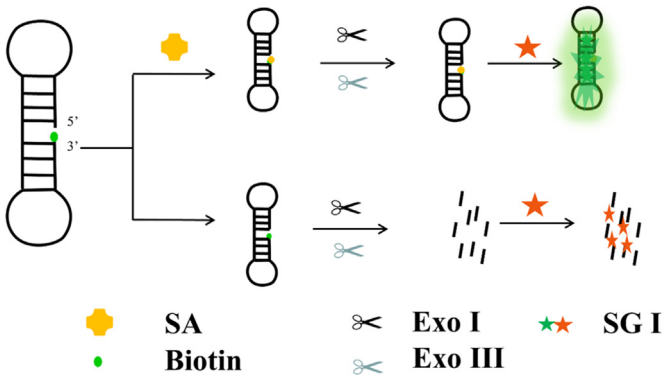
3. Results and discussion

3.1. Design principle of the strategy

In this work, we designed a label-free, fluorescence assay for SA detection based on the terminal protection of SA-biotin-DNA against exonucleases digestion. The sensing mechanism is illustrated in Scheme 1. In this sensing design, the DNA probe was designed as a single-stranded molecule that formed a self-complementary structure at both ends with two stem–loop structures, and biotin was covalently conjugated to the 3′-end. In the absence of SA, DNA-small molecule chimeras could be completely digested to mononucleotides after the addition of Exo I and Exo III. Because of the properties of Exo I and Exo III, the DNA probe section of double strands was degraded stepwise from the blunt 3′-end to 5′-end direction by Exo III at first, and then Exo I catalyzed the removal of 3′-end mononucleotides from the single-stranded DNA fragments. Both Exo I and Exo III were applied to digest the DNA probe to minimize the fluorescence background to an extremely low level. In the presence of SA, the biotin conjugated DNA probe was bound to SA with high affinity, the complex of SA-biotin DNA have steric hindrance effect to protect the DNA probe from degradation by Exo I and Exo III, and maintained the double-strand structure. A strong fluorescence signal was obtained after staining with SG I, and the fluorescence intensity was positively related to the concentration of the protein target SA.

3.2. Assay validation

To demonstrate the feasibility of this proposed approach, the fluorescence intensities of the SA-biotin-DNA probe solution in the absence/presence of the target SA, and with the addition of Exo I and Exo III, were first measured. As shown in Fig. 1(A), in the presence of SA, bright fluorescence of the DNA probe solution was observed (curve a) upon the addition of SG I. This result indicated the effective binding of SA to the ds-DNA probe, which resisted the



Scheme 1. Schematic illustration of the label-free fluorescence strategy for the selective detection of SA based on terminal protection of small molecule-linked loop DNA.

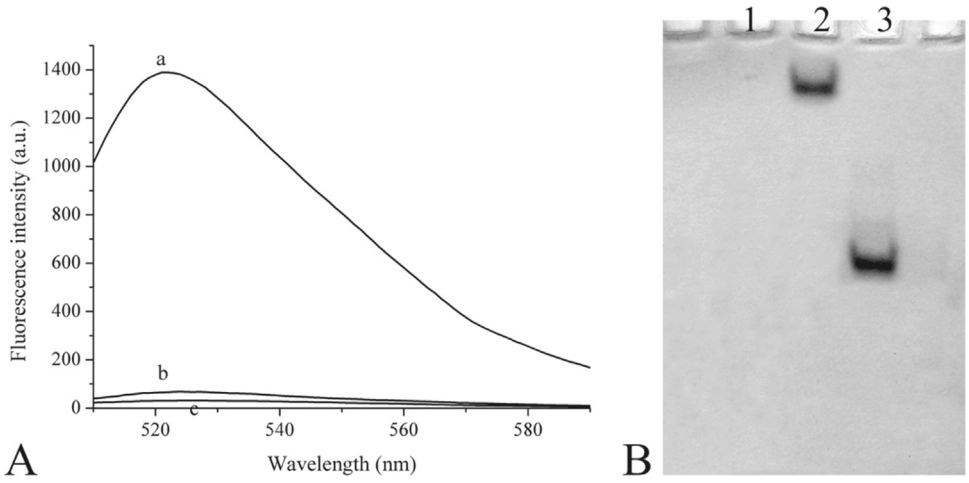


Fig. 1. (A) Typical fluorescence responses of small molecule-protein interaction assay. (a) 500 nM DNA+200 nM SA+Exo I+Exo III+SG I; (b) 400 nM DNA+Exo I+Exo III+SG I; (c) SG I. (B) Gel electrophoresis image for the label-free assay of SA compared with the control experiment. Lane 1: DNA probe digested by Exo I and Exo III without SA; Lane 2: DNA probe with SA following with Exo I and Exo III; Lane 3: DNA probe with no treatment.

digestion of Exo I and Exo III. However, in the absence of SA, a small increase of fluorescence intensity (curve b) could be found compared with that of SG I in the blank solution (curve c). In order to further verify the terminal protection strategy for SA, native PAGE was performed. In the gel electrophoresis image (Fig. 1(B)), an apparent band was observed for the ds-DNA probe (lane 3) without any other treatment, while no band (lane 1) was found with the addition of Exo I and Exo III due to the effective digestion of dsDNA to mononucleotides by the Exo I and Exo III. In contrast, after the incubation of ds-DNA with SA, a band (lane 2) with a decreased migration shift was observed compared to lane 3. The decreased migration shift resulted from the biotin-dsDNA bound to SA, indicating that SA bound to DNA could protect the dsDNA from digestion by Exo I and Exo III, and the decrease of migration shift was caused by the molecular weight increase from the SA bound to the dsDNA. These results revealed that the label-free and terminal protection strategy for SA detection was effective.

3.3. Optimization of the experimental parameters

In order to obtain the best analytical results, several vital experimental parameters were optimized including the amount of the DNA probe, the reaction time of the DNA probe with SA, and the concentration and digestion time of exonucleases. Experimental results indicated that the concentration of the DNA probe obviously influenced the signal and the blank fluorescence response. As shown in Fig. S1, the fluorescence intensity was increased gradually with the concentration of the DNA probe from 0.1 to 0.8 nM and the blank fluorescence intensity also increased. It was observed that the S/N ratio reached the highest value when the DNA probe concentration was selected at 500 nM, and this concentration was therefore used throughout the experiments. The reaction time of the DNA probe with SA was investigated, and 20 min was selected as the optimal time (see Fig. S2). Since efficient degradation was the crucial step, the effects of the concentration and digestion time of the exonucleases on the fluorescence intensity were evaluated. We first investigated the background noise when only use Exo I or Exo III, and results shown that the background noise was lowest in the presence of Exo I and Exo III (Fig. S3). And then we explored the amount and incubation time of Exo I and Exo III. As shown in Fig. S4a, the fluorescence intensity decreased obviously with the increased amount of Exo I and Exo III, and then lower intensity change was obtained. Further increase of the exonuclease concentration caused little effect on the final fluorescence intensity. An exonuclease concentration of 0.5 U/μL was finally selected as the optimal condition in this study. The fluorescence intensity also decreased with the digestion time and then reached a plateau (Fig. S4b). As a result, an optimal digestion time of 60 min was chosen as the digestion time of exonucleases.

3.4. Analytical performance of SA by terminal protection fluorescence assay

Under the optimized conditions, we further characterized the detection range of this sensing strategy. Fig. 2(A) shows the fluorescence response in the presence of different concentrations of SA from 0 to 800 nM. The fluorescence intensity increased dramatically with the increase of SA concentration from 0 to 400 nM; Fig. 2(B) shows the relationship between the fluorescence response and the concentration of target SA under the optimized conditions. The insert graph in Fig. 2(B) shows the calibration curve for the SA detection and the linear range was found to be from 0 to 200 nM with a linear equation of $Fi = 101.629 + 6.532\ C_{SA}$, where Fi represents the fluorescence intensity of SG I at 520 nm and C_{SA} is the concentration of SA (regression coefficient $R_2 = 0.9979$). The detection limit was 0.4 nM according to $3\sigma/k$, in which σ is the standard deviation for the blank solution and k is the slope of the calibration curve. The detection limit was comparable to the other previously reported methods (Chen et al., 2014; Wang et al., 2013b; Wu et al., 2011; Zhou et al., 2013) (Table S1). For each concentration of the protein, the measurement was repeated at least three times independently. The relative standard deviations for the series of repetitive measurements with different concentrations of SA were all within 10% and the average of the relative standard deviation was 4.48%, indicating the acceptable precision and reproducibility.

To evaluate the specificity of the sensing approach towards SA, some other nonspecific proteins, such as thrombin, BSA and FR, which cannot specifically bind to biotin, were selected for the control study under the optimized detection conditions. In the absence of SA, the nonspecific proteins could not bind to the probe DNA, and which was completely digested by Exo I and Exo III, leading to no dye being inserted into the duplex DNA. As shown in Fig. 3 the fluorescence intensities of these irrelevant proteins were slightly higher than that of the control experiment. However, as expected, a significant fluorescence enhancement was observed only in the presence of SA with the same concentration as these

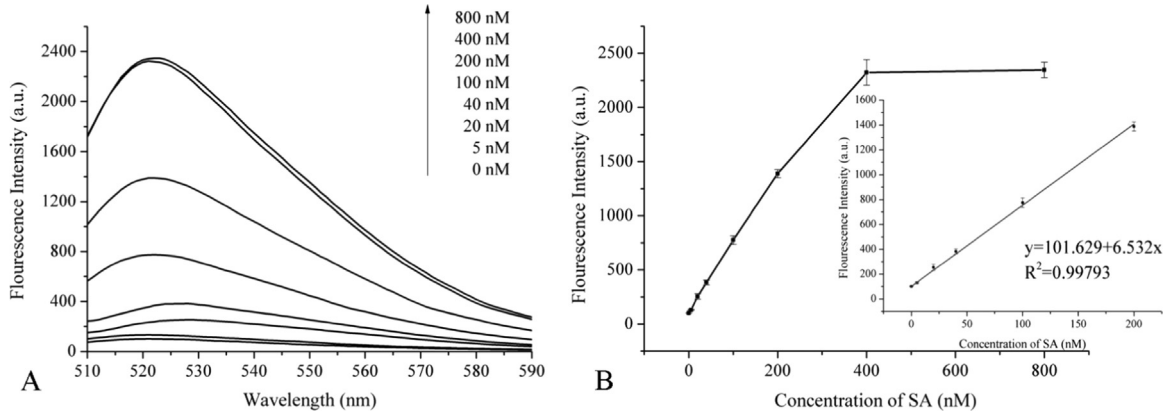


Fig. 2. (A) Fluorescence responses at different SA concentrations. (B) Fluorescence intensity upon the addition of different concentrations of SA at 522 nm. Insert: linear relationship between the fluorescence intensity and the concentration of SA in the range from 0 to 200 nM. Error bars show the standard deviation of three experimental results.

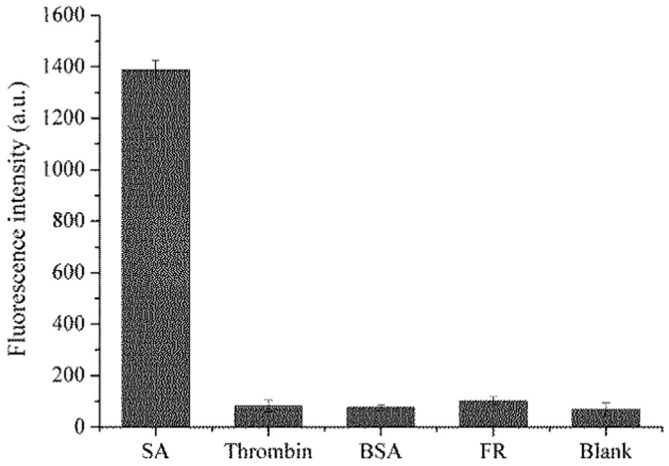


Fig. 3. Fluorescence intensity of the loop DNA biosensor in the presence of different proteins: SA, thrombin, BSA and FR, plus blank control. The concentration of these proteins was 200 nM. Error bars indicate the standard deviation of the three independent experiments.

proteins (200 nM). These results clearly demonstrated the high specificity of the proposed method for SA detection.

To further confirm the practicability of this strategy in real sample analysis, we applied the above biosensor to SA detection in serum. Different concentrations of SA in diluted human serum (obtained from the Affiliated Hospital of Xiamen University, China) and cell lines were monitored (Table S2 and Fig. S5). As listed in Table S2, the recoveries for SA using this approach were in the range 96–118%. These results indicate that the reaction could proceed in complex biological fluids and this strategy was therefore promising for use in the bioanalysis of practical samples.

4. Conclusions

In summary, we successfully produced a simple, rapid, novel and label-free fluorescence biosensor for protein detection based on the terminal protection of a small molecule linked DNA. This method relied on the terminal protection mechanism. Using this approach, when the 3′-end of the ds-DNA was conjugated to a small molecule moiety that was specifically bound to its target protein the ds-DNA was protected from degradation by Exo III and Exo I. Coupled with Exo I and Exo III, the DNA probe was digested completely in the absence of target proteins, thus avoiding incomplete digestion influence when using only Exo III, resulting in a low background signal. The combination of SG I improved the sensitivity and effectively decreased the background noise. As a model target, a detection limit of 0.4 nM was achieved. In addition, this method displayed favorable detection specificity compared with other nonspecific proteins, and can be used to detect DNA, small molecules or other proteins that have good affinity with small molecules. It is predicted that this method based on TPSMLD could open a wide avenue with the discovery of various small molecules capable of specific binding to proteins with high affinity. These advantages endowed this protein sensing strategy with a great potential for practical applications without sample purification, and its applications in the environmental and biomedical fields could be expanded.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.04.038.

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