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A fluorescent biosensor for protein detection based on poly(thymine)-templated copper nanoparticles and terminal protection of small molecule-linked DNA

Hai-Bo Wang*, Hong-Ding Zhang, Ying Chen, Yan-Ming Liu

College of Chemistry and Chemical Engineering, Xinyang Normal University, Xinyang 464000, PR China

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ABSTRACT

In this paper, a fluorescent biosensor has been developed for protein detection based on poly(thymine) (poly T)-templated copper nanoparticles (Cu NPs) and terminal protection of small molecule linked-DNA. This strategy was demonstrated by using small molecule biotin and its binding protein streptavidin (SA) as a model case. In this assay, biotin-linked poly T (biotin-T30) probe was specifically bound to the target protein SA with strong affinity in the presence of SA. The selective binding events confirmed that biotin-T30 probe was protected against the hydrolysis by exonuclease I (Exo I), which could effectively template the formation of fluorescent Cu NPs. The results revealed that the developed strategy was highly sensitive for detecting SA in the concentration range from 0.5 to 1000 nM with a detection limit of 0.1 nM. In addition, the relative standard deviation was 3.6% in 5 repetitive assays of 50 nM SA, which indicated that the reproducibility of the method was acceptable. Besides desirable sensitivity, the developed biosensor also showed high selectivity, low cost, and simplified operations. Thus, it could hold considerable potential to construct a simple, selective and sensitive fluorescent platform for detection of small molecule–protein interactions in molecular diagnostics and genomic research.

1. Introduction

Due to their unique physical, electrical, and optical properties for use in vivo imaging and in vitro chemical/biological detection, ultrasmall fluorescent nanoparticles including several noble metal nanoclusters, such as Au, Ag, and Pt nanoclusters, have attracted special research interest in the past decade as promising substitutes to common fluorophores like organic dyes and quantum dots (Zheng et al., 2007). Particularly, DNA or oligonucleotide templated fluorescent metal nanoparticles have showed great potential as fluorescent probes for biochemical applications because of their advantages of easy synthesis, tunable fluorescence emission, low toxicity, high photo-stability, good biocompatibility, as well as suitability as a diagnostic tool for biological concerns (Richards et al., 2008). For example, cytosine-rich (C-rich) single-stranded DNA has been screened to be able to serve as an ideal template for the formation of highly fluorescent silver nanoclusters, which have been successfully applied for detection of various targets, such as single-nucleotide mutation, proteins, micro-RNA, cancer cells, and so on (Guo et al., 2010; Li et al., 2012; Liu et al., 2012; Sharma et al., 2011; Tian et al., 2015). Lately, it was found that random ds-DNA could act as an efficient template for the formation of Cu nanoparticles (Cu NPs) at a low concentration of $CuSO_4$ and the formed Cu NPs possessed excellent fluorescence, whereas the random ss-DNA template or triplex DNA did not support the formation of Cu NPs (Hu et al., 2013a; Rotaru et al., 2010). The ds-DNA templated Cu NPs have been used as fluorescent probes for protein, small molecules, metal ions and enzyme assays (Chen et al., 2012; Hu et al., 2013b; Yang et al., 2014; Zhang et al., 2013; Zhou et al., 2011).

Very recently, Qing et al. found that single-stranded poly(thymine) (poly T) could also be employed as a promising template for the formation of fluorescent Cu NPs (Qing et al., 2013a). The Cu NPs formation templated by poly T was due to high affinity between thymine and Cu^{2+}, and the thymine-complexed Cu^{2+} was chemically reduced to Cu^0 by the reducing agent along the shape of the poly-T scaffold. Additionally, the fluorescence intensity of the Cu NPs could be regulated by the length of poly T. More importantly, the poly T-templated fluorescence produced was highly efficient and completed within several minutes after the reaction beginning under ambient conditions, which was much faster and more convenient than the synthesis produced by other fluorescent metal nanoparticles using DNA as template, such as ds-DNA tempaled Cu NPs (>1 h) (Rotaru et al., 2010; Yang et al., 2014),

* Corresponding author. Fax: +86 376 6391172.
E-mail address: wanghaibohn@163.com (H.-B. Wang).

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silver nanoclusters (24 h in the dark at 4 °C) (Xie et al., 2009), and gold nanoclusters (2 days) (Gwinn et al., 2008). Thus, the poly T-templated Cu NPs as a fluorescence probe was more propitious to construct novel biosensing platform for chemical/biological analysis. Qing et al. developed a novel ultrasensitive label-free method for the nuclease (S1 nuclease as a model system) assay, and its inhibitors screening by using the poly T-templated Cu NPs as fluorescent probes (Qing et al., 2013b). Ou et al. reported that poly T-templated Cu NPs could be used as novel and sensitive fluorescence probes for the detection of Pb^{2+} based on the fluorescence quenching effect (Ou et al., 2014). They also utilized poly T-templated Cu NPs as effective fluorescent probes to construct a simple and sensitive fluorescence strategy for the trypsin assay based on Cu NPs and its different fluorescence response toward trypsin-catalyzed hydrolysis of cytochrome c (Cyt c) (Ou et al., 2015). Zhang et al. have developed a label-free nanosensor for detection of biothiols based on poly T-templated fluorescent Cu NPs, which were controlled through thymine-Hg(II)- thymine coordination (Zhang et al., 2015). Despite the above studies, exploration of poly T-templated Cu NPs for biochemical application was still at its very early stage.

It was crucial importance for sensitive and selective identification and quantification of small molecules capable of binding to protein receptors with reasonable affinity and specificity in chemical genetics, molecular diagnostics and drug developments (Gao et al., 2004; Michnick et al., 2007; Stockwell, 2004). The selective bindings between small molecules and particular protein receptors could perturb the function of the proteins and provide great potentials in clinic diagnosis, molecular therapeutics and biomedical research. The development of effective methods for detection of small molecule/protein interactions had attracted considerable attention over recent years (Fei et al., 2011). Small molecule linked DNA was emerging as a versatile tool for detecting the interaction between small molecules and their protein receptors. Recently, Jiang and colleagues found that the binding the target protein to small molecule-linked single stranded DNA (ss-DNA) could protect the conjugated ss-DNA from degradation by 3′-termini specific exonulease I (Exo I) (Wu et al., 2009). Based on the finding, they reported a series of studies on terminal protection for protein detection (Wang et al., 2013b; Wu et al., 2013; Zhen et al., 2012). When the small molecule moiety was bound to its target protein, the small molecule linked-DNA was protected from the digestion by exonuclease (Exo I and Exo III) or restriction endonuclease, because the bound protein macromolecules might convey a steric hindrance preventing nuclease from approaching and cleaving the phosphodiester bond of DNA (Wang et al., 2013b; Wu et al., 2009, 2013). Relying on the terminal protection mechanism and exonuclease catalyzed digestion of ss-DNA, several sensitive and specific biosensing strategies have been successfully developed for detection the interactions between small molecules and the corresponding protein receptors (Cao et al., 2012; Chen et al., 2014; Li et al., 2014; Wang et al., 2013a, 2014; Wei et al., 2012; Zhu et al., 2015).

In this study, based on the specific small molecule-target–protein interaction that could prevent DNA enzymolysis by Exo I (Wang et al., 2013b, 2014; Wu et al., 2013), we designed a convenient fluorescent biosensor for protein detection by using poly T-templated Cu NPs as fluorescent probe. In our proposed sensing strategy, small molecule biotin and its binding protein streptavidin (SA) were chosen as the model system. The SA could protect the biotin-T30 probe from the degradation of Exo I through the interaction between SA and biotin. And biotin-T30 probe could be used as template for the formation of Cu NPs and the system would show strong fluorescence. Thus, the SA concentration could be identified by Cu NPs' fluorescence changes. Compared with existing protein-binding and nucleic acid assay technologies that commonly involved separation steps, surface-based operations or complicated modifications (Michnick et al., 2007; Stockwell, 2004), this strategy was able to offer desirable sensitivity, high selectivity, increased throughput, low cost, and simplified operations.

2. Experimental

2.1. Reagents

Streptavidin (SA), bovine serum albumin (BSA), human serum albumin (HSA) immunoglobulins G (IgG), thrombin were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Exonuclease I (Exo I) was obtained from New England Biolabs (Ipswich, MA). All other chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and without further purification. All the solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance > 18.2 MΩ. All the oligonucleotides used in this work were synthesized from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The sequences of oligonucleotide probes were shown in Table S1 in Supporting Information.

2.2. Apparatus

The fluorescence measurements were performed on a Hitachi F-7000 fluorescence spectrometer (Hitachi Co. Ltd., Japan) equipped with a Xenon lamp excitation source and with a R928F photomultiplier tube detector. Both the excitation and emission slit were set at 5.0 nm. A quartz fluorescence cell with an optical path length of 10 mm was used. The fluorescence emission spectra of Cu NPs were collected from 500 nm to 660 nm at room temperature with a 340 nm excitation wavelength. All fluorescence measurements were carried out at room temperature unless stated otherwise. Transmission electron microscopy (TEM) was performed using a Tecnai G2 F20 S-TWIN field emission transmission electron microscopy.

2.3. Synthesis of poly T templated Cu NPs

Poly T templated Cu NPs were synthesized according to the previous reported methods (Qing et al., 2013a, 2013b; Ou et al., 2014, 2015). Briefly, a mixture solution containing 1 μM T30 DNA probe and 3 mM ascorbic acid in MOPS buffer (20 mM MOPS, 300 mM NaCl, pH 7.5) was firstly prepared. After blending completely, 500 μM $CuSO_4$ solutions were added to the mixture solution and kept in dark for 5 min at room temperature to form Cu NPs. Finally, the fluorescent spectrum of the mixture was recorded by F-7000 spectrophotometer (Hitachi Co. Ltd., Japan) immediately. The fluorescence excitation and emission spectra of the poly T templated Cu NPs were shown in Fig. S1 in Supporting Information. The morphology of poly T-templated Cu NPs was characterized by TEM (shown in Fig. S2 in Supporting Information).

2.4. Fluorescence assay of terminal protection

Firstly, 10 μL 10 μM biotin-T30 DNA probe was added into 20 μL of the sample solution (20 mM Tris–HCl buffer, 50 mM NaCl, pH 7.5) containing different concentration of SA. The mixture was incubated at room temperature for 10 min to allow complete interaction between SA and biotin. Then, 10 U Exo I was added in the mixture and incubated at 37 °C for 10 min to perform the digestion

reaction. Subsequently, the resulting solution was transferred to 100 μL reaction system (20 mM MOPS, 300 mM NaCl, pH 7.5, and containing 3 mM ascorbic acid). After that, 500 μM $CuSO_4$ solutions were added to the mixture solution and incubated in dark for 5 minutes at room temperature. Finally, the fluorescent spectrum of the mixture was recorded by F-7000 spectrophotometer (Hitachi Co. Ltd., Japan) immediately. The excitation wavelength was 340 nm, and the emission wavelengths were in the range from 500 to 660 nm with both excitation and emission slits of 5 nm.

2.5. Gel electrophoresis experiment

In the gel electrophoresis experiment, FAM-biotin-T30 probe with FAM labels at its 5′ terminus was used in the digestion reaction, and the product obtained was loaded on the gel for electrophoresis. The gel electrophoresis was performed using the DNA sample on a 3% agarose gel in 50 mM Tris-borate running buffer (pH 9.2) containing 2 mM EDTA at 100 V for 1 h. After electrophoresis, the gel was visualized via fluorescence detection using a Tocan 240 gel imaging system.

3. Results and discussion

3.1. The design principle of the proposed biosensor

On the basis of terminal protection mechanism of small molecule-linked ss-DNA against Exo I hydrolysis, we have developed a novel fluorescent biosensor for detecting the interaction between protein and small molecule by using poly T-templated fluorescent Cu NPs as signal reported probes. Biotin and its binding protein streptavidin (SA) were chosen as a model case. The mechanism of this method was illustrated in Scheme 1. Firstly, the biotin-T30 probe was designed with a small molecule recognition element (biotin) at its 3′ terminus and could be as a template for fluorescent Cu NPs formation. In the absence of SA, the biotin-linked T30 probe was hydrolyzed from the 3′ terminus by Exo I to yield mononucleotides. Therefore, the fluorescent Cu NPs could not be formed due to the lack of poly T template, which resulting in low fluorescence intensity. In the presence of binding protein SA, the biotin-T30 probe was specifically bound to the target protein (SA) with strong affinity, forming the complex of SA/biotin-T30 probe. In this case, biotin-T30 probe was protected from the degradation by Exo I because the bound SA macromolecules might convey significant steric hindrance, preventing Exo I from approaching and cleaving the phosphodiester bond adjacent to the 3′ end. Upon the reduction of Cu^{2+} by ascorbic acid, the poly T templated Cu NPs was formed and exhibited high fluorescence intensity. In addition, the fluorescent response of the biosensor increased gradually with increasing SA binding events, suggesting that this assay could be exploited to detect the interaction of biotin with its target protein SA.

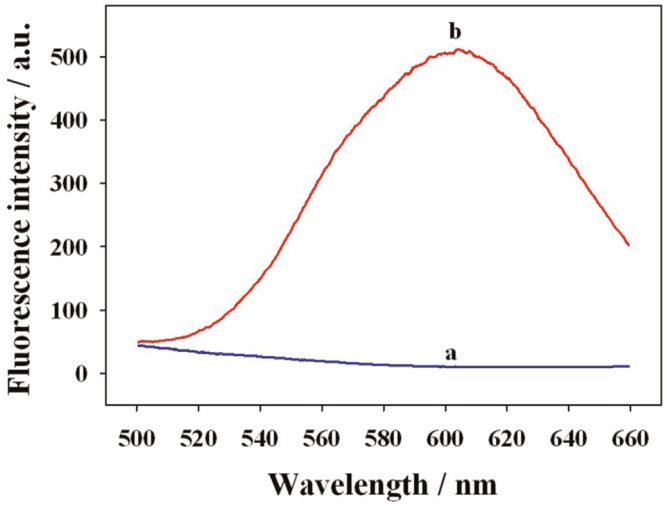


Fig. 1. Fluorescence spectra of biotin-T30 templated Cu NPs in the absence (curve a) and presence of 500 nM streptavidin (curve b), respectively.

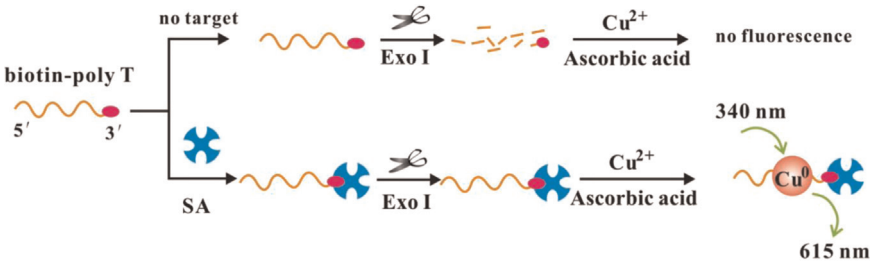
3.2. Fluorescent characterizations

Following the design, the feasibility of the terminal protection biosensor for SA assay was investigated. First, we compared the fluorescence intensity responses in the absence and presence of SA. Fig. 1 showed the fluorescence spectra of the biotin-T30 in the absence and presence of SA. In the absence of SA, low fluorescence intensity was observed (curve a in Fig. 1). This indicated that the fluorescent Cu NPs could not be formed due to the lack of poly T template, which was degraded into mononucleotides by Exo I. However, the fluorescence intensity obviously increased in the presence of 500 nM SA (curve b in Fig. 1). It was mainly attributed to that biotin linked poly T template was protected by SA from the degradation of Exo I, which forming fluorescent Cu NPs.

To further explore the feasibility of our novel strategy in constructing poly T Cu NPs sensing platform, control experiment was carried out under the same experimental conditions. Three 30-mer ss-DNA homopolymers (poly T, poly A, poly C) and guanine (G)-rich oligonucleotides were tested as templates for Cu^{2+} reduction and Cu NPs formation. As shown in Fig. 2, it was found that only T30 exhibited high fluorescence response. However, by using A30, C30 and G-rich DNA as template, there would no obvious fluorescent signal be observed, which demonstrated that poly A, poly C and G-rich ss-DNA would not be used as effective template for the formation of fluorescent Cu NPs.

3.3. Gel electrophoresis characterizations

Gel electrophoresis analysis was carried out to understand the sensing mechanism of the terminal protection strategy. Considering that only double-stranded DNA oligonucleotides could be selectively stained by ethidium bromide, 5′ terminus labeled



Scheme 1. Schematic illustration of the novel biosensing strategy for protein detection based on poly T templated Cu NPs and terminal protection assay.

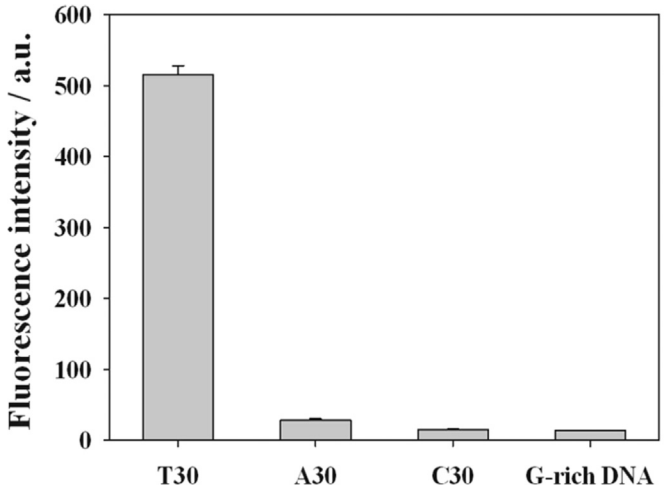


Fig. 2. Fluorescence intensity of Cu NPs templated by different kinds of ss-DNA. The error bars were the standard deviation of three repetitive measurements.

fluorescein amidite (FAM) biotin-T30 probe was used in this experiment. The results were shown in Fig. S3 in Supporting Information. In the absence of SA, it was observed that there was no bright band in lane 2 with the treatment of Exo I. This phenomenon might be attributed to the single-stranded biotin-T30 probe was hydrolyzed into mononucleotides from the 3′ terminus by Exo I, and the mononucleotides would migrate out of the gel. After biotin-T30 probe was incubated with SA followed by Exo I hydrolysis, a bright band was observed on lane 3 in Fig. S3 in Supporting Information. The result revealed that the interaction with SA made biotin-T30 probe resistant to the digestion by Exo I, confirming the terminal protection of biotin-linked ss-DNA. However, bright band was observed on lane 4 where biotin-T30 probe was not subjected to Exo I treatment. These results further demonstrated the biosensing mechanism of terminal protection of small molecule-linked DNA.

3.4. Optimization of the experimental conditions

The assay performance of the developed method for SA detection was greatly depended on the fluorescence intensity of the poly T templated Cu NPs, so the effects of the synthetic conditions of Cu NPs were investigated. Poly T was used as the template for the formation of fluorescent Cu NPs. Thus, the fluorescence intensity of the Cu NPs was dependent on the lengths of poly T. Firstly, the fluorescence intensity of a series of poly T with different lengths were studied for templating fluorescent Cu NPs. As shown in Fig. 3A, the fluorescence intensity signals increased with a increasing length of poly T. When the length of poly T was less than 15 bases, it almost failed to induce fluorescence practically. However, T30 probe exhibited high fluorescence intensity and could satisfy the detection requirements. Therefore, 30 bases of poly T was used throughout all further experiments.

Since the concentration of Cu^{2+} was an important parameter influencing the fluorescent intensity of poly T templated Cu NPs, the effect of the concentration of Cu^{2+} was investigated. As shown

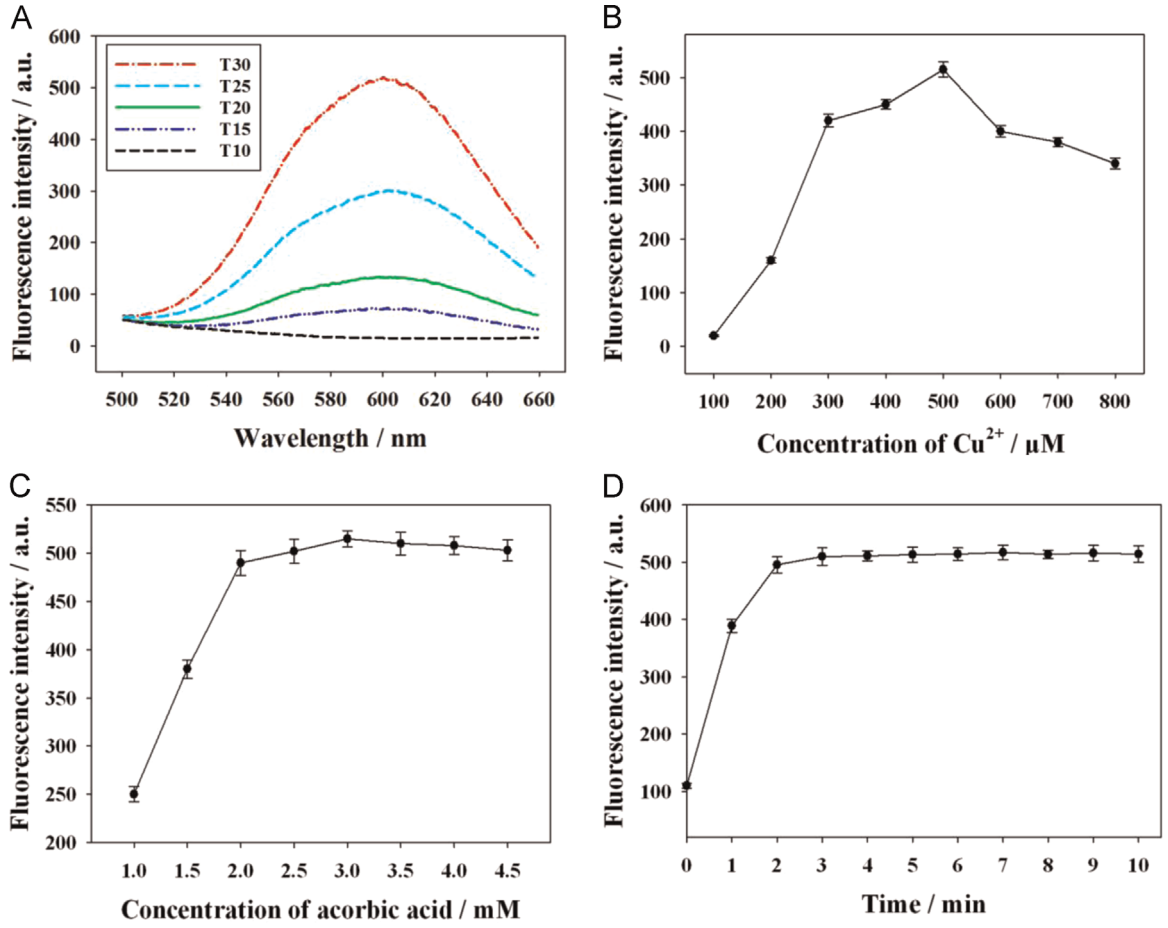


Fig. 3. (A) Fluorescence spectra of Cu NPs templated by poly T with different length. (B) The effect of Cu^{2+} concentration on the fluorescence intensity of poly T templated Cu NPs. (C) The effect of ascorbic acid concentration on the fluorescence intensity of the poly T templated Cu NPs. (D) The effect of incubation time of ascorbic acid on the fluorescence intensity of the poly T templated Cu NPs. The error bars were the standard deviation of three repetitive measurements.

in Fig. 3B, the fluorescence intensity of Cu NPs increased rapidly with the increasing Cu^{2+} concentration firstly and then decreased when the Cu^{2+} concentration was higher than 500 μM. The highest fluorescence intensity was obtained when the final concentration of Cu^{2+} was 500 μM. The result was possibly attributed to the fact that a relatively high Cu^{2+} concentration might degrade the poly T template through oxygen-based radicals after being activated by a reducing agent. As a result, 500 μM Cu^{2+} was selected as the optimal concentration.

Furthermore, the concentration of ascorbic acid had great effect on the fluorescence signal of the Cu NPs. As can be seen from Fig. 3C, it was observed that the fluorescence intensity increased notably with the increasing concentration of ascorbic acid and then reached the highest when the concentration of ascorbic acid was 3 mM. Therefore, 3 mM ascorbic acid was used in this study. In addition, the incubated time of ascorbic acid was investigated. It can be seen from Fig. 3D that the fluorescence intensity increased rapidly and then approached a plateau within 5 min. Thus, in order to obtain a high efficiency of Cu NPs formation and a short reaction time, 3 mM ascorbic acid and 5 min incubation time of ascorbic acid were used throughout all further experiments.

3.5. Analytical performance of the fluorescent biosensor

In order to further demonstrate the ability of the present poly T-templated Cu NPs-based fluorescence assay to sensitively quantify target protein SA, the fluorescence intensity of different concentrations of SA was recorded under the optimized condition.

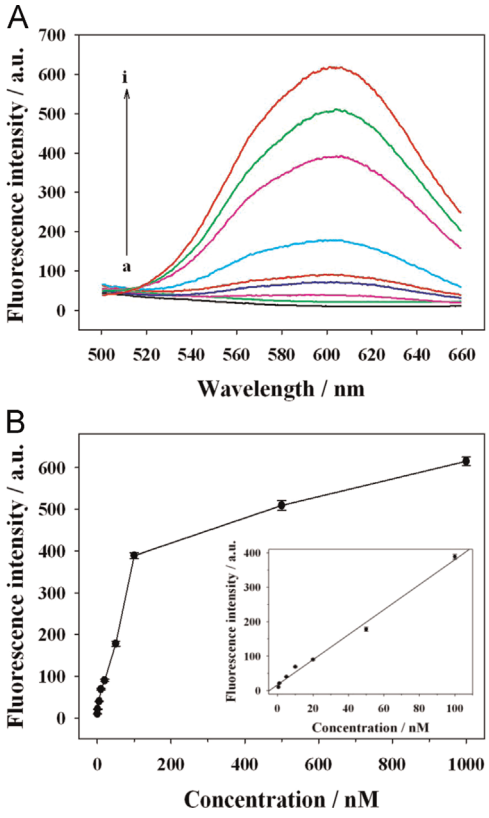


Fig. 4. (A) Fluorescent spectra of biotin-T30 templated Cu NPs in the presence of increasing streptavidin concentrations, the curves from a to i, the concentration of streptavidin are 0.5, 1, 5, 10, 20, 50, 100, 500, 1000 nM, respectively. (B) The relationship between the fluorescence intensity and the concentration of streptavidin. Inset of (B) is the calibration curve between the fluorescence intensity and the concentration of streptavidin. The RSD were 4.8%, 3.2%, 4.2%, 3.3%, 3.5%, 3.6%, 2.7%, 3.8%, 4.0%, respectively. The error bars were the standard deviation of five repetitive measurements.

Fig. 4 depicted typical fluorescence spectra of the poly T-templated Cu NPs-based terminal protection strategy to SA of varying concentration after SA-biotin affinity interaction. As shown in Fig. 4A, the fluorescence intensity increased gradually as the concentration of SA increased from 0.5 to 1000 nM. Fig. 4B illustrated the relationship between the SA concentration and the fluorescence intensity at the maximum emission wavelength. In the concentration range from 0.5 to 100 nM, the fluorescence intensity exhibited a linear correlation to SA concentration (inset of Fig. 4B). According to the rule of three times standard deviation over the blank response, the detection limit was estimated to be 0.1 nM, which was comparable to that of the previously reported methods (shown in Table S2) (Chen et al., 2014; González-García et al., 2000; Wang et al., 2013a; Wu et al., 2011). In addition, the relative standard deviation of 5 repetitive experiments for 50 nM SA detection was 3.6%, which indicated that the reproducibility of the method was acceptable. These results confirmed that the proposed biosensor can be easily applied for quantitative analysis of the target proteins with a good performance.

Additionally, the specificity of the fluorescence biosensor was also investigated by testing the fluorescence response of other non-specific proteins including bovine serum albumin (BSA), human serum albumin (HSA), immunoglobulins G (IgG), and thrombin. From Fig. 5, it could be seen that the fluorescence responses of BSA (5 μM), IgG (5 μM), HSA (5 μM) and thrombin (5 μM) were slightly higher than that of the control experiment with the absence of SA (blank). However, in the presence of 500 nM target SA, a remarkably higher fluorescence response was obtained compared to the non-specific proteins. It revealed that these proteins had little effect on the Exo I hydrolysis reaction for biotin-T30 probe. The result further verified that the protection of biotin-linked DNA was specific to the SA binding event, implying excellent selectivity of terminal protection assay for probing the interaction between small molecules and their target proteins.

To further demonstrate the applicability of the proposed method in real-life samples, various concentrations of streptavidin was spiked in normal human serum samples and assayed according to the above method. The standard addition method was used to complete the experiment and the results were given in Table S3. The relative standard deviations were less than 4%, indicating that detection of SA in serum samples was also feasible. Moreover, the proposed sensor was applied to determinate the SA concentrations in culture broth of *Streptomyces avidinii* (shown in Table S4).

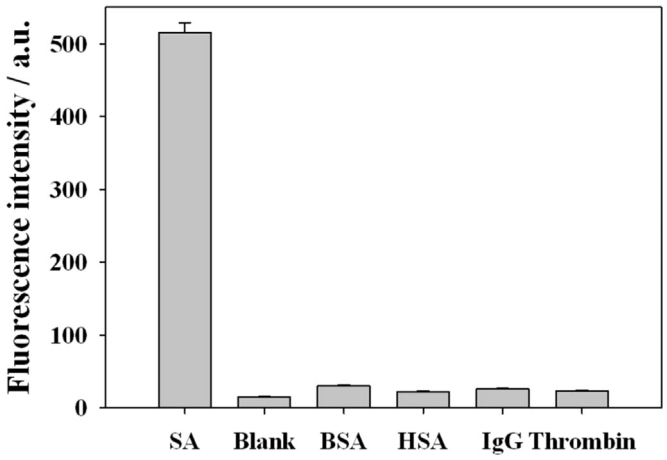


Fig. 5. Fluorescence responses of the biotin-T30 templated Cu NPs in the presence of 500 nM streptavidin or 5 μM other analytes. The error bars represent the standard deviation of three measurements.

4. Conclusions

In conclusion, we have developed a simple, rapid, sensitive fluorescent biosensor for protein detection by using poly T-templated Cu NPs as fluorescent probe. This strategy was relied on the terminal protection mechanism that single stranded DNA terminally tethered to a small molecule was protected from the degradation by Exo I when the small molecule moiety was bound to its target protein. The novel strategy offered several advantages. (1) it was free of any conjugation or labeling process, and without any other complex design and purification process. (2) it operated at mild conditions, all the process were performed within several minutes at room temperature. (3) it provided high specificity of SA detection against other non-specific protein at a high concentration. Therefore, it could indeed hold great potential as a simple, cost-effective, selective and sensitive platform for detection of small molecule–protein interactions.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.07.021.

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