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Dual-color determination of protein via terminal protection of small-molecule-linked DNA and the enzymolysis of exonuclease III

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ABSTRACT

We have developed a new dual-color fluorescent biosensor for protein detection based on terminal protection of small-molecule-linked DNA and the enzymolysis of exonuclease III (Exo III). The determination of streptavidin (SA) was realized via fluorescence signals of the green color from quantum dots (QDs) and the red from $[Ru(phen)_2(dppx)]^{2+}$. In the absence of SA, biotin-DNA was degradated by the Exo III, thus making the $[Ru(phen)_2(dppx)]^{2+}$ employed as a fluorescence quencher to the QDs. With the addition of SA, dual-color response appeared because of the specific binding between SA and biotin so that the biotin-dsDNA was protected and combined with $[Ru(phen)_2(dppx)]^{2+}$, leading to the QDs recovery and the generating of $[Ru(phen)_2(dppx)]^{2+}$ fluorescence. This sensor exhibited high sensitivity with a low detection limit (2.11 ng/mL) and firstly introduced dual-color QDs–ruthenium complex dyads to protein assay.

1. Introduction

The study of the interaction between small molecules and protein is of great importance for the understanding of chemical genetics, molecular diagnostics and drug development (Strausberg and Schreiber, 2003; Stockwell, 2004; Gao et al., 2004). In recent years, Jiang's group has developed a novel method to study small molecule–protein interaction based on the binding of small-molecule-linked DNA to target protein. The principle is that, the small molecule-linked single-stranded DNA (ssDNA) can avoid enzymolysis by exonuclease I upon the small molecule binding to its target protein. This phenomenon, which is called terminal protection of small-molecule-linked DNA (TPSMLD) (Wu et al., 2009), has encouraged many researchers to develop different analytical strategies applied to the detection of H5N1 antibody (Cai et al., 2012; Wei et al., 2013), FITC antibody (Wu et al., 2011) and folate receptor (Wu et al., 2011; Cao et al., 2012; Wei et al., 2012; He et al., 2013). However, these detection methods require expensive and complicated fluorescent labeling (Wang et al., 2014) or deliberate modifications of electrode surface (Wu et al., 2009; Cao et al., 2012).

For fluorescence analysis, single-response of fluorescence technique for protein detection in the complex samples is likely to produce false-positive signals and the inaccuracy of quantification due to non-specific interactions (Chen et al., 2007). The dual-color fluorescence technology is favored in avoiding the above disadvantages. As we know, QDs have the potential to serve as an ideal and bright fluorescent signal generator due to their unique optical features such as photostability and broad excitation spectra, narrow, symmetric and tunable emission spectra (Bruchez et al., 1998; Michalet et al., 2005). On the other hand, it can be quenched by ruthenium complexes of phenazine, called nucleic acid molecular light-switches, through a photoinduced electron transfer process. Interestingly, nucleic acid molecular light-switches, such as $[Ru(phen)_2(dppx)]^{2+}$, are not photoluminescent in water but can generate red fluorescence when they are inserted in the dsDNA (Choi et al., 2009; Jiang et al., 2004; Liu et al., 2009). Compared with ethidium bromide (EB), they are nontoxic. Based on these phenomena, our research group has developed a series of dual-color fluorescent biosensors for dsDNA detection in tube and on chip via nucleic acid molecular light-switches as both quencher of QDs and reporter of dsDNA (Zhao et al., 2009; Xiang et al., 2012). Moreover, this method was further extended to living-virus-genome labeling (Huang et al., 2012) by using the excellent advantages, such as the strong binding to dsDNA, low background, and the red-shifted emission of nucleic acid molecular light-switches. Unfortunately, this dual-color method is confined to DNA biosensor because of the difficulty in labeling between nucleic acid molecular light-switches and protein.

In order to take advantages of the QDs–ruthenium complex for protein detection, a dual-color detection of protein in homogeneous

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assay was demonstrated by combining the cleavage function of Exo III on dsDNA with the TPSMLD assay. In the system, Exo III can eliminate the interference of dsDNA due to the enzymolysis of Exo III on ssDNA or dsDNA with 3'-end protrusion is limited, and the enzyme's preferred substrates are dsDNA of blunt 3'-end (Richardson et al., 1964; Lee et al., 2005). Using small-molecule-linked dsDNA as probe, the concentrations of target protein can be determined by the dual-color fluorescence enhancement.

2. Materials and methods

2.1. Chemicals and reagents

Exo III was purchased from Takara Biotechnology (Dalian) Co., Ltd. Streptavidin (SA) was purchased from Amresco (USA). Bovine Serum Albumin (BSA) was obtained from Roche (USA). Tris (hydroxymethyl) aminomethane hydro chloride (Tris) and thrombin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The other chemicals not mentioned here were of analytical-reagent grade or better. All oligonucleotides with different sequences were synthesized and HPLC purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). The sequences of the oligonucleotide used in this work are as follows:

Biotin-DNA: 5'-AATACCACATCATCCATATA-biotin-3';
Complement DNA (C_{DNA}): 5'-TATATGGATGATGTGGTATTAAAAA-3';

The synthesis of $[Ru(phen)_2(dppx)]^{2+}$ and 2.46 nm water-soluble N-acetylcysteine (NAC)-capped CdTe quantum dots (QDs) (Fig. S1) were performed according to previous reports (Amouyal et al., 1990; Zhao et al., 2011). The stock solution of $[Ru(phen)_2(dppx)]^{2+}$ (5 μM) was prepared in ultrapure water. The DNA, SA and QDs (0.28 μM) solutions were prepared in 10 mM Tris–HCl buffer (10 mM NaCl and 5 mM $MgCl_2$, pH 8.0). Fluorimetric spectra were obtained with a RF-5301PC spectrophotometer (Shimadzu, Japan) equipped with a 150 W xenon lamp (Ushio Inc, Japan). Transmission electron microscopy (TEM) image was recorded on a JEOL Ltd. JEM 2100 electron microscope (Japan).

2.2. Procedure for dual-color detection of SA

A one-step hybridization reaction was performed by biotin-DNA (60 μL, 1 μM), C_{DNA} (20 μL, 1 μM) and different concentrations of SA in Tris–HCl buffer for 25 min at room temperature. Then 30 U Exo III was added to the hybridization solution and the mixtures were kept at 37 °C for 30 min with gentle shaking. After that, $[Ru(phen)_2(dppx)]^{2+}$ (90 μL, 5 μM) and QDs (50 μL, 0.28 μM) were added, and the Tris–HCl buffer was added immediately to a final volume of 300 μL. After incubating 15 min, the fluorescence signals of the QDs and $[Ru(phen)_2(dppx)]^{2+}$ were measured with scanning fluorescence spectrometry. Excitation wavelength was 390 nm and the scan range was from 450 nm to 700 nm.

3. Results and discussions

3.1. The principle and feasibility of TPSMLD-based dual-color sensor for SA detection

As illustrated in Fig. 1, SA/biotin-DNA are selected as the model of protein detection. The DNA labeled by biotin at 3'-end is hybridized with the C_{DNA} to form dsDNA. When dsDNA is mixed with Exo III, the biotin-DNA in dsDNA is degraded from its blunt 3'-end. Thus, the fluorescence of QDs is quenched by $[Ru(phen)_2(dppx)]^{2+}$. However, due to the specific binding between

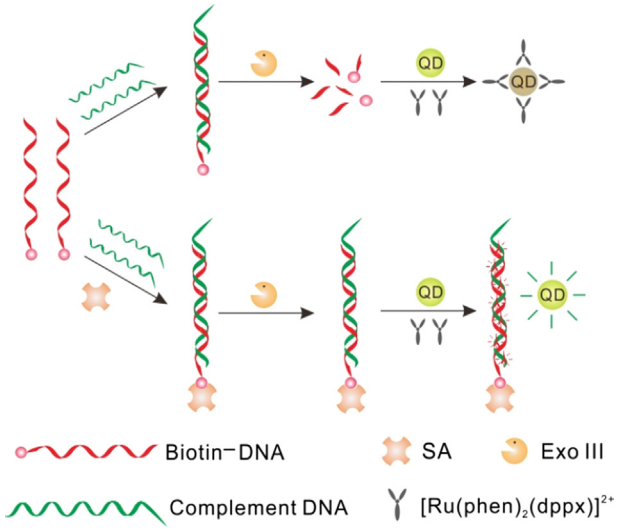

Fig. 1. The mechanism of the dual-color biosensor for SA.

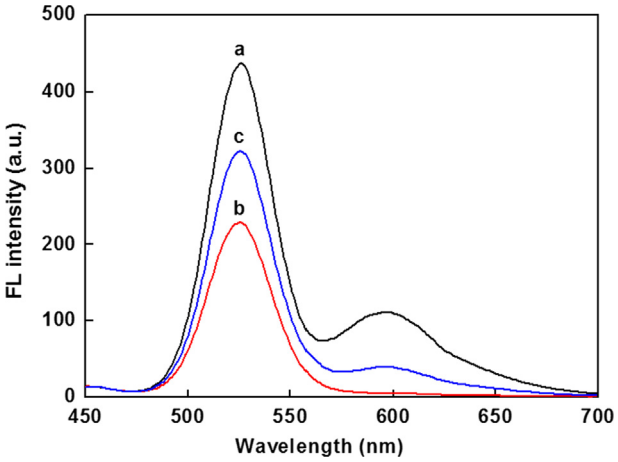

Fig. 2. Fluorescence spectra of the QDs and $[Ru(phen)_2(dppx)]^{2+}$ with (a) biotin-DNA and C_{DNA}; (b) biotin-DNA, C_{DNA} and Exo III; (c) biotin-DNA, C_{DNA}, Exo III and SA. Experimental conditions: QDs, 46.7 nM; $[Ru(phen)_2(dppx)]^{2+}$, 1.5 μM; biotin-DNA, 200 nM; C_{DNA}, 200 nM; Exo III, 50 U; and SA, 50 ng/mL.

biotin and SA, the dsDNA is protected from Exo III digestion. As a result, $[Ru(phen)_2(dppx)]^{2+}$ inserts into the biotin-dsDNA to emit red fluorescence at 599 nm (Fig. S2), and keeps far away from the surface of QDs, making the fluorescence recovery of QDs. The fluorescence intensity is related to SA concentration in the assay solution.

To verify this design, the feasible experiment had been carried out. As shown in Fig. 2, in the absence of Exo III and SA, because $[Ru(phen)_2(dppx)]^{2+}$ could effectively intercalate into dsDNA, the strong green fluorescence of QDs and red fluorescence emitted by the $[Ru(phen)_2(dppx)]^{2+}$ appeared simultaneously (Fig. 2, curve a). In the presence of Exo III, only a weak fluorescent signal of QDs was exhibited (Fig. 2, curve b) owing to the degradation of dsDNA by Exo III and the quenching effect of $[Ru(phen)_2(dppx)]^{2+}$. Compared with curve b, with Exo III and SA involved, an apparently increased signal of QDs was observed and a new fluorescent emission peak at 599 nm of $[Ru(phen)_2(dppx)]^{2+}$ was also appeared (Fig. 2, curve c). Evidently, the binding of biotin-dsDNA to SA could protect it from the digestion of Exo III. Therefore, dual-color detection of small molecule–protein interaction via TPSMLD and enzymolysis of Exo III is feasible.

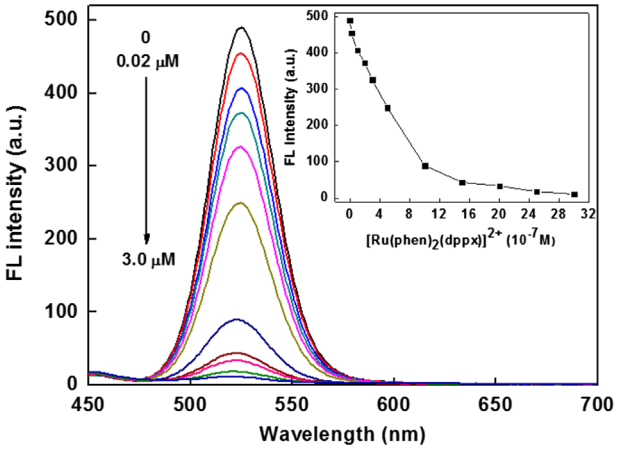

Fig. 3. Changes in the fluorescence spectra of QDs (46.7 nM) upon increasing the concentration of $[Ru(phen)_2(dppx)]^{2+}$: 0, 0.02, 0.10, 0.2, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 (μM) (from top to bottom). Inset: the plot of the fluorescence intensity of QDs vs the concentrations of $Ru[(phen)_2(dppx)]^{2+}$.

3.2. Effective quenching of QDs fluorescence by $[Ru(phen)_2(dppx)]^{2+}$

We further explored the quenching effect of QDs by $[Ru(phen)_2(dppx)]^{2+}$. To ensure dual-color detection, green luminescent QDs with emissive peak at 525 nm were chosen for this investigation. As shown in Fig. 3, the fluorescence intensity of QDs was reduced with the increase of the concentration of $[Ru(phen)_2(dppx)]^{2+}$. The linear range was from 2.0×10^{-8} to 1.0×10^{-6} M. To obtain a good response sensitivity of the biosensor, 1.5 μM $[Ru(phen)_2(dppx)]^{2+}$ was chosen in the following experiments.

3.3. Optimization of the variables

The reaction conditions were optimized successively in order to obtain obvious fluorescence changes. The concentration of C_{DNA} was studied first. With a fixed concentration of biotin-DNA, the fluorescence intensity of dual-color (QDs and $[Ru(phen)_2(dppx)]^{2+}$) was in enhancement with the increasing concentration of C_{DNA} from 0 to 66.7 nM and there was no obvious increase when the concentration was higher than 66.7 nM (Fig. S3). Hence, 66.7 nM was chosen as the optimal concentration. Besides, the influence of the amount of Exo III used in assay on the fluorescence intensity was investigated. The results were shown in Fig. S4. With the increase of Exo III, the fluorescence increased at first and then decreased gradually. The reason might be that the increased background made signal-to-noise level reduce when Exo III was greater than 30 U. Further, we studied the fluorescence signals at different digestion times in the presence of 30 U Exo III. It was observed that the $(F–F_0)$ was increased progressively in 30 min and then reached a plateau due to the complete degrading of dsDNA by Exo III (Fig. S5). Therefore, 30 U Exo III for 30 min was chosen for SA analysis.

3.4. The dual-color sensor for SA determination

Under the optimized conditions, we studied the fluorescence signals in the presence of different concentrations of the SA. Fig. 4a displayed that the fluorescence intensity was increased with the increase of SA concentration, and tended to be stable at high SA concentration. The plots of the dual-color fluorescence intensity versus SA were shown in Fig. 4(b and c). For QDs, the linear range was 10–100 ng/mL with the linear equation $y=0.6052x+179.6042$ ($R^2=0.9946$). The limit of detection (LOD) was calculated to be 6.25 ng/mL (3σ). Simultaneously, the LOD determined by $[Ru(phen)_2(dppx)]^{2+}$ was to be 2.11 ng/mL (3σ), which revealed that this dual-color method for SA detection held a good sensitivity.

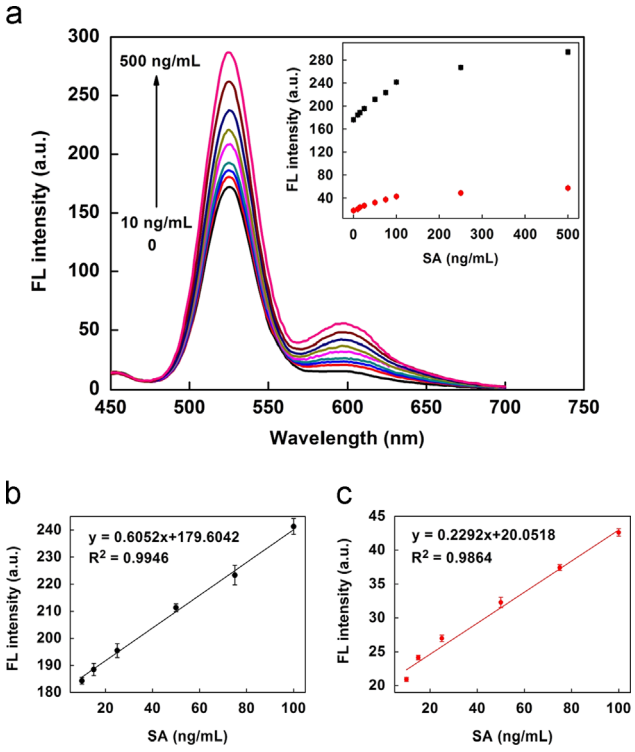

Fig. 4. (a) Changes in the fluorescence spectra of the QDs and $[Ru(phen)_2(dppx)]^{2+}$ upon the addition of increasing concentration of SA: 0, 10, 15, 25, 50, 75, 100, 250, and 500 ng/mL. Inset: the linear curve of determination for SA. (b) The calibration curves by QDs and (c) $[Ru(phen)_2(dppx)]^{2+}$ in the presence of different concentrations of SA. Experimental conditions: biotin-DNA, 200 nM; C_{DNA}, 66.7 nM; Exo III, 30 U; QDs, 46.7 nM and $[Ru(phen)_2(dppx)]^{2+}$, 1.5 μM.

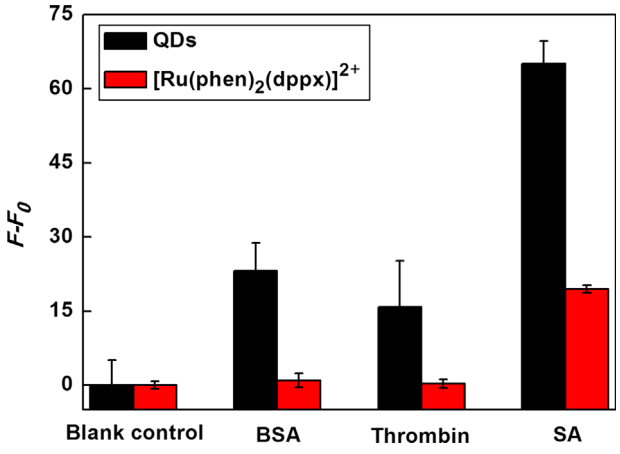

Fig. 5. Bar chart of the fluorescent responses in the presence of different proteins: blank control, BSA (100 ng/mL), thrombin (100 ng/mL), SA (100 ng/mL). Experimental conditions: biotin-DNA, 200 nM; C_{DNA}, 66.7 nM; Exo III, 30 U; QDs, 46.7 nM and $[Ru(phen)_2(dppx)]^{2+}$, 1.5 μM.

3.5. The specificity of the biosensor

Finally, the specificity of this sensor was evaluated. As depicted in Fig. 5, an obvious fluorescence increase was observed in the presence of SA, whereas the fluorescence changed less in the presence of BSA and thrombin. This was attributed to the good specific binding between biotin and SA. Meanwhile, for $[Ru(phen)_2(dppx)]^{2+}$, it has been the first time to avoid the interference of dsDNA with the

introduction of Exo III (blank control). The observations indicated that this biosensor can be applied in the detection of SA with high specificity.

4. Conclusions

In conclusion, we have successfully developed a novel and universal dual-color fluorescent biosensor for protein detection via TPSMLD. On one hand, with the introduction of Exo III, this biosensor avoids the interference of dsDNA to ruthenium complex. On the other hand, the QDs–ruthenium complex dyads is first applied to protein assay in dual-color without sophisticated experimental techniques and instruments. Besides, the reagents are easy to obtain and low-cost in contrast to a fluorescent probe. Taking the SA–biotin system as a model, SA is detected in a range from 10 to 100 ng/mL with a low LOD (2.11 ng/mL) and high selectivity. This approach can be further expanded to detect a wide range of small molecules' binding proteins in general with appropriate modification of the corresponding small molecule on DNA, showing great potential in biochemical detections.

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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.2014.02.060.

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