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An ultrasensitive label-free biosensor for assaying of sequence-specific DNA-binding protein based on amplifying fluorescent conjugated polymer

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ABSTRACT

Sensitive, reliable, and simple detection of sequence-specific DNA-binding proteins (DBP) is of paramount importance in the area of proteomics, genomics, and biomedicine. We describe herein a novel fluorescent-amplified strategy for ultrasensitive, visual, quantitative, and "turn-on" detection of DBP. A Förster resonance energy transfer (FRET) assay utilizing a cationic conjugated polymer (CCP) and an intercalating dye was designed to detect a key transcription factor, nuclear factor-kappa B (NF-κB), the model target. A series of label-free DNA probes bearing one or two protein-binding sites (PBS) were used to identify the target protein specifically. The binding DBP protects the probe from digestion by exonuclease III, resulting in high efficient FRET due to the high affinity between the intercalating dye and duplex DNA, as well as strong electrostatic interactions between the CCP and DNA probe. By using label-free hairpin DNA or double-stranded DNA containing two PBS as probe, we could detect as low as 1 pg/μL of NF-κB in HeLa nuclear extracts, which is 10000-fold more sensitive than the previously reported methods. The approach also allows naked-eye detection by observing fluorescent color of solutions with the assistance of a hand-held UV lamp. Additionally, a less than 10% relative standard deviation was obtained, which offers a new platform for superior precision, low-cost, and simple detection of DBP. The features of our optical biosensor shows promising potential for early diagnosis of many diseases and high-throughput screening of new drugs targeted to DNA-binding proteins.

1. Introduction

DNA-binding proteins (DBP) are critically important in the regulation of a variety of essential cellular processes, such as genome replication, gene transcription, cell division, and DNA repair (Ren et al., 2000; Sen and Baltimore, 1986; Helin, 1998; Aboussekhra et al., 1995). Transcription factors (TFs) are one of the largest class of DBP; they have long been recognized as attractive markers or targets for disease diagnosis and drug development (Pandolfi, 2001), because the dysregulation of TFs signaling has a close relationship with a number of cancers, developmental disorders, inflammation and autoimmunity (Jr, 2002; Libermann and Zerbini, 2006). Currently, most detection methods for DBP are still based on DNA footprinting (Galas and Schmitz, 1978), electrophoresis mobility shift assay (EMSA) (Garner and Revzin, 1981), Western blotting (Bowen et al., 1980), and enzyme-linked immunosorbent assay (ELISA) (Renard et al., 2001). These protocols are not only laborious, DNA-consuming, and inaccurate but they also require radioisotope or fluorescence labels, which makes them impractical for routine application. Recently, several new approaches for measuring specific protein–DNA interactions have been developed, including DNA microarray technology (Bulyk, 2006a; Bulyk et al., 2006b; Wang et al., 2007; Boger et al., 2009; Wang et al., 2011), an electrical approach based on DNA-modified electrode (Barton et al., 2002, 2008; Ricci et al., 2009), AFM imaging (Menotta et al., 2010), surface-enhanced resonance Raman scattering (Reich et al., 2007), and surface plasma resonance chips (Wang et al., 2009). Despite advances in these techniques, their need for surface-immobilized DNA strands, complicated labeling of the target protein and/or DNA strands is an obstacle. In this context, homogeneous, simple and reliable approaches that require neither surface-based operations nor protein labeling are highly desirable for the detection of DBP.

Förster resonance energy transfer (FRET) has been widely used for the detection of biological interactions because of its inherently high sensitivity, versatile detection capability, and multichannel signal collection capability (Clegg, 1995). In general, labeled protein (Giannetti et al., 2006; Dinant et al., 2008) or oligonucleotides (Li et al., 2000; Heyduk and Heyduk, 2002; Wang et al., 2005; He et al., 2007; Xu et al., 2008) are needed for

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assaying of protein–DNA interactions. However, protein labeling is not always easy as it may affect the native features of the protein, and dual labeling of oligonucleotides is expensive. So far, most of the FRET assays for the detection of DBP work in a "turn-off" mode by monitoring the emission of a fluorophore quenched by a quencher, which may give false positive results because of non-specific quenching caused by environmental factors. Recently, several new label-free fluorometric strategies were developed using label-free DNA and duplex-intercalating dye or a luminescent transition metal complex, which may simplify the analysis and decrease the cost to some extent (Chen et al., 2006; Ma et al., 2011; Leung et al., 2012). However, these approaches still suffer from low sensitivity and poor precision.

Conjugated polymers (CPs) have attracted great interest in biological analysis (Gaylord et al., 2002; Feng et al., 2008; Wang and Liu, 2011; Liu et al., 2011), molecular electronics (Malhotra and Saxena, 2003), and organic electronics (Ong et al., 2008; Zhang et al., 2011). Water-soluble CPs possess excellent biocompatibility and optical characteristics have attracted particular attentions in the biosensor community. Recently, CPs have been widely used as sensitive and convenient optical platforms for the detection of DNA (Xu et al., 2004; He et al., 2005; Pu and Liu, 2009; Liu et al., 2011), proteins (Xu et al., 2010; Yu et al., 2008), proteases (Zhang et al., 2009), small molecules (Ho et al., 2005), cell (Feng et al., 2010), and metal ions (Ho and Leclerc, 2003; Liu et al., 2007) because of the amplified signal (up to million fold) compared with their monomers (Gaylord et al., 2002; Thomas III et al., 2007). It is reported that the quenched fluorescence of CP can be recovered by the existence of the detection target (Ho et al., 2005; Liu et al., 2007); the fluorescence of CP can also be transferred to the dye (intercalates into or be labeled on DNA probe) via FRET process when the target exists (Xu et al., 2004; Wang et al., 2004; He et al., 2005; Yu et al., 2008). Both two features of CPs offer good ideas to design biosensors work in a "turn-on" mode. More recently, we developed an optical-logic system based on the cationic conjugated polymer (CCP)/DNA/intercalating dye assembly for the label-free detection of conformational conversion of DNA i-motif structure (Huang et al., 2011). Intercalating dyes, such as ethidium bromide (EB), thiazole orange (TO), and Sybr Green (SG), are known to exhibit different affinities for different conformations of DNA (Guo et al., 1992; Duhamel et al., 1996; Nygren et al., 1998; Cosa et al., 2001). Using an intercalating dye as an energy acceptor in a CP-based biosensor can obviate the need for oligonucleotide labeling, reduce the cost, and simplify the analysis. Moreover, CPs were verified to be a potential tool for visual detection of biomolecules. All these inspired us to design a highly sensitive, label-free, and colorimetric sensing protocol to detect DBP by utilizing highly efficient FRET arising from the interactions between the CCP and intercalating dye.

Nuclear factor-kappa B (NF-κB) is a family of TFs that play a central role in the immune system regulating the expression of several cytokines and growth factors (Caamaño and Hunter, 2002). Herein, a cancer-related p50 protein, a subunit of NF-κB was used as a target model to demonstrate our CCP-based FRET approach for the highly sensitive and label-free detection of DBP. Surprisingly, a limit of detection (LOD, be calculated based on three times the standard deviation over the blank response) for the activated target protein in nuclear extracts was estimated to be 1 pg/μL, which is a 10000-fold improvement over a previous report (Wang et al., 2009). To the best of our knowledge, it is the most sensitive optical method for the detection of NF-κB p50 protein. Additionally, much better precision, expressed as relative standard deviation (RSD) was obtained for the quantitation of the target protein. The biosensor also allows naked-eye detection by observing fluorescent color of solutions with the assistance of a hand-held UV lamp. We posit that our direct FRET strategy for the label-free, sensitive, visual, ratiometric, and "turn-on" detection of DBP such as NF-κB might have promising potential for the early diagnosis of many diseases and high-throughput screening of new drugs that target DBP.

2. Material and methods

2.1. Materials

All oligonucleotides as well as bovine serum albumin were purchased from Sangon Biotechnology Inc. (Shanghai, China). Concentrations of the oligonucleotides were determined by measuring their absorbance (also referred to as optical density (OD)) at 260 nm. Purified recombinant human nuclear factor-kappa B (rhNF-κB) and transcription factor Sp1 (rhSp1) were purchased from Promega (Madison, WI, USA). Interferon-gamma was obtained from Peprotech Inc. (USA). Myoglobin, cytochrome C, and hemoglobin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Exonuclease III was purchased from Fermentas (Hanover, MD). Serum was obtained from Acon Biotechnology Inc. (Hangzhou China). HeLa cell nuclear extract stimulated with TNF-α (20 ng/mL) was purchased from Takara Active Motif (Carlsbad, CA). Sybr Green I was obtained from Invitrogen (Carlsbad, CA).

2.2. Methods

Purified rhNF-κB, rhSp1, and other proteins used as negative controls were pre-incubated at 37 °C for 10 min in DNA-binding buffer solution. The water-soluble CCP was synthesized by the previously reported method (Ricci et al., 2009). The concentration of the CCP refers to the concentration of the polymer structure unit, which was calculated from the molecular and monomer weights of the neutral polymer. The molecular weight and poly dispersity of the neutral polymer were 12600 and 1.28, respectively. SG was used at a concentration of 500 times diluted from the original stock.

All other reagents were analytical grade and purchased from the Chemical Company (China). All solutions were prepared using Milli-Q water (18.2 MΩ cm) from a Millipore system. Buffer solutions used in this study include a hybridization buffer (10 mM Tris-HCl, 100 mM NaCl, pH 8.0), a DNA-binding buffer (10 mM Tris-HCl, 50 mM NaCl, 3 mM $MgCl_2$, 10% (v/v) glycerol, 0.5 mM EDTA, 0.5 mM DTT, pH 7.5), a nuclease cleavage buffer (66 mM Tris-HCl, 0.66 mM $MgCl_2$, pH 8.0), and a detection buffer (10 mM Tris-HCl, 100 mM NaCl, pH 7.5).

DNA probe was prepared by adding DNA solution of 0.5 μM concentration (stock solution) in 7 μL of the hybridization buffer. The mixture was heated at 90 °C for 5 min and slowly cooled to 25 °C. The probe was then incubated with protein in DNA-binding buffer at 37 °C for 30 min. After that, ExoIII (40 U) in the concentrated nuclease cleavage buffer was added and incubated at 37 °C for additional 5 min. EDTA solution was then added at a final concentration of 25 mM to terminate the nuclease digestion. Then 1 μL of the diluted SG was added followed by incubation at 37 °C for 30 min. The final mixture was further diluted with the detection buffer solution to a final volume of 1 mL, and a fluorescence emission spectrum was recorded at an excitation wavelength of 480 nm. Then, 5 μL of the 5.5 μM conjugated polymer solution was added to the above mixture, and the fluorescence emission spectrum was immediately measured at an excitation wavelength of 404 nm.

Absorbance of oligonucleotides were measured using a UV-vis 3150 spectrophotometer (Shimadzu, Japan). All fluorescence and FRET measurements were obtained at room temperature in 3 mL

quartz cuvette using a RF-5301 fluorometer (Shimadzu, Japan). Fluorescence images were obtained using a GDS8000 UV transilluminator.

3. Results and discussion

3.1. Mechanism

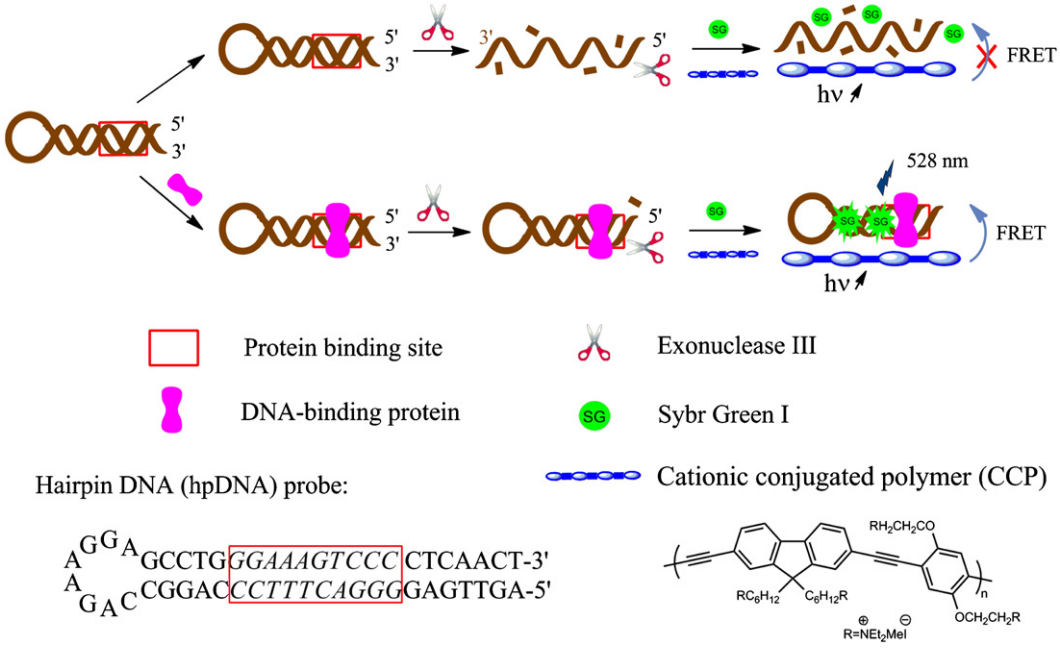
Scheme 1 illustrates the principle of our direct FRET biosensor for the detection of DBP. Oligonucleotide containing NF-κB-binding site (5′-GGGACTTTCC-3′) (Barton et al., 2002) was used as probe for specific recognition of the target protein. SG, an intercalating dye that features a substantial fluorescence enhancement upon binding to double-stranded DNA (dsDNA), was used as an energy acceptor. CCP act as an energy donor to amplify the fluorescence signal emitted from the intercalating dye because the absorption spectrum of the SG (acceptor) overlaps well with the emission of the CCP (donor) and thus satisfies the conditions for efficient FRET (Fig. S1).

We first designed a hairpin DNA (hpDNA) containing one protein-binding site (PBS) to investigate the feasibility of the label-free strategy, as shown in Scheme 1. In the absence of a target protein, the hpDNA probe will be cleaved by the ExoIII from the blunt 3′ terminus (Rogers and Weiss, 1980), which leads to a single-stranded DNA (ssDNA) and some DNA fragments. The distance between the CCP and the SG dye is far because the dye cannot intercalate into ssDNA, although there exist electrostatic interactions between the negatively charged ssDNA and positively charged CCP. We observed an inefficient FRET from the CCP to the dye, and a dark blue-green color was observed with the assistance of a UV transilluminator. However, in the presence of the target protein, the strong bond between the protein and the consensus sequence sterically hinder the ExoIII to be close to the 3′-terminus, which protects the hpDNA probe from the ExoIII-mediated digestion. In this case, the intercalating dye may intercalate into the groove of the hpDNA probe. The strong electrostatic interactions between the CCP and the DNA allow the CCP and the dye into close proximity, causing an efficient energy transfer from the CCP to the dye, which corresponds to a high FRET signal and a brilliant green color under a UV lamp.

3.2. Experimental verification of the assay

As reported, electrostatic interactions between a cationic or anionic conjugated polymer and protein can be used for probing specific biomolecules (Gu et al., 2010; Li and Liu, 2010). Before assaying DBP with our direct FRET strategy, we investigated the influence of the protein's charges on the biosensor by monitoring the quenching of the CCP with the addition of purified human recombinant NF-κB p50, nuclear extracts, and ExoIII, respectively. Results (Fig. S2) demonstrated that fluorescence of the CCP was quenched approximately 5% with the addition of these substance even at high concentrations. So we conclude that these proteins almost have no effect on the optical properties of the CCP.

Results for the detection of purified target protein using hpDNA probe were shown in Fig. 1. We observed remarkable differences in emission spectra, FRET efficiency, and fluorescence color under a UV lamp for two assays without or with the target protein. The fluorescence spectrum of the pure CCP solution exhibits a strong emission at 450 nm, which corresponds to a bright blue fluorescence color (a in Fig. 1A). In the absence of the target protein, emission at 450 nm decreased slightly and a weak emission at 528 nm was observed, which corresponds to a dark blue-green fluorescence (b in Fig. 1A). However, a strongly quenched emission at 450 nm, a dramatically increased emission at 528 nm, and a bright green fluorescence color were observed in the presence of the target protein due to a highly efficient FRET from the CCP to the SG (c in Fig. 1A). These results suggest that the specific combination of the target protein within the probe effectively protects the probe from ExoIII digestion and leads to the formation of a probe/protein/dye/CCP complex via the intercalation of SG in the groove of hpDNA probe, as well as strong electrostatic interactions between the CCP and probe. The close proximity between the CCP and SG thus leads to an obvious FRET signal and an amplified emission of dye. After normalization of the fluorescence spectra at λ_{em}=450 nm, a 6-fold increase in FRET efficiency was achieved when comparing the target protein with the blank (Fig. 1B). Furthermore, We observed almost the same



Scheme 1. Scheme for the detection of DNA-binding protein (transcription factor) using hairpin DNA (hpDNA) and cationic conjugated polymer (CCP) .

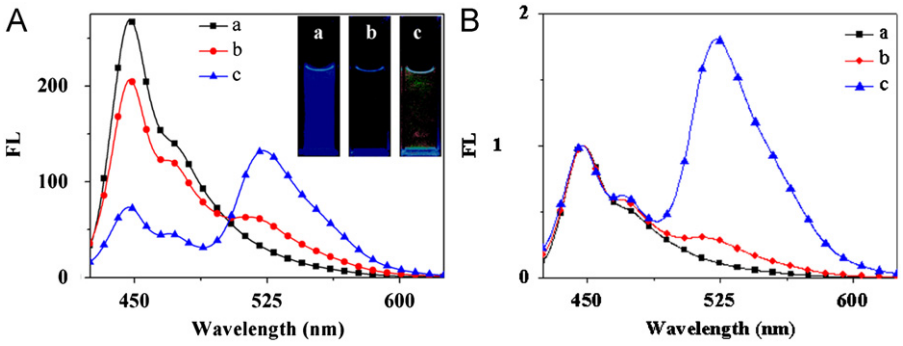


Fig. 1. (A) Fluorescence spectra and images of a: CCP; b: DNA/ExoIII/SG/CCP; c: DNA/NF-κB/ExoIII/SG/CCP. (B) Normalized fluorescence spectra of the samples in (A). [DNA]=0.5 nM; [NF-κB] =10 nM; [CCP]=27.5 nM. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

high level FRET signal from the protein/DNA/SG/CCP complex and DNA/SG/CCP complex when the ExoIII was absent, indicating that conjugation of the protein to the probe had no effect on either the intercalation of SG into the hpDNA probe, or the FRET process from the CCP to the SG (Fig. S3).

3.3. Effect of probe structure on the performance of biosensor

It has been reported that structure of oligonucleotide probe could influence the digestion rate of ExoIII (Xodo et al., 1991), and the number of binding sites as well as the length of DNA sequence have a great influence on the feasibility of the protein detection assays (Ma et al., 2011). To further investigate the effect of the probe structure on the protein detection assay and optimize the structure of the NF-κB probe, three different probes were designed to detect the target protein of same concentration (10 nM): dsDNA with one binding site (1BS-dsDNA), dsDNA with two binding sites (2BS-dsDNA), and dsDNA without a binding site (0BS-dsDNA). It is noted that all the three probes have two flat termini, which allows a digestion from two 3′-termini of the probe by the ExoIII.

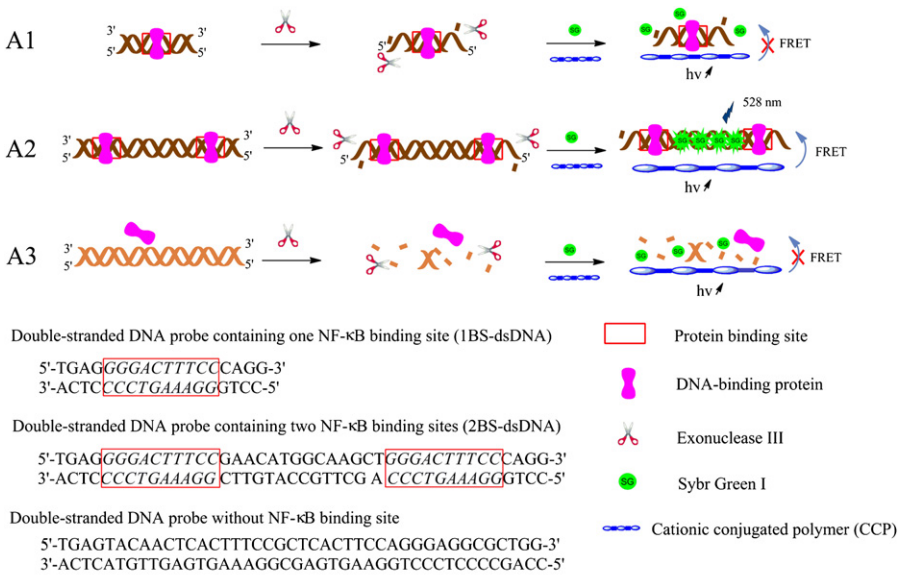
Mechanisms of the biosensor employing probes with different structures were shown in Fig. 2A. For the 1BS-dsDNA probe, when it combines with the target protein, naked duplex (sequences unbound with protein) of the 1BS-dsDNA/protein complex would be digested by ExoIII from two flat 3′-termini into some fragments, results a failure of SG to intercalate. An inefficient FRET signal will be observed, indicating too much distance between the SG and CCP (routine A1). However, for the 2BS-dsDNA probe, combination of the target protein on the two binding sites would effectively protect the probe from complete digestion by ExoIII, and the duplex structure in the middle of the probe allows the dye to intercalate. Strong electrostatic interactions between the probe and CCP will lead to a close proximity, enabling highly efficient FRET to occur (routine A2). In comparison, the 0BS-dsDNA probe could not bind with the target protein, thus the probe will be cleaved into many small DNA fragments after the ExoIII digestion, leads to an inefficient FRET signal (routine A3).

Fig. 2B shows the results for the detection of the target protein with four different probes. We observed that the hpDNA and 2BS-dsDNA probes can generate distinguishable signals compared to the corresponding blanks, and the ratios of FRET efficiencies are 3.2 and 4.5, respectively. However, the 1BS-dsDNA and 0BS-dsDNA probes produce very weak FRET signals no matter whether the target protein was added or not, although there exist strong electrostatic interactions between the CCP and DNA. We considered that it is mainly due to the different structure of the probe. In detail, the hpDNA probe consists of a 22-base-length stem and a loop. After the combination of the protein on the PBS and subsequent digestion by ExoIII, there left a duplex which include five base-pairs for SG to intercalate. For the 2BS-dsDNA probe, there are fourteen base-pairs in the middle of the probe remained after the nuclease digestion, which is favorable for the dye to intercalate. In comparison, the length of the 1BS-dsDNA probe is shorter (eighteen bases), and the only one PBS is in the middle of the probe, no duplex for dye to be intercalated was left after the protein binding and subsequent ExoIII digestion. For the 0BS-dsDNA probe, no matter how long is the sequence, the probe was completely digested into small fragments after the addition of ExoIII because the probe cannot be protected from the nuclease cleavage by combining with protein. So we conclude that the specific detection of the target protein may be attributed to three key factors: the specific DNA-protein binding, ExoIII-protected intercalation of SG in the duplex, and a high efficient FRET from CCP to SG as a result of the first two factors.

Our recent investigation of protein detection based on target-mediated FRET shows that dsDNA probe has five protruding nucleotides at one 3′-terminus that might protect the probe from nuclease digestion from a second 3′-terminus (Liu et al., 2012). Therefore, we may also design a feasible 1BS-dsDNA probe by adding more than five nucleotides at any 3′-terminus to form a sticky end. In this case, a duplex with four base pairs will be left after the protein binding and ExoIII digestion, which provides local base pairing for the dye to intercalate and an efficient FRET will occur. From this we can see that the structure of the probe is of key importance in the strategy.

3.4. Specificity and sensitivity of the assay

To further explore the specificity of this assay for protein detection, a series of control experiments were performed by using six non-cognate proteins, interferon-gamma (INF-γ), bovine serum albumin (BSA), myoglobin (Mb), hemoglobin (Hb), cytochrome C (Cyt C), and recombinant human transcription factor Sp1 (rhSp1). Effects of the protein surface charges on the fluorescence of the CCP were investigated first. Results (data not shown) illustrated that the emission of the CCP was quenched less than 5% when these proteins at high concentration were added. So we propose that influence of the protein's charges on the optical properties of the CCP can be ignored. Fig. 3 shows the results for the detection of the target protein (1 nM) and six non-cognate proteins (each at 10 nM) with probes hpDNA and 2BS-dsDNA. We observed approximately 6∼7-fold signals of the target protein over the negative proteins with two specific probes. The results confirm that proteins that cannot bind to the recognition sequence do not protect the probe from ExoIII-mediated digestion, which results in a low FRET signal.



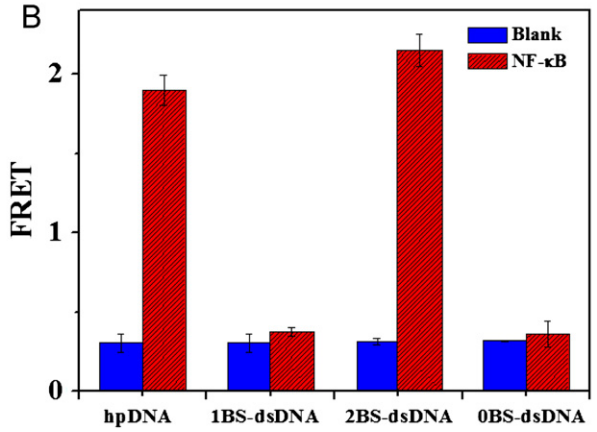


Fig. 2. (A) Schematic representation for transcription factor detection using oligonucleotides as probes (A1: 1BS-dsDNA; A2: 2BS-dsDNA; A3: 0BS-dsDNA). (B) Comparison of the FRET efficiencies for the detection of target protein (10 nM NF-κB) employing four different probes.

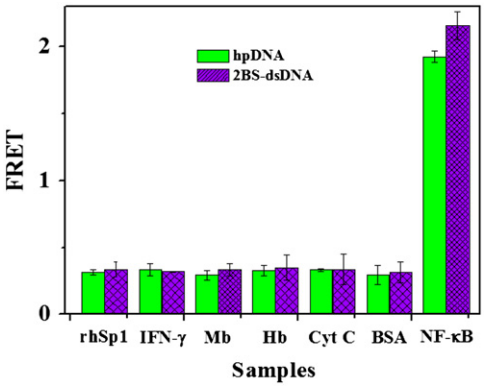


Fig. 3. Comparison of FRET efficiencies for the detection of different proteins (NF-κB at 1 nM; rhSp1, IFN-γ, Mb, Hb, Cyt C and BSA, each at 10 nM) with hpDNA probe and 2BS-dsDNA probe.

Developing easy-to-perform and cost-effective techniques to accurately quantify NF-κB activity is important for the diagnosis of diseases. The capability of our strategy for the quantitative detection of purified recombinant NF-κB p50 protein was investigated next using probes hpDNA and 2BS-dsDNA. Dose-dependent FRET signals were observed for two assays; each exhibited different linear range, saturation point, and LOD. A saturation point of 1 nM, a linear range (R=0.9928) from 0 to 0.5 nM, and a LOD of 0.1 nM were obtained for the hpDNA probe assay (Fig. S4A). In contrast, a saturation point of 5 nM, a linear range (R=0.9943) from 0 to 1 nM, and a LOD of 0.2 nM were observed for the 2BS-dsDNA probe assay (Fig. S4B). The sensitivity of our strategy for the purified human NF-κB was improved at least one order of magnitude or comparable to those of existing homogeneous assays based on the fluorescence techniques (Xu et al., 2008; Wang et al., 2005; Chen et al., 2006). Additionally, the precision of our approach was evaluated by RSD, average RSDs for all eight concentration points are 8.3% and 5.5%, which are smaller than that of the reported methods (detailed information are shown in Table 1).

3.5. HeLa cell nuclear extracts detection

To test the applicability of the assay on real biological samples, we challenged the performance of the biosensor in HeLa cell nuclear extracts, which was stimulated with recombinant human tumor necrosis factor-alpha (TNF-α, 20 ng/mL) for 10 min at 37 °C.

Table 1
Comparison of the performance of recently reported optical approaches for the detection of NF-κB p50.

Method	Labeling	Number of binding sites	LOD[a]		Work mode	RSD[b](%)		Reference
			A[c] (nM)	B[d] (pg/μL)		A	B	
FL[e]	Label-free	One	0.1	1	Turn-on	8.3	2.95	Our work
		Two	0.2	1		5.5	3.8	
FL	Label-free	One	2	50000	Turn-on	~11	~13	Chen et al. (2006)
LUM[f]	Label-free	One or two	30 nM for purified expressed p50 protein	Turn-on	~9 (one site) ~8 (two sites)			Ma et al. (2011)
FL	Double	One	0.4	1000	Turn-off	~15	~11	He et al. (2007)
FL	Double	Two	5	50000	Turn-off	~10	~12	Wang et al. (2005)

[a] Limit of detection.
[b] Average relative standard deviation (RSD), which was calculated based on the average RSDs for given concentration points in an experiment.
[c] A refers to purified human recombinant NF-κB p50.
[d] B refers to HeLa cell nuclear extracts.
[e] Fluorometric method.
[f] Luminometric method.

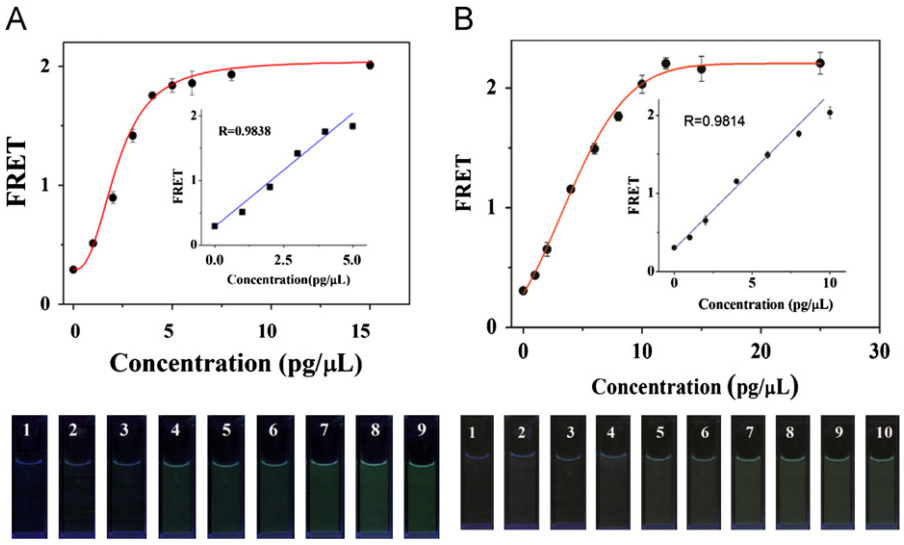


Fig. 4. Relationship between FRET efficiency and protein concentration and images for the detection of HeLa cell nuclear extracts with hpDNA probe (A) and 2BS-dsDNA probe (B). The amounts of HeLa cell nuclear extracts in A are 0, 1, 2, 3, 4, 5, 6, 8, and 15 pg/μL, corresponding to the pictures 1–9; the amounts of HeLa cell nuclear extracts in (B) are 0, 1, 2, 4, 6, 8, 10, 12, 15 and 25 pg/μL, corresponding to the pictures 1–10.

As shown in Fig. 4, the FRET signals appeared to be dose-dependent over the given concentration ranges (0~15 pg/μL for the hpDNA assay; 0~25 pg/μL for the 2BS-dsDNA assay). A LOD of 1 pg/μL for the detection of NF-κB in nuclear extracts was obtained with two probes hpDNA and 2BS-dsDNA. Compared with the previously reported method (Chen et al., 2006), the sensitivity was improved for 10000-fold. To the best of our knowledge, ours is the most sensitive optical method for the detection of activated NF-κB in HeLa cell nuclear extracts. Furthermore, the LOD for the target protein in nuclear extracts with naked eyes was estimated to be 10 pg/μL. More importantly, average RSDs of 2.95% and 3.8% were achieved with the hpDNA probe and 2BS-dsDNA probe, whereas the average RSDs of the previous methods were generally more than 10% (Table 1). Thus, the excellent reproducibility of our strategy allows the quantitative and accurate detection of the target protein from crude extracts.

To further address the advantages of our FRET assay, we provide figures of merit for recently reported optical methods with label-free or labeled DNA probes for the detection of NF-κB p50 for comparison (Table 1). Our approach has the advantages of a much lower LOD and lower average RSD compared to the label-free methods. As compared with methods that require labeling, our approach is simple, inexpensive, and works in "turn-on" mode in addition to having a low LOD and excellent precision.

3.6. Evaluation of signal amplification with the CCP

Finally, we tried to understand the reasons for the improved sensitivity of our label-free approach, we compared the fluorescent response of the assay with and without CCP was not used (Fig. S5). In these assays, the 2BS-dsDNA probe of 0.5 nM was used for the detection of 10 nM purified NF-κB p50. In the absence of the CCP, the samples were excited at 480 nm, and a ratio of 2.2 for the emission intensities at 525 nm of the target protein over the blank was obtained. In contrast, in the presence of the CCP, the solutions were excited at 404 nm and emitted at both 448 nm and 525 nm, a ratio of 7.3 for the FRET signals ($I_{525\ nm}/I_{448\ nm}$) of the target protein over the blank was obtained. These results indicated that the ratio of the positive sample over the blank control was increased for 3.3-fold. In addition, when the CCP was used as an energy donor, the fluorescence intensity at 525 nm increased for 5.8-fold and 5.1-fold for the positive sample and blank control, respectively. Compared with the previously reported method using DNA intercalating dye only (Chen et al., 2006), the LOD of our CCP-amplified strategy for the detection of

NF-κB p50 was lowered for 10000-fold. In the past decade, researchers have found that the fluorescence response of conjugated polymer-based biosensors can be improved tens, hundreds or even thousands fold (Feng et al., 2008; Gaylord et al., 2002). Recently, we found that the twisted conjugated backbone and the higher charge density of the CCP is more favorable to the interaction between the CCP and DNA, which may improve the sensitivity of both DNA hybridization and protein detection (Huang et al., 2009). Taken together, we attribute the sensitivity of our label-free strategy to the "molecule wire" property and twisted structure of the CCP, as well as highly efficient FRET from the CCP to the intercalating dye.

4. Conclusion

In conclusion, we demonstrated a highly sensitive optical platform for the detection of DNA-binding protein. Our approach presents several advantages over currently available assays. First, compared with the previous studies where chromophore- or quencher-labeled DNA substrate is needed, this method is label-free, cost efficient, and offers a convenient protocol for "turn-on", homogeneous, and ratiometric detection. Second, the CCP used here offers significant amplification of the detection signal, leading to a much lower LOD than that of the previous reports. Third, this assay is easy to implement for visual detection with the assistance of a UV transilluminator. So we posit that the label-free, sensitive, and economical method may have potential applications in a wide range of fields such as biomedical research, medical diagnosis, and drug discovery.

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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.2012.08.027.

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