



# An aptameric molecular beacon-based “Signal-on” approach for rapid determination of rHuEPO- $\alpha$

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## ABSTRACT

The aim of this work is to describe the first example of aptameric molecular beacon (MB)-based probe for the detection of recombinant human erythropoietin (rHuEPO- $\alpha$ ) in physiological buffer, using a novel 35 nt ssDNA aptamer (807–35 nt) originally isolated by Systematic Evolution of Ligands by Exponential enrichment (SELEX) technique in our laboratory. Both “Signal-on” and “Signal-off” MB modes were developed, respectively, in which the conformational alteration of aptamer before and after binding to rHuEPO- $\alpha$  can be demonstrated in terms of the correspondingly fluorescent changes. Comparing with “Signal-off” mode, “Signal-on” mode provided higher sensitivity, while with the addition of target rHuEPO- $\alpha$ , quenching between fluorescent 807–35 nt aptamer (F-Apt) and a short quencher-labeled complementary sequence (QDNA) was disturbed by the specific binding between rHuEPO- $\alpha$  and F-Apt. QDNA was thus loosened and released from F-Apt, leading to a consequently full fluorescent restoration. Systematic optimization of parameters in “Signal-on” mode were carried out, the choice of QDNA length, the hybridization site of a small supplementary DNA (SDNA) stabilizer, and the existence of Mg<sup>2+</sup> cation played essential roles for the performance characterization. A convenient and sensitive determination of rHuEPO- $\alpha$  with a LOD of 0.4 nM was achieved.

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## 1. Introduction

Erythropoietin is an endogenously hematopoietic growth factor, which can regulate erythropoiesis production by promoting the proliferation and differentiation of erythroid progenitor cells and maintains the red blood cell mass at an optimum level [1]. Recent development of biological recombinant techniques has allowed large scale production of biologically active erythropoietin called recombinant human EPO (rHuEPO) as pharmaceuticals for anaemia treatment in clinic. Meanwhile, rHuEPO was a kind of famous doping hormone which was strictly prohibited by International Olympics Committee [2,3], due that it can enhance the performance of athletes in endurance competition sports by stimulating more production of new red blood cells and boosting delivery of oxygen to the tissues. Immunological assays have the main applications in monitoring this widespread recombinant glycoprotein drug [4–6], but how to overcome the interference and narrow the specificity of anti-rHuEPO antibodies is still a challenge. For example, anti-rHuEPO monoantibody (mAb) AE7A5 was widely adopted in the assays as the capturing or probing molecules, but it has a broad cross-reactivity with eukaryotic or bacterial proteins, even human

urothelium tissue [7]. In comparison with antibody, aptamers, known as “chemical antibodies”, have a number of advantages such as higher specificity and affinity, non-immunogenic, stable, robust against denaturation, ease of chemical modification and ease of being synthesized, which would offer an alternative and novel way for the detection of rHuEPO- $\alpha$  in the analytical field.

Aptamers are the artificial single-stranded (ss) DNA or RNA sequences generated by an *in vitro* selection technique known as SELEX from an oligonucleotide pool with immense combinatorial random sequences [8–11]. They have attracted much attention as biosensors for many important biomedical targets, such as metal ions, peptides, proteins and even intact cell [12,13]. The oligonucleotide aptamers are one kind of excellent recognition module, they exert specific binding capability for the targets by the shape fit, *i.e.*, by forming special three-dimensional structures. These spatial structures are the natural result of their complicated secondary structures including partial Watson-Crick, Hoogsteen hydrogen bonds, hairpins, loops and bulges, *etc.* Particularly, aptameric MB sensors attract great concern in the diagnostics area in recent years, because the recognition events can be easily reflected by the Signal changes of an oligonucleotide ssDNA aptamer, and the fast, simple and cost-effective determination of targets can thus be achieved [14]. It is reported that MB are DNA sequences composed of one target recognition region of about 30 bases flanked by two short complementary stem sequences, which force the entire

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**Table 1**  
Sequences of constructed oligonucleotides.

| ssDNA               | Sequence (5' → 3')                                      |
|---------------------|---|
| FQ-Apt1 (807–39 nt) | 5'-FAM-GATTGAAAGGTCGTGTTTTGGGGTTGGTTGGGTCAATA-DABCYL-3' |
| FQ-Apt2 (807–35 nt) | 5'-FAM-TTGAAAGGTCGTGTTTTGGGGTTGGTTGGGTCAA-DABCYL-3'     |
| FQ-Apt3 (807–33 nt) | 5'-FAM-TGAAAGGTCGTGTTTTGGGGTTGGTTGGGTCA-DABCYL-3'       |
| F-Apt (807–35 nt)   | 5'-TTGAAAGGTCGTGTTTTGGGGTTGGTTGGGTCAA-FAM-3'            |
| QDNA1 (7 nt)        | 5'-DABCYL-TTGACCC-3'                                    |
| QDNA2 (8 nt)        | 5'-DABCYL-TTGACCCA-3'                                   |
| QDNA3 (10 nt)       | 5'-DABCYL-TTGACCCAAA-3'                                 |
| QDNA4 (12 nt)       | 5'-DABCYL-TTGACCCAAACC-3'                               |
| SDNA1 (8 nt)        | 5'-GACCTTTC-3'  |
| SDNA2 (5 nt)        | 5'-AAAAA-3'   |
| SDNA3 (7 nt)        | 5'-AAACCAA-3'   |

sequence to form a stem-loop structure in the absence of a target [15].

In our recent work, we have firstly *in vitro* isolated a series of anti-rHuEPO- $\alpha$  ssDNA aptamers, in which aptamer 807 is a prevalent sequence (55% in 42 clones) [16]. The binding capacity of random sequence 807–39 nt was almost identical with the full sequence 807 (Fig. S1). The secondary hairpin structure of 807–39 nt meets the prerequisite of MB probe quite well, its loop would function as a recognition site, and its stem as a support. That makes aptamer 807–39 nt as an ideal framework to build signal transduction module. Therefore, a convenient aptameric MB approach was constructed in this paper, both “Signal-off” and “Signal-on” MB modes were investigated. Fluorescent conformational changes could be observed when the specific binding between aptamer and rHuEPO- $\alpha$  occurred. Systematic optimization on conditions in the case of “Signal-on” MB mode were undertaken by examining the quencher-labeled DNA (QDNA) length and concentration, the hybridization site of a short supplementary DNA (SDNA), the role of metal ions, and the concentration of glycerol as a fluorescence stabilizer. Finally a LOD of 0.4 nM for rHuEPO- $\alpha$  was achieved.

## 2. Experimental

### 2.1. Chemical and reagents

rHuEPO- $\alpha$  (3.89 mg/mL, purity higher than 98.5%) was donated by SCIPROGEN Bio-pharmaceutical Company (Shenzhen, China). Fluorophore and quencher dual end-labeled MB, fluorophore single end-labeled MB, QDNA and SDNA were synthesized in Shanghai Sangon Biological Engineering Technology & Services Company (Shanghai, China) (Table 1). In the dual end-labeled MB (FQ-Apt) and single end-labeled MB (F-Apt/QDNA) sequences, 6-carboxyfluorescein (6-FAM) and 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL) are used as the fluorophore and quencher groups, respectively. HSA, hemoglobin and cytochrome C were obtained from Sigma-Aldrich (St. Louis, Mo, USA). BSA and lysozyme were purchased from Shanghai Sangon Biological Engineering Technology & Services. Ig G was purchased from Tianlai Bioengineering Company (Beijing, China). Globin was purified by our laboratory. All other reagents were of analytical purity and obtained from Beijing Chemical Company (Beijing, China). The physiological buffer as well as the selection buffer consisted of 20 mM Tris, 140 mM NaCl, 5 mM MgCl<sub>2</sub> and 5 mM KCl (pH 7.4). Deionized, distilled water was purified from a Milli-Q water purification system (Millipore, France).

### 2.2. Measurements

All fluorescence experiments were performed on a HITACHI F4010 spectrofluorometer, using a 0.5 mL quartz cuvette as the sample cell. The fluorescence emission spectra of the aptameric MB

were obtained from 510 nm to 600 nm with an excitation wavelength fixed at 494 nm (“Signal-on” mode) or 488 nm (“Signal-off” mode), respectively. The slit widths of excitation and emission were set at (5 nm and 10 nm) and (10 nm and 10 nm) for “Signal-on” and “Signal-off” modes, respectively. The concentrations of F-Apt and FQ-Apt in all samples were 10 nM. Prior to the fluorescent determination, the aptameric MB was denatured at 94 °C for 5 min and annealed at the optimum condition, then mixed with rHuEPO- $\alpha$  in selection buffer and incubated at room temperature for 1 h. The titration curves were plotted as quenching percentage ( $y = (\bar{F}_0 - F)/\bar{F}_0$ ) or as  $\Delta F(y = F - \bar{F}_0)$  versus increasing concentration of rHuEPO- $\alpha$  ( $x$ ) for “Signal-off” and “Signal-on” mode, respectively. To examine the specificity of the aptameric molecular beacon for “Signal-on”, other proteins were used, including HSA, BSA, IgG, hemoglobin, globin, lysozyme and cytochrome C.

### 2.3. Gel electrophoresis

Electrophoretic gel mobility shift assay was used to confirm the binding between rHuEPO- $\alpha$  and the MB. Aptamer 807–35 nt, FQ-Apt2 and F-Apt (1  $\mu$ M) were incubated with rHuEPO- $\alpha$  (1  $\mu$ M) in physiological buffer for 1 h at room temperature, respectively. Electrophoresis was performed on 12% non-denatured polyacrylamide gel electrophoresis at 22 V/cm for 1 h at 4 °C. The gel was stained in ethidium bromide and analyzed by UVP VisionWorks<sup>TM</sup>LS Image System.

### 2.4. Determination of $K_d$ and $T_m$

To calculate the  $K_d$  values, the changes of fluorescence intensity or the quenching percentage versus increasing rHuEPO- $\alpha$  concentrations ( $x$ ) was plotted, and the data points were fitted by the non-linear regression analysis based on one-site direct binding model with the following equation using Originpro 7.5 software (OriginLab, USA):

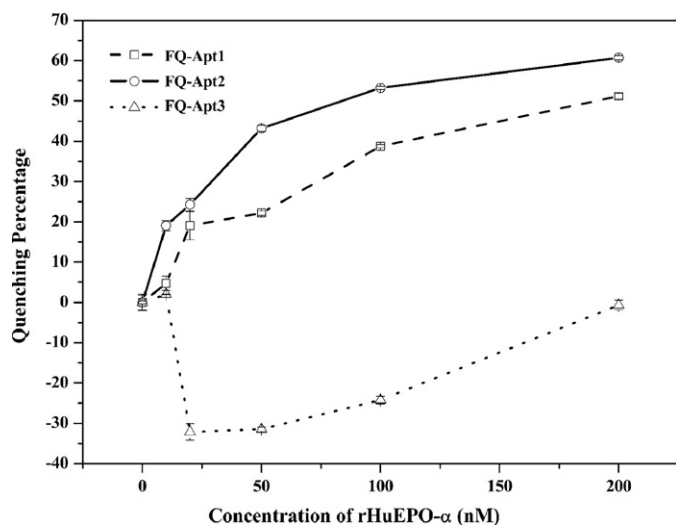
$$y = \frac{B_{\max} \cdot x}{K_d + x} \quad (1)$$

where  $B_{\max}$  is the degree of saturation, and  $K_d$  is the dissociation constant.

$T_m$  values for short probe (<15 nt) were determined by the Wallace rule [17]:

$$T_m = 2^\circ\text{C}(A + T) + 4^\circ\text{C}(C + G) \quad (2)$$

where  $T_m$  = temperature (°C) at which 50% of the oligonucleotides are annealed to its complementary sequence. The number of each particular nucleotide in the sequence is inserted into the equation in place of the letters. When the sequence is free in solution, add 7–8 °C to  $T_m$ .



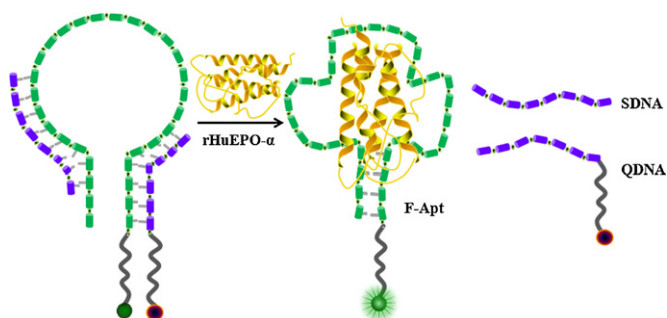
**Fig. 1.** The titration curves of the “Signal-off” aptameric MB mode. FQ-Apt (10 nM) of different stem length was titrated with increasing concentration of rHuEPO- $\alpha$  from 0 nM to 200 nM in the physiological buffer (20 mM Tris, 140 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM KCl; pH 7.4). Data are reported as mean  $\pm$  SD values with RSD < 5.0% ( $n=3$ ).

### 3. Results and discussion

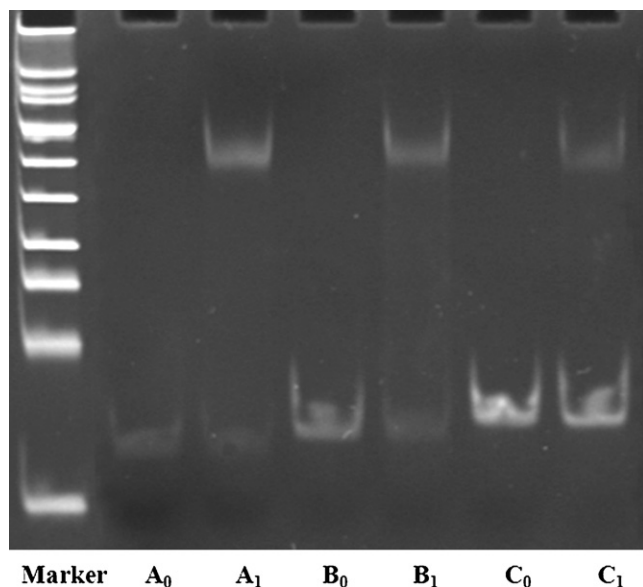
#### 3.1. Design of “Signal-off” and “Signal-on” aptameric MB

Based on the simulated secondary stem-loop structure of our selected aptamer 807–39 nt by M-fold [18] (Fig. S2), a dual end-labeled design was chosen as a readily start to test the possibility in formation of MB alike structure [19]. Binding of dual end-labeled MB with rHuEPO- $\alpha$  target brought the fluorophore and quencher in close proximity, resulted in the decrease of fluorescence. The stems with 3 bp (FQ-Apt3)/4 bp (FQ-Apt2)/5 bp (FQ-Apt1) in the dual end-labeled MB form were investigated, respectively (sequences shown in Table 1), and 4 bp (FQ-Apt2) in the stem was found to be the minimum length to achieve the specific recognition. FQ-Apt2 (807–35 nt) was then considered as the shortest motif for binding with rHuEPO- $\alpha$ . The addition of rHuEPO- $\alpha$  mainly facilitates the forming and stabilizing of stem-loop structure of FQ-Apt2, drawing the distance closer between fluorophore and quencher. Because the fluorescent change is limited to the close proximate extent of fluorophore and quencher groups, a detection concentration of 10 nM rHuEPO- $\alpha$  was merely obtained by “Signal-off” mode (Fig. 1).

Another single-labeled “Signal-on” mode was followed on the fully utilization of the stem and loop binding ability of 807–35 nt aptamer for the target protein, it can further increase the detection sensitivity as expected (Scheme 1). In this “Signal-on” design,



**Scheme 1.** Illustration of “Signal-on” mode using 807–35 nt aptamer.



**Fig. 2.** Binding experiments of aptamer 807–35 nt with or without labeling group and rHuEPO- $\alpha$  in PAGE. Lanes X<sub>0</sub> and X<sub>1</sub> represent aptamer alone and aptamer with the addition of rHuEPO- $\alpha$  (1  $\mu$ M). X represents A, B and C. A: 807–35 nt (1  $\mu$ M); B: FQ-Apt2 (1  $\mu$ M); C: F-Apt (1  $\mu$ M), QDNA1 (10  $\mu$ M).

toward a single 3′ end-labeled F-Apt, a short 5′-end quencher-labeled complementary QDNA sequence was employed and the fluorescence background became “darken”. When the “Signal-on” system was exposed to rHuEPO- $\alpha$ , QDNA was departed from F-Apt and the fluorescent signal was restored. This kind of structural-switching approach is obviously leading to an increase of a fluorescent signal.

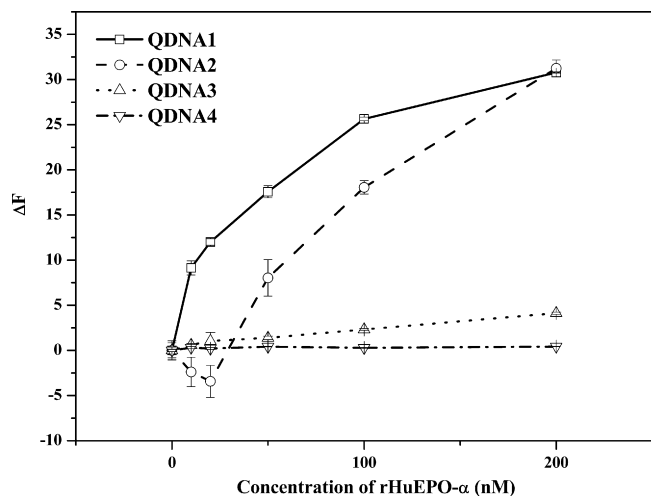
The fluorescent signal change either in “Signal-off” or “Signal-on” mode was indeed induced by the aptamer-target complexation by using binding experiments in gel electrophoresis. As shown in Fig. 2, aptameric MBs both adopted in “Signal-off” and “Signal-on” modes still maintain the binding ability with rHuEPO- $\alpha$ . The complex of MB with rHuEPO- $\alpha$  shifted slowly than free MB, and the binding capacity is not weakened by the labeling in comparison with the non-labeled aptamer 807–35 nt. Furthermore, since the degree of fluorescence recovery is depended on the binding ability of aptamer for rHuEPO- $\alpha$ , which is a reflection of the aptamer’s spatial structure. Several factors that can affect the folding and binding efficiency of aptamer should be optimized firstly.

#### 3.2. Systematic optimization on “Signal-on” mode

To achieve the most sensitivity of this aptameric MB-based fluorescent “Signal-on” mode, how to eliminate the background fluorescence and how to fully regenerate the inherent fluorescence of F-Apt in the presence of rHuEPO- $\alpha$  are both the key considerations. Such parameters as length and concentration of QDNA, the hybridization site of a short SDNA stabilizer, the role of metal ions, and the concentration of glycerol as a fluorescence stabilizer are systematically investigated.

##### 3.2.1. The length and concentration of QDNA

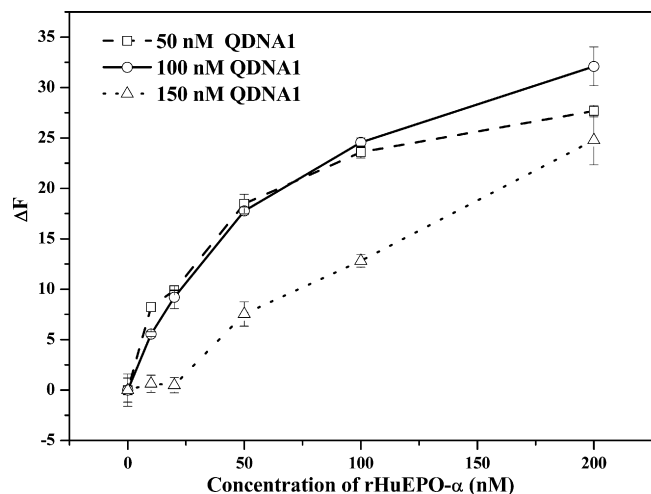
QDNA adopted here should meet two points. One point is to hybridize with the 3′-end stem of F-Apt so as to compose a fluorescent quenching resonance energy transfer, and the other is to be a displaceable element when the rHuEPO- $\alpha$  as a competitor added. Only the suitable length of QDNA can offer both low background fluorescence in the absence of the target and a possibility for fast structural switching when the target is present [20]. In a series of



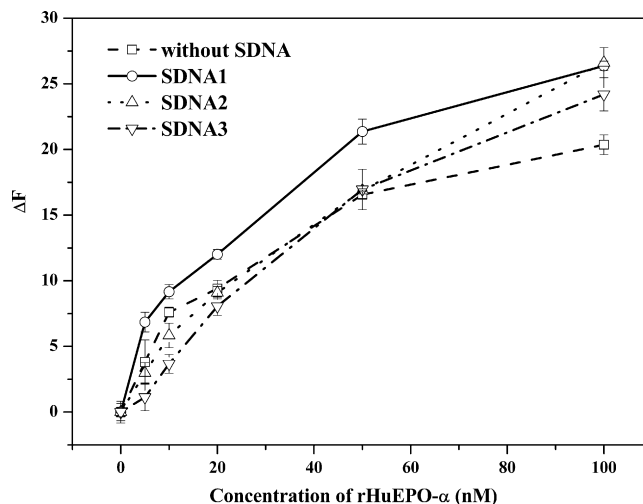
**Fig. 3.** Effects of QDNA length on the “Signal-on” aptameric MB mode. F-Apt (10 nM) and QDNA (100 nM) with different length were titrated with increasing concentration of rHuEPO- $\alpha$  from 0 to 200 nM in the physiological buffer. Data are reported as mean  $\pm$  SD values with RSD < 7.0% ( $n = 3$ ).

QDNAs with different lengths, QDNA1 (7 nt), QDNA2 (8 nt), QDNA3 (10 nt) and QDNA4 (12 nt), QDNA1 shows the best signal response. The background of fluorescence was more decreased when QDNA with excess length (QDNA2–4) was added, but the specific competition ability of F-Apt was obviously weakened in some extent (Fig. 3). A possible explanation is QDNAs with excess complementary bases would produce a more tight duplex structure in MB, which induce the partially hybridized F-Apt is hardly to effectively bind with rHuEPO- $\alpha$ .

The fluorescent intensities of system background and the signal restoration with target addition were also influenced by the concentration of QDNA. The results showed that addition of 50 nM or 100 nM QDNA1 had the almost same contribution to the recovery of fluorescence, and lower background was obtained with 100 nM QDNA1. Although background of fluorescence is decreased by the higher concentration of QDNA1 (150 nM), the addition of excess QDNA1 would lead to fluorescence self-quenching, which affected the restoration of fluorescence (Fig. 4).



**Fig. 4.** Effects of QDNA1 concentration on the “Signal-on” aptameric MB mode. F-Apt (10 nM) and various concentration of QDNA1 (50 nM, 100 nM and 150 nM) were titrated with increasing concentration of rHuEPO- $\alpha$  from 0 to 200 nM in the physiological buffer. Data are reported as mean  $\pm$  SD values with RSD < 5.0% ( $n = 3$ ).



**Fig. 5.** Effects of the hybridization site of SDNA on the “Signal-on” aptameric MB mode. Concentrations of F-Apt, QDNA1 and SDNA were 10 nM, 100 nM and 100 nM, respectively. The MB solution was titrated against increasing concentration of rHuEPO- $\alpha$  (0–100 nM) in physiological buffer. Data are reported as mean  $\pm$  SD values with RSD < 4.0% ( $n = 3$ ).

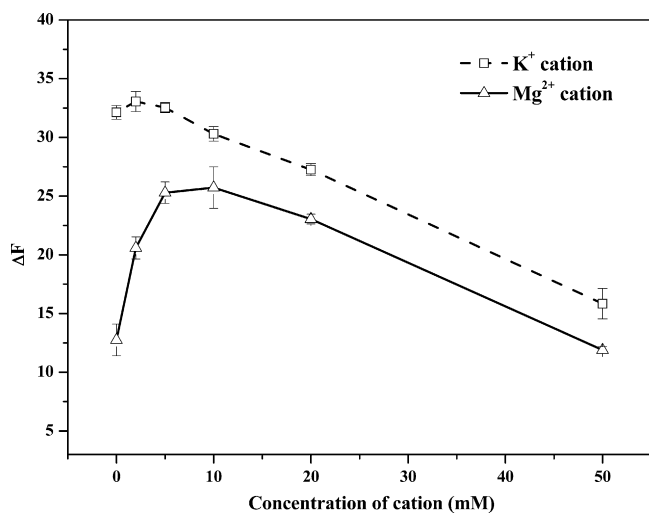
### 3.2.2. The hybridization site of SDNA

An aptamer with a hairpin motif, the loop part is generally considered as the key motif for recognizing rHuEPO- $\alpha$  [15]. In the “Signal-on” mode, F-Apt was partially hybridized with QDNA1 in a 7 bp from its 3'-end, other 28 nt sequence would remain a free coiling status. A short supplemental sequence which form a complementary duplex with the 5'-end stem of F-Apt is expected to aid the other 28 nt sequence in an order status and expose full recognition sites of loop for target rHuEPO- $\alpha$ . Different SDNA sequences with different binding sites of F-Apt were synthesized to confirm this presumption. SDNA2 and SDNA3, which were partly hybridized with the loop part in F-Apt, might interfere the binding between rHuEPO- $\alpha$  and F-Apt, so less QDNA1 was departed, and the fluorescent change was lower than the case of SDNA1. Only SDNA1 with 8 nt sequences participated in the 5'-end stem hybridization can achieve the most signal change by stabilizing the “Signal-on” mode and exposing the loop with possible recognition site (Fig. 5). Another possible reason is that the higher  $T_m$  value of SDNA1 (32 °C) than that of SDNA2 (18 °C) and SDNA3 (26 °C), which could make the aptameric MB system more stabilizer [21].

### 3.2.3. Concentration of metal ions

It is known that the presence of alkali and alkaline-earth metal ions has important effect on the aptamer–target interaction. For example, it is reported that the cations  $K^+$  and  $Mg^{2+}$  can directly induce a dominant structural formation of nucleotides through a size-selective coordination mechanism in free solution [22]. In this work, the maximum fluorescence signal can be kept well in the range of 0–10 mM  $K^+$ , while the specific binding between aptamer and rHuEPO- $\alpha$  lowered when  $K^+$  cation was higher than 10 mM (Fig. 6). It is assumed that under higher concentration of  $K^+$ , formation of complex was reduced by the possible distortion of aptamer spatial structures [23].

The concentration of divalent  $Mg^{2+}$  cation was also investigated. In the range of 0–50 mM  $Mg^{2+}$ , the fluorescent signal recovery was steadily increased at first and then significantly decreased at the concentration higher than 10 mM (Fig. 6), indicating that the formation ability of aptamer–target complex was greatly enhanced at lower  $Mg^{2+}$  concentration [24,25]. However, at the higher  $Mg^{2+}$  level, the fluorescence was quenched in some extent [26]. Since the existence of  $Mg^{2+}$  is reported to be helpful to form and stabilize the duplex of oligonucleotide [22], it is assumed that the spatial

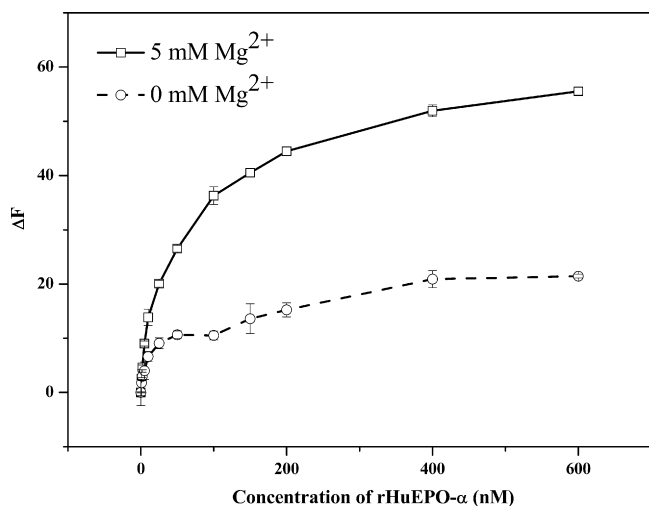


**Fig. 6.** Effects of cation concentration on the “Signal-on” aptameric MB. Concentrations of F-Apt, QDNA1 and SDNA1 were 10 nM, 100 nM and 100 nM, respectively. The MB solution was titrated with 100 nM rHuEPO- $\alpha$  in the physiological buffer contained various concentration of metal ion. Data are reported as mean  $\pm$  SD values with RSD < 3.0% ( $n = 3$ ).

recognition of F-Apt and rHuEPO- $\alpha$  was also benefited from the addition of Mg<sup>2+</sup>. As shown in Fig. 7, specific recognition between F-Apt and target protein was deteriorated without Mg<sup>2+</sup>. This behavior is in accord with other Mg<sup>2+</sup>-dependent aptamer-ATP or OTA target binding [27,28]. Moreover, for such a negatively charged rHuEPO- $\alpha$  and the likelihood negatively charged aptamers, it is supposed that proper concentration of Mg<sup>2+</sup> cation could shield the negative charges of DNA and act as the electrostatic attractive cation bridge, which probably facilitate the approaching of the negatively charged rHuEPO- $\alpha$  to the surface of the oligonucleotide [29]. Since higher Mg<sup>2+</sup> concentration is disadvantageous for fluorescence intensity, the Mg<sup>2+</sup> concentration kept at 5 mM was selected in the “Signal-on” mode.

### 3.2.4. Other parameters have effect on hybridization and complex formation

The hybridization of oligonucleotides complementary sequences is following a slow kinetics process, which requires a



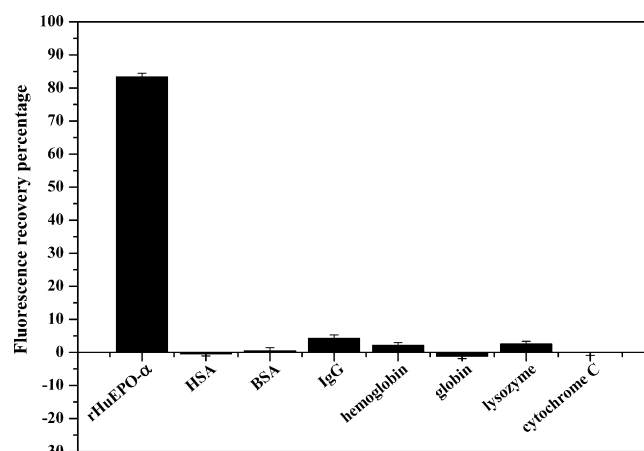
**Fig. 7.** The titration curves of the “Signal-on” aptameric MB against the increasing concentration of rHuEPO- $\alpha$  (0–600 nM) in physiological buffer contained 1.5% (v/v) glycerol with or without 5 mM Mg<sup>2+</sup>. Data are reported as mean  $\pm$  SD values with RSD < 5.0% ( $n = 3$ ). Other conditions are same as in Fig. 6.

relative long annealing period [30]. Since the extent of quenching efficiency is directly depending on the Förster distance in such a fluorescent resonance energy transfer format, the tighter base-pairing of oligonucleotides, the more decreased background of fluorescence, and the best result was achieved in an annealing condition of 4 °C overnight. The background of fluorescence under other conditions such as room temperature 30 min, room temperature overnight and ice bath for 10 min are higher 27%, 15% and 30% than that under the condition of 4 °C overnight, respectively.

As a kind of additives with high viscosity and good biological compatibility, glycerol was tested to increase the viscosity of solution and stabilize the formation of F-Apt-QDNA hybridization or F-Apt-target protein complex. Although 0, 0.5%, 1.5% and 3% (v/v) glycerol have no significant effect on the change of fluorescence, addition of 0, 0.5% and 3% glycerol made the increasing extent of fluorescence lower 26%, 29% and 13% than addition of 1.5% glycerol, respectively, at a low concentration of rHuEPO- $\alpha$  (50 nM). So 1.5% glycerol was chosen to facilitate the improvement of sensitivity by stabilizing the MB system when low concentration of rHuEPO- $\alpha$  was added.

### 3.3. Determination of rHuEPO- $\alpha$ in “Signal-on” mode

Best performance of our “Signal-on” mode was found in the physiological buffer of 140 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM KCl, and with 1.5% glycerol added. Titration experiment was then performed with increasing concentration of rHuEPO- $\alpha$  from 0 to 600 nM under above optimized conditions. The fluorescence signal was first rapidly increased with an increased rHuEPO- $\alpha$  concentration from 0 to 50 nM, indicating that the added rHuEPO- $\alpha$  was largely bound to the F-Apt, and then steadily increased when the concentration of rHuEPO- $\alpha$  was beyond 50 nM. In the presence of 600 nM rHuEPO- $\alpha$ , full fluorescence was recovered. Using the “Signal-on” mode, 1 nM of rHuEPO- $\alpha$  could be detected without any pre-treated procedure in the physiological buffer. The linear range was 1–10 nM ( $y = 1.976 + 1.229x$ ,  $r = 0.9905$ ) (Fig. 7), and a LOD could be calculated according to the equation of  $LOD = 3SD_{blank}/k$  (where  $k$  represents the slope of linear curve, the repeated number of blank is 11), so the LOD is 0.4 nM. The RSD values of high and middle concentration in the linear curve are determined as 3.3% and 4.2%, respectively. Also the dissociation constant ( $K_d = 50 \pm 7$  nM) was



**Fig. 8.** Binding specificity of the “Signal-on” aptameric MB. Different proteins HSA, BSA, IgG, hemoglobin, globin, lysozyme and cytochrome C were compared with rHuEPO- $\alpha$  in their capability to bind with F-Apt in physiological buffer contained 5 mM Mg<sup>2+</sup> and 0.5  $\mu$ M BSA. Other conditions are same as in Fig. 7. The concentration of rHuEPO- $\alpha$  is 200 nM, other proteins is fivefold of rHuEPO- $\alpha$ . Data are reported as mean  $\pm$  SD values with RSD < 2.0% ( $n = 3$ ).

determined by the titration curve of “Signal-on” based on one-site direct binding model (Fig. S4), which is in agreement with the dissociation constant ( $K_d = 33 \pm 4$  nM) determined by “Signal-off” mode (Fig. S3).

#### 3.4. Binding specificity of “Signal-on” MB

The specificity of the “Signal-on” MB mode was demonstrated in the physiological buffer containing 0.5  $\mu$ M BSA as fluorescence sensitized stabilizer. Here the full recovery of 100% was defined as the fluorescence intensity reached the value of FDNA alone, when equivalent amount of F-Apt-QDNA (combined with SDNA) was considered as the background in the “Signal-on” mode. rHuEPO- $\alpha$  brought about 80% of fluorescence recovery, while fivefold of HSA, BSA, IgG, hemoglobin, globin, lysozyme and cytochrome C showed no cross binding toward the F-Apt. It is indicated that this “Signal-on” MB probe has good selectivity when compared with other interfering proteins (Fig. 8).

#### 4. Conclusion

Here we reported an aptameric MB-based probe for direct determination of rHuEPO- $\alpha$  in homogeneous physiological buffer for the first time. The structural switchable “Signal-on” strategy demonstrated here using 807–35 nt aptamer as a proof of principle, the effective signal transduction module showed a potential practical utility of our new selected ssDNA aptamer for rHuEPO- $\alpha$  detection with a LOD of 0.4 nM. Improved sensitivity can be achieved when coupling preconcentration procedures and signal amplification strategies in the further work. Aptamer 807–35 nt was also expected to be a powerful biosensor probe or a potential clinic diagnosis element to facilitate new methodological developments for rHuEPO- $\alpha$ , including *in vitro* diagnostics, or high-throughput screening of abused samples in doping control area.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2009.08.028.

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