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Terminal protection of small molecule-linked ssDNA for label-free and sensitive fluorescent detection of folate receptor

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ABSTRACT

In this work, based on terminal protection of folate-linked ssDNA (FA-ssDNA) and the SYBR Gold fluorescent dye, we describe the development of a label-free fluorescent strategy for the detection of folate receptors (FRs). The binding between the target FR and the FA moiety of the FA-ssDNA protects the FR bound FA-ssDNA from digesting by Exo I. The binding of SYBR Gold to the terminal protected, undigested FA-ssDNA leads to enhanced fluorescent emission for the monitoring of FR with a detection limit of 30 pM. Besides, the developed method also shows high selectivity toward FR against other control proteins. Moreover, our approach avoids the labeling of the probes with fluorescent tags and achieves label-free detection of FR. With these advantages, the proposed method thus holds promising potential for the development of simple and convenient strategies for the detection of other proteins by using different small molecule receptor/protein ligand pairs.

1. Introduction

Folate receptors (FRs) are cysteine-rich, glycosyl-phosphatidylinositol-linked membrane glycoproteins that bind folate (FA) with high affinity to mediate cellular uptake of FA [1]. Although expressed at very low levels in most tissues, it was found that FRs were overexpressed at relatively high levels in various kinds of human tumors to meet the demand of folate for cell dividing [2–4]. Studies have confirmed that overexpression of FRs are related to myelogenous leukemias, ovarian, lung, kidney, brain, endometrial and breast tumors [5–8]. On the other hand, FRs can bind FA or FA-linked drugs with a high binding affinity at 1:1 stoichiometry [9], and can enter the inner of the cell via a receptor-mediated endocytosis for efficient delivery of chemotherapeutic agents, drug carriers, photosensitizers and diagnostic reporters [10,11]. Because of these two important biological functions of FRs as tumor biomarkers and therapeutic targets, it is therefore critical to monitor FRs for the diagnosis and treatment of cancers and chronic inflammatory diseases.

Conventional methods for FR monitoring primarily rely on radioimmunoassays, which involve the interactions between FR and the corresponding antibodies [12,13]. However, these methods are time-consuming and involve the use of hazardous radioactive labels. Alternatively, by coupling the specific and highly selective interaction between FR and FA with electrochemical [14–16], diffractometric [17] and quartz crystal microbalance [18] signal transduction means, several other strategies for the detection of FR have been demonstrated in recent years. Very recently, a new strategy, called the terminal protection of small molecule-linked ssDNA, has been proposed by the Jiang group for monitoring the interactions between small molecules and proteins [19–21]. The small molecule-linked ssDNA, a very useful tool for highly sensitive in vitro selection of small molecules with protein binding affinity and specificity [22,23], has been employed as effective probes for the detection of different kinds of proteins. The detection mechanism is based on the fact that the association of the target proteins with the small molecules can protect the ssDNA from digesting by exonuclease due to the steric hinderance of the bound proteins. Owing to the ease modification and amplification of the ssDNA, this terminal protection strategy has advanced the design of sensitive sensing methods for proteins, including FRs. The reported terminal protection assay methods for FR commonly involve the labeling of the ssDNA with fluorescent tags [24,25] or the immobilization of the probes on the electrode sensing surface [26,27], which potentially increases the complexity and cost of the detection methods. Therefore, the development of sensitive and label-free terminal protection strategies will significantly facilitate

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the monitoring of FR and other proteins. Recently, two label-free fluorescent terminal protection assays based on the formation of G-quadruplex/quinaidine red complexes have been reported for the detection of FR [28] and H_5N_1 antibody [29], respectively. However, these approaches are limited by the structural selectivity of G-quadruplex to quinaidine red and the stability of the G-quadruplex/quinaidine red complexes.

Based on the use of a universal fluorescent dye, SYBR Gold (SG), as the signal indicator, we report herein a label-free and sensitive fluorescent terminal protection approach for the detection of FR. SG is one of the most commonly used commercial fluorescent nucleic acid gel stain dyes for nucleic acid detection in gels [30]. SG exhibits significant fluorescence enhancement when bound to nucleic acids other than mononucleotides, which makes it potentially useful fluorescent signal indicator in small molecule-linked ssDNA assay of proteins. The association of FR with folate-linked ssDNA (FA-ssDNA) can protect the ssDNA from digesting by exonuclease into mononucleotides and the binding of SG to the protected ssDNA can thus emit intense fluorescence to achieve label-free and sensitive detection of FR.

2. Experimental

2.1. Materials and reagents

FR was purchased from Beijing Biosynthesis Biotechnology CO., Ltd. (Beijing, China). Thrombin (Th), mouse immunoglobulin G (IgG), carcinoembryonic antigen (CEA) and bovine serum albumin (BSA), FA, Tris–HCl, *N*-(3-dimethylamminopropyl)-*N*′-ethylcarbodiimidhydrochloride (EDC), *N*-hydroxysulfosuccini-mid sodium salt (NHS) were bought from Sigma (St. Louis, MO). SG (10,000 × concentration) was obtained from Invitrogen (Beijing, China). SG was firstly diluted to 500 × concentration with dimethyl sulfoxide (DMSO) to make a stock solution and was freshly diluted to a suitable concentration (50 × concentration) with buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) before usage. Exonuclease I (Exo I) and 3′-NH_2 modified oligonucleotide (5′-AGTCCGTGGTAGGG-CAGGTTGGGGTGACT-NH_2-3′) were supplied by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). Other reagents were ordered from Kelong Chemical Inc. (Chengdu, China). All reagents were of analytical grade and used without further purification, and ultrapure water (specific resistance of 18.3 MΩ cm) was used throughout the whole process.

2.2. Preparation of FA-ssDNA

FA was conjugated to the 3′-NH_2 terminus of the ssDNA using the succinimide coupling method [19]. Briefly, 0.5 mL of 20 μM ssDNA was incubated with 0.5 mL of 10 mM phosphate buffer solution (PBS, pH 7.4) containing 10 mM FA, 1 mM EDC and 5 mM NHS at 37 °C for 2 h in the dark. Then, the mixture was centrifuged to remove excessive, unreacted FA by using the Illustra MicroSpin G-25 Columns (GE Healthcare) to collect the FA-linked ssDNA probes.

2.3. Terminal protection, fluorescent detection of FR

Various concentrations of FR were incubated with the FA-ssDNA probes in the reaction buffer (50 μL, 20 mM Tris–HCl, 3 mM $MgCl_2$, 50 mM NaCl, pH 7.5) at 37 °C for 1 h in dark to form the FR/FA-ssDNA complexes. Next, Exo I (7 U) was added to the mixture and the solution was further incubated for 25 min at 37 °C in dark to hydrolyze the unprotected FA-ssDNA. This was followed by the addition of 20 mM EDTA to terminate the hydrolysis reaction. Finally, SG (3 μL) was added to the mixture to incubate for 1 min at room temperature. Fluorescence measurements were performed on a RF-5301PC fluorescence spectrophotometer (Shimadzu, Tokyo, Japan) by diluting the solution to a final volume of 200 μL with reaction buffer. A 150 W xenon lamp was used as the excitation source. The excitation wavelength was set at 494 nm, and the emission wavelengths were collected in the range from 500 nm to 600 nm with excitation and emission slits of 10 nm and 5 nm, respectively.

2.4. Native polyacrylamide gel electrophoresis

The sample solutions were transferred to the electrophoretic system with 16% native polyacrylamide gel, which was freshly prepared at laboratory. The electrophoresis was carried out in 1 × TBE at a constant voltage of 100 V for 90 min and the gel was stained with ethidium bromide for 10 min, followed by photographing with a digital camera under UV irradiation.

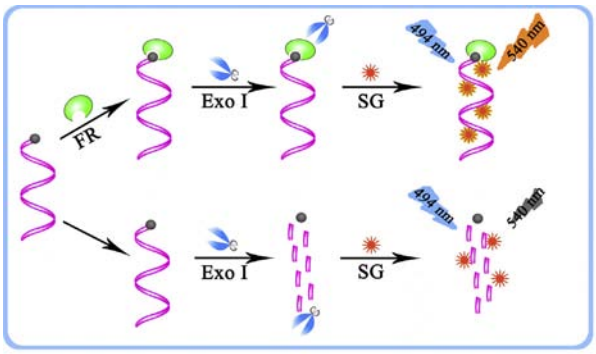
3. Results and discussion

3.1. Principle for fluorescent detection of FR

Scheme 1 illustrates our label-free, fluorescent approach for FR detection based on SG indicator and terminal protection of FA-ssDNA against Exo I digestion. In the sensing design, the small molecule receptor, FA, is first covalently conjugated to the 3′-NH_2 modified ssDNA by using the succinimide reaction (in the presence of EDC and NHS). In the absence of FR, the FA-ssDNA can be successively hydrolyzed into mononucleotides from the 3′ terminus of the ssDNA by Exo I. Upon the introduction of SG to the solution, a very weak fluorescent signal is expected because SG cannot bind the digested mononucleotides. On the contrary, when the target protein, FR, is introduced into the probe solution, it associates with the FA moiety of the FA-ssDNA and prevents Exo I from approaching and cleaving the ssDNA in the 3′ to 5′ direction because of the significant steric hindrance rendered from the protein-ligand interaction [19]. Subsequently, SG binds the terminal protected FA-ssDNA and generates enhanced fluorescent emission. Therefore, with the presence of increasing amount of FR, more ssDNA can be protected from digestion by Exo I, and stronger fluorescent signals can be expected for the monitoring of FR

3.2. Assay validation

For proof-of-concept demonstration of our proposed approach for FR detection, the fluorescence intensities of the FA-ssDNA probe solutions in the absence/presence of the target FR with/without the addition of Exo I were first monitored. As displayed in Fig. 1A, the FA-ssDNA probe solution (20 nM) shows intense



Scheme 1. Principle for terminal protection of FA-ssDNA for label-free and sensitive fluorescent detection of FR using the SG dye.

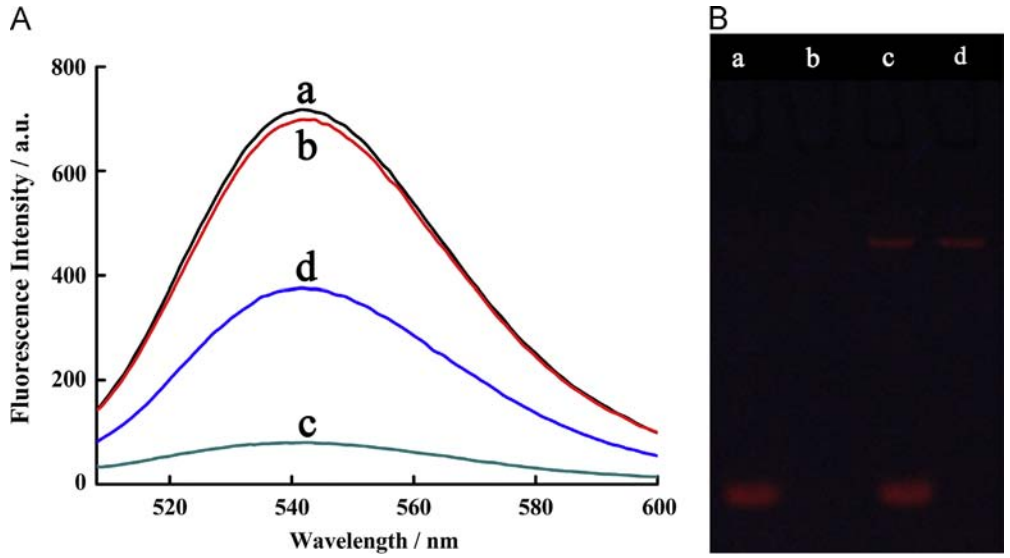


Fig. 1. (A) Typical fluorescence emission spectra of different solutions: (*a*) FA-ssDNA (20 nM) and SG (5 μL), (*b*) FA-ssDNA (20 nM), FR (10 nM) and SG (5 μL), (*c*) FA-ssDNA (20 nM), Exo I (10 U) and SG (5 μL) and (*d*) FA-ssDNA (20 nM), FR (10 nM), Exo I (10 U) and SG (5 μL). (B) Native polyacrylamide gel electrophoresis image for (a) FA-ssDNA (5 μM), (b) FA-ssDNA (5 μM) and Exo I (50 U), (c) FA-ssDNA (5 μM) and FR (1 μM) and (d) FA-ssDNA (5 μM), FR (1 μM) and Exo I (50 U).

fluorescence intensity (curve *a*) upon the addition of SG (5 μL) and the presence of FR (10 nM) exhibits minimal effect on the fluorescence intensity (curve *b*) due to the effective binding between SG and the FA-ssDNA. However, when Exo I (10 U) is added to the FA-ssDNA probe solution without the presence of FR, significant decrease in fluorescence intensity is observed (curve *c* vs. *a* and *b*). Such decrease is basically due to the generation of mononucleotides by Exo I-catalyzed digestion of FA-ssDNA and the low fluorescent background signal of SG. When the FA-ssDNA probe solution is incubated with FR followed by the addition of Exo I, increase in fluorescence intensity is observed (curve *d*). This fluorescence increase phenomenon can be ascribed to the association of FR with the FA moiety of the FA-ssDNA and terminal protection of FA-ssDNA from digestion by Exo I as discussed previously. Besides, native polyacrylamide gel electrophoresis was performed to further verify our terminal protection assay strategy for FR. According to the gel electrophoresis image shown in Fig. 1B, an apparent band is observed for FA-ssDNA (5 μM, lane a) while the addition of Exo I (50 U) leads to the disappearance of this band (lane b) due to the effective digestion of the FA-ssDNA by Exo I. In contrast, after the incubation of the FA-ssDNA with the target FR (1 μM) in the absence of Exo I, two bands with different migration shifts are observed. The decreased migration shift results from the FA-ssDNA bound to FR (lane c). When Exo I is added after the incubation of FA-ssDNA with FR, we only detect the band with decreased migration shift (lane d), indicating the digestion of the free FA-ssDNA by Exo I and the protection of the FR/FA-ssDNA from digestion by Exo I. These results reveal that our label-free, terminal protection strategy can be used to monitor FR.

3.3. Optimization of the assay conditions

To achieve optimal conditions for fluorescent detection of FR, several factors, including the volume of SG, the amount of Exo I and the enzymatic digestion time, were examined. Various volumes of SG (50 ×) were first added to the FA-ssDNA probe solution (20 nM) and the fluorescence intensities were monitored (Note: the binding between SG and FA-ssDNA is quite fast at room temperature, and it can reach equilibrium in 1 min). As can be seen from Fig. 2A, the fluorescence intensity of the mixture increases with increasing volume of SG from 1 μL to 3 μL and reaches a plateau from 3 μL to 7 μL, indicating that 3 μL of SG is enough to bind the FA-ssDNA probes to generate strong fluorescence emission. The amount of Exo I was optimized by incubating the FA-ssDNA with Exo I from 1 U to 10 U at 37 °C for 30 min followed by the addition of 3 μL of SG (50 ×). As shown in Fig. 2B, the fluorescence intensity progressively decreases with increasing amount of Exo I until 7 U and then levels off thereafter. Therefore, 7 U of Exo I was selected for subsequent experiments. In order to obtain the optimized enzymatic digestion time for Exo I, Exo I (7 U) was mixed with the FA-ssDNA probe solution (20 nM) and 3 μL of SG (50 ×), and the fluorescence intensity was measured at a time interval of 5 min from 5 min to 30 min. According to Fig. 2C, the fluorescence intensity of the mixture decreases in the range from 5 min to 25 min and remains almost unchanged thereafter. Thus, 25 min was selected as the optimal digestion time for Exo I.

3.4. Sensitivity and selectivity of the proposed method for the detection of FR

Quantitative detection of FR by using the proposed terminal protection and label-free method was investigated. The FA-ssDNA probes were first incubated with different concentrations of FR from 0 nM to 10 nM for 1 h at 37 °C in dark. Then, 7 U of Exo I was added and incubated for another 25 min. The mixtures were subjected to fluorescence measurements after the addition of 3 μL of SG. Fig. 3A displays the typical fluorescence spectra of the assay method for various concentrations of FR. It can be seen that the fluorescence intensity gradually increases with increasing concentration of FR from 0 nM to 10 nM (curve *a* to *h*) and the fluorescence intensity at 540 nm exhibits a linear correlation to the concentration of FR in the range of 0.1–10 nM with a coefficient of correlation of 0.992 (Fig. 3B). Based on the 3σ rule, the detection limit of our approach for FR is calculated to be 30 pM. Six repetitive measurements of FR at the concentration of 10 nM yielded a relative standard deviation of 7.1%, which indicates our method is coupled with good reproducibility.

To evaluate the selectivity of our terminal protection strategy for label-free fluorescent FR detection, other control proteins, including Th, IgG, CEA and BSA, were tested by using the developed method. As shown in Fig. 4, the presences of the control proteins (100 nM) at 10-fold excess concentrations of the target have insignificant effects on the fluorescence intensities of the probe solutions compared with that of the blank test (with the

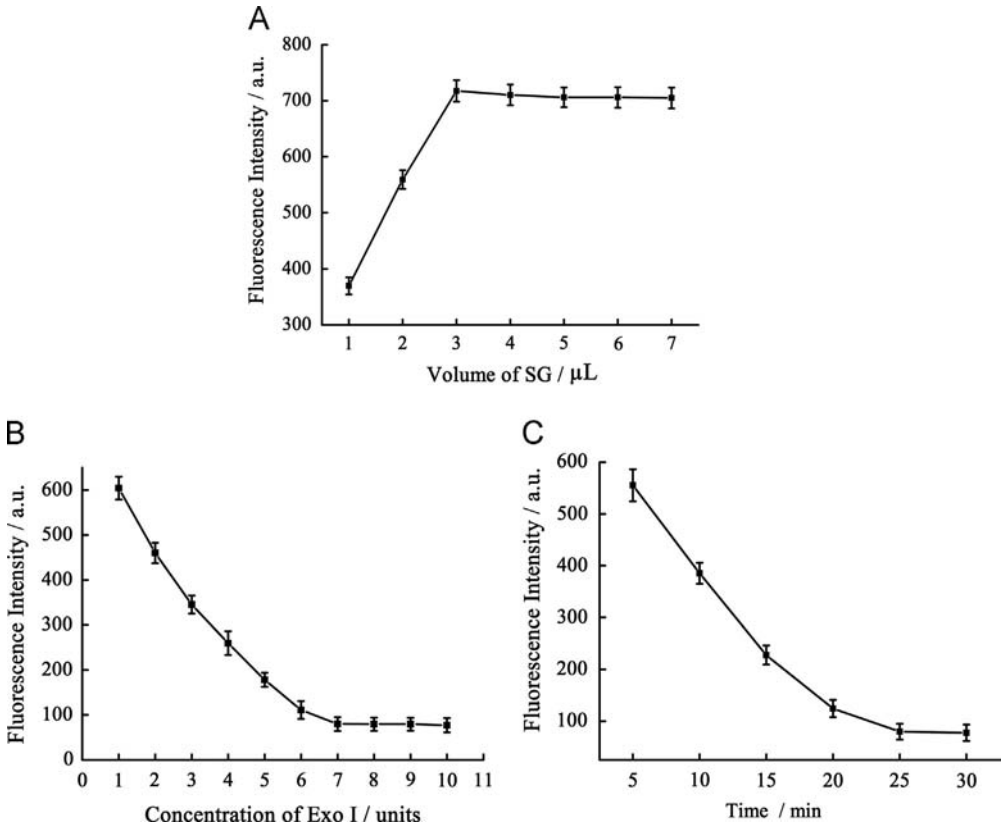


Fig. 2. Effect of the (A) volume of SG (50 ×), (B) amount of Exo I and (C) enzymatic digestion time on the fluorescence intensity of the FA-ssDNA probe solution.

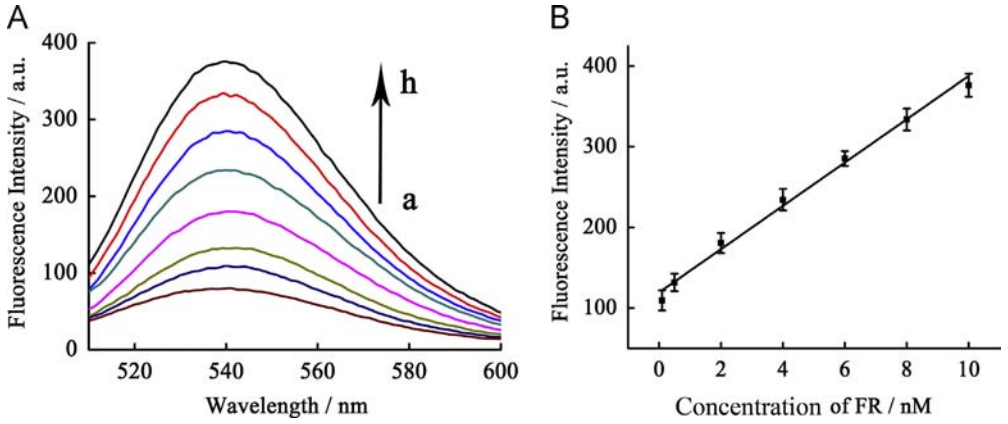


Fig. 3. (A) Typical fluorescence emission spectra of the FA-ssDNA probe solution after the incubation with various concentrations of FR, followed by further incubation with 7 U of Exo I and the addition of 3 μL of SG. The concentration of FR (from (*a*) to (*h*)): 0 nM, 0.1 nM, 0.5 nM, 2 nM, 4 nM, 6 nM, 8 nM, 10 nM. (B) The corresponding calibration plot of the concentration of FR (form 0.1 nM to 10 nM) vs. the fluorescence intensity. Error bars, SD, $n=3$.

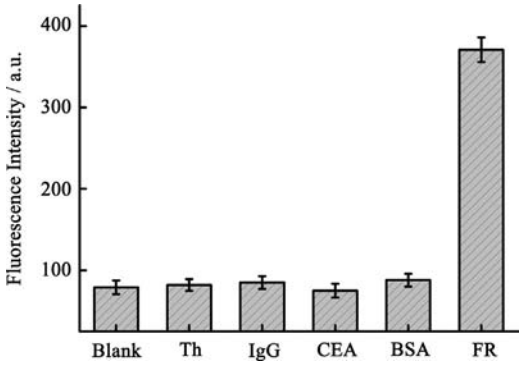


Fig. 4. Selectivity investigation of the proposed method for FR (10 nM) against other control proteins: Th, IgG, CEA and BSA (100 nM).

absence of FR). However, the presence of even 10-fold less concentration of the target FR (10 nM) leads to substantial increase in the fluorescence intensity. This comparison clearly demonstrates that the proposed method has high selectivity toward FR against other control proteins, which is related to the highly specific binding capability between FA and FR.

3.5. Detection of FR in serum samples

To evaluate the potential application of the proposed method for real samples, various concentrations of FR in diluted human serums (obtained from the 9th People's Hospital of Chongqing, China and diluted 10 times by reaction buffer) were monitored. As listed in Table 1, the recoveries for FR by using the developed

Table 1
Recovery tests for the detection of FR in diluted serum samples ($n=3$).

Samples	FR added (nM)	Found (nM)	Recovery (%)
1	0.5	0.48	96.0
2	6.0	5.65	94.2
3	10.0	9.85	98.5

approach are in the range of 94.2–98.5%, indicating the applicability of the sensing strategy for FR in serum samples.

4. Conclusion

In summary, we have demonstrated a label-free fluorescent strategy for the detection of FR based on the terminal protection of FA-ssDNA and the SG fluorescent indicator. The association of FR with the FA moiety of the FA-ssDNA prevents the FA-ssDNA probes from digesting by Exo I. Quantitative signal generation relies on the enhanced fluorescent emission of SG upon binding to the protected FA-ssDNA and a detection limit of 30 pM can be achieved in a label-free format. Besides, the developed method is highly selective for the target FR. With the successful demonstration of the developed method for label-free detection of FR, this terminal protection, label-free fluorescent approach can be readily extended for the detection of other proteins (e.g., streptavidin and H_5N_1 antibody) by replacing the FA moiety of the FA-ssDNA to the corresponding receptors (e.g., biotin and the H_5N_1 M2 fragment of peptide), making our approach a useful addition to the arena of protein detection.

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