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Sensitive fluorescence biosensor for folate receptor based on terminal protection of small-molecule-linked DNA†

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Based on the specific folic acid–folate receptor (FA–FR) interaction, macromolecular FR can bind with FA-linked DNA–small molecule chimeras, which can prevent enzymolysis by exonuclease III (Exo III), enabling a novel fluorescence biosensor for FR to be developed using quinaldine red as a G-quadruplex-binding probe.

Folate receptor (FR), commonly known as a magnetic tumor-selective target, is expressed at high frequency in tremendous kinds of malignant tumors, such as ovarian, breast, brain, lung, colorectal, endometrial, renal carcinomas and so on.[1–3] Peculiarly, the epithelial carcinoma of an ovarian tumor is deemed to have a higher FR expression level, which elevates to about 90%.[4] On the contrary, scarcely any FR exists in the vast majority of normal tissues and organs.[3] In addition, FR can combine cohesively with the vitamin folic acid and its conjugates, thus entering the inner of the cell *via* a receptor-mediated endocytosis.[5] In recent years, referring to imaging and therapy direction, many reports focus on the potential exploitation of FR-binding-folic acid (FA)-targeted therapy, for instance, tumor-specific drug delivery, radio-imaging of therapeutic agents,[6] fluorescence imaging of cancer cells,[7] magnetic resonance imaging (MRI) contrast agents[8] and gadolinium liposomes[9] *etc*.

Undoubtedly, FR-binding-FA-targeted therapy is extensively applied for the treatment and diagnosis of the above disorders, but the detection of FR as the tumor biomarker is also important since this can be applied to evaluate the evolution of malignancy. Certain thought is that there may exist the overexpressed FR of rhinitis cancer (KB) cells against the FA chimaeras (FA-targeted drugs or agents), and that diagnosis can be performed through monitoring the consistency of FR in the cancer pathogenesis at different stages when the FR biosensor is used for the clinical therapy.

At present, the acquired methods for the detection of small molecules (*e.g.* FA) or their protein receptor (*e.g.* FR) are capillary electrophoresis,[10,11] fluorescence resonant energy transfer,[12,13] fluorescence anisotropy,[14] protein-fragment complementation,[15] biosensors[16,17] and surface plasmon resonance (SPR).[16,18] These methods are authenticated to be effective approaches and it is noteworthy that they have some insufficiencies, such as the necessities of the exorbitant apparatus, specific labeling and surface-based molecular immobilization. Thus, to develop novel strategies to overcome the above limitations is important. Wu[19] recently developed a sensitive biosensor for electrochemical detection of FA small molecule–FR protein interactions in consideration of the so-called terminal protection of single strand DNA.

In this study, based on the specific FA–FR interaction that can prevent enzymolysis by exonuclease III (Exo III), a convenient fluorescence biosensor for FR is proposed. A 55-mer oligonucleotide (sequence is shown in Fig. 1A) with the $-NH_2$ label at the 3′-end was hybridized at 37 °C in metal saline solution for 2 h to form a stem–loop secondary DNA structure, which contains the 5′-end G-rich region as the source of the G-quadruplex. Then, the 3′-end of the hairpin DNA combined with a FA molecule *via* a conjugate link. Quinaldine red (QR, the structure is shown in Fig. S1 in the

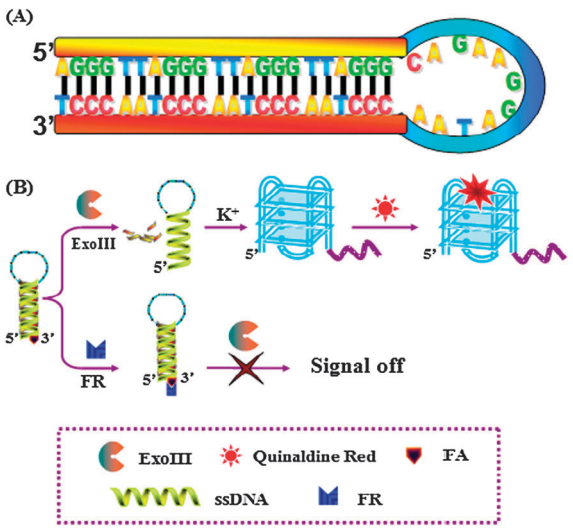

Fig. 1 (A) The sequence of the 55-mer oligonucleotide. (B) A schematic representation of the proposed fluorescence biosensor for folate receptor based on terminal protection of small-molecule-linked DNA.

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supporting information†) itself only gives off a weak fluorescence intensity, but the presence of G-quadruplex can enhance the fluorescence intensity greatly. In the presence of FR, macromolecular FR can bind with FA in DNA–small molecule chimeras, which prevent the conjugated DNA from degradation by the 3′-end double-strand-specific Exo III. Sequentially, scarce G-rich DNA was left. So few G-quadruplexes formed and a weak fluorescence signal was recorded. Conversely, in the absence of FR, Exo III hydrolyzed DNA specifically from 3′-end (red in Fig. 1A) in the spiral-stem area and the reaction was suspended at the loop area (blue in Fig. 1A), since Exo III is incapable of digesting single-stranded DNA. The G-rich sequence (yellow in Fig. 1A) is released and folds into a stable G-quadruplex in the presence of potassium ions. Therefore, an intense fluorescence signal was recorded due to the special response between QR and the G-quadruplex secondary DNA structure. Based on this, a fluorescent biosensor for FR can be developed.

A simple experiment was done to verify our proposal. Fig. 2A shows the fluorescence spectra in different conditions. If the solution contained only QR, a weak fluorescence signal was detected (line e). If the DNA was not linked with FA, a strong fluorescence was detected, no matter whether there was FR or not (lines b and c). The reason lies in that DNA had been degraded and that the G-quadruplex structure formed, which binds with QR to give off strong fluorescence. In the solution that contained FA-linked DNA and QR (line a), the fluorescence is nearly the same as that of lines b and c. But if FR had been added into the above mentioned solution, the fluorescence intensity decreased greatly (line d); this indicated that the FR had reacted with FA and the DNA degradation had been prevented, and no G-quadruplex formed.

One part of the initial G_{55} single-stranded DNA is the guanine-rich oligonucleotides (5′-end yellow bar in Fig. 1A), which may form G-quadruplex and bind with QR and causing interference to the fluorescence detection (see Fig. S2 in the supporting information†). If the structure shown in Fig. 1A forms, no G-quadruplex can be formed and no interference present. It has been reported that the presence of metal saline solution (Mg^{2+}) can accelerate the DNA hybridization.[19] So the effect of Mg^{2+} was studied. The results are shown in Fig. 2B. We firstly attempted to hybridize the G_{55} oligonucleotides

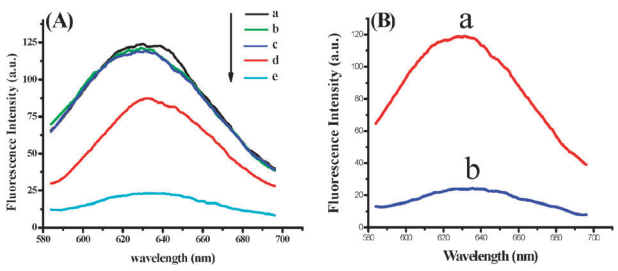

Fig. 2 (A) Fluorescence spectra at different conditions: (a) the solution contained FA labeled DNA and QR; (b) the solution contained DNA and QR; (c) the solution contained DNA, FR and QR; (d) the solution contained FA-labeled DNA, FR and QR; (e) the solution contained only QR. $[G_{55}] = 3.6 \times 10^{-7}$ M; $[Mg^{2+}] = 100$ mM; $[FA] = 3.6 \times 10^{-7}$ M; $[FR] = 1.0 \times 10^{-7}$ M; $[QR] = 10^{-5}$ M; $[K^+] = 100$ mM; [Exo III] = 500 U mL^{-1}. (B) The effect of 100 mM Mg^{2+} on the fluorescence intensity: (a) without 100 mM Mg^{2+}; (b) with 100 mM Mg^{2+}; $[G_{55}] = 3.6 \times 10^{-7}$ M; $[QR] = 10^{-5}$ M; $[K^+] = 100$ mM.

without magnesium ions at 37 °C in the dark for 2 h. A strong fluorescence signal was detected, which indicates that the hybridization is incomplete and some G_{55} single-stranded DNA present and the G-quadruplex formed, which binds with QR to give off a fluorescence signal. After adding the metal salt, a weak fluorescence signal was detected (see Fig. 2B) and the fluorescence was nearly the same as that from QR (line e in Fig. 2A). This shows that the DNA hybridization is complete and there has little residual single-stranded DNA. The effect of Mg^{2+} concentration was also studied. The results showed that the fluorescence intensities decrease with the increase of Mg^{2+} concentration and would not change after 100 mM. So in this study, the optimal signal was reached in the presence of 100 mM Mg^{2+}.

The effect of the concentration of Exo III and digestion time on the fluorescence intensity was studied. As shown in Fig. 3A, the fluorescence intensity increased with the increasing of Exo III concentration from 0 to 5000 U mL^{-1} and then reached a constant. We detected the fluorescence signals at different digestion times in the presence of 500 U mL^{-1} Exo III. The fluorescence intension increased progressively until 3 h and then reached a plateau (Fig. 3B). Hence, 500 U mL^{-1} of Exo III and 3 h digestion time were chosen as the optimized conditions.

Fig. 4A depicts the typical fluorescence responses at different FR concentrations. The fluorescence signals decreased gradually with increasing FR concentration. There is a linear relationship between the fluorescence intensities and the logarithm of FR concentrations in the range 10^{-9}–10^{-7} mol L^{-1}, with a detection limit of 3.0×10^{-10} mol L^{-1} ($S/N = 3$) (shown in Fig. 4B).

In addition, to verify the repeatability of the proposed method, the enzymolysis at the same FR concentration (1.0×10^{-8} mol L^{-1}) was detected five times. The relative standard deviation (RSD) of the fluorescence signals is 5.02%, which shows that the developed approach has high reproducibility. Some other proteins were used to replace FR to test the selectivity of the biosensor and the results showed that they could not interact with FA-linked DNA and prevent the digestion from Exo III, so the fluorescence did not change. The reason lies in that FR and the FA-linked DNA are specifically conjugated in this reaction. Hence, the proposed biosensor has remarkable selectivity.

In summary, through the digestion of unprotected DNA strands by 3′–5′ Exo III, a strong fluorescence signal had been detected since QR can bind with G-quadruplex DNA to give off high fluorescence signal. The digestion can be prevented if we modified the DNA with macromolecular FR at 3′-end and the

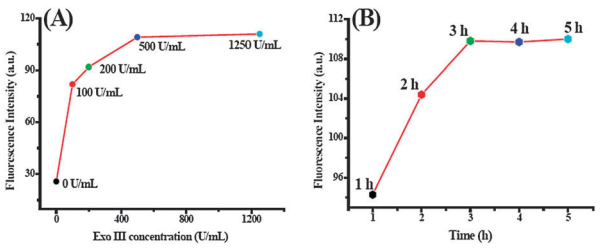

Fig. 3 (A) Fluorescence intensity at different Exo III concentrations, incubated for 4 h at 37 °C in dark. (B) Fluorescence intensity at different enzymolysis time, incubated at 37 °C in dark in 500 U mL^{-1} Exo III; $[G_{55}] = 3.6 \times 10^{-7}$ M; $[Mg^{2+}] = 100$ mM; $[FA] = 3.6 \times 10^{-7}$ M; $[QR] = 10^{-5}$ M; $[K^+] = 100$ mM.

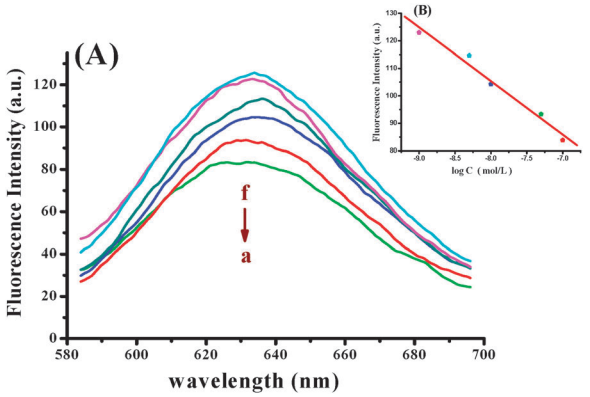

Fig. 4 (A) Fluorescence spectra after incubation with varying concentrations of FR: (a) 1.0×10^{-7}; (b) 5.0×10^{-8}; (c) 1.0×10^{-8}; (d) 5.0×10^{-9}; (e) 1.0×10^{-9} mol L^{-1}; (f) in the absence of FR) followed by digestion of 500 U mL^{-1} Exo III. (B) A plot of fluorescence intensities *versus* the FR concentrations. $[G_{55}] = 3.6 \times 10^{-7}$ M; $[Mg^{2+}] = 100$ mM; [FA] = 3.6×10^{-7} M; [QR] = 10^{-5} M; $[K^{+}] = 100$ mM.

fluorescence signal will decrease. Based on this, a selective, oligonucleotide-based fluorescence biosensor for folate receptor had been developed. The proposed method provides a satisfactory strategy for small molecule–protein interaction investigations.

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