Electronic Supplementary Information

Detection of cancer cells using triplex DNA molecular beacons based on expression of enhanced green fluorescent protein (eGFP)

Dongmei Xi,[a] Xindong Wang,[b] Shiyun Ai[b] and Shusheng Zhang[a *]

[a] *Shandong Province Key Laboratory of Detection Technology for Tumor Makers, College of Chemistry and Chemical Engineering, Linyi University, Linyi 276005, P. R. China.*

[b] *College of Chemistry and Material Science, Shandong Agricultural University, Taian 271018, P. R. China.*

* Corresponding author. Fax: +86-539-8766867; Tel: +86-539-8766867

E-mail: shushzhang@126.com

EXPERIMENTAL SECTION

Apparatus and Materials

Apparatus: Measurement of Fluorescence spectra was performed on F-4600 fluorescence spectrophotometer (HITACHI, Japan). Ultraviolet spectra were measured on a Cary 60 UV-Vis spectrometer (Agilent Technologies, USA). Circular plasmid DNA was obtained by UNlQ-500 Column Plasmid Maxi-Preps Kit (Sangon Biotech Co., Ltd, China). Enhanced green fluorescent protein (eGFP) was expressed in S30 T7 High-Yield Protein Expression System (Promega, USA). Internal reflection fluorescence images were recorded with a MultiColor Total Internal Reflection Fluorescence (TIRF) Leica AM TIRF MC.

Reagents: Magnetic Fe_3O_4-Au core-shell nanoparticles (Fe_3O_4@Au) were obtained from Xi'an GoldMag Nanobiotech Co. Ltd. Coralyne chloride (CORA) was purchased from Sigma. Tri (2-carboxyethyl) phosphine hydrochloride (TCEP, 98%) was purchased from Alfa Aesar (Massachusetts). 10X Reaction Buffer for Klenow Fragment was obtained from Thermo Scientific (USA). All of oligonucleotides were custom-ordered from Sangon Biotech Co., Ltd. (Shanghai, China), and their sequences are listed in Table S1. Plasmid pEHISEGFPTEV was kindly provided by Huanting Liu (the University of St Andrews). Phosphate buffered saline (PBS, 0.1 M, pH 7.0) was prepared by mixing the stock solutions of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 and adjusting the pH with 0.1 M H_3PO_4 or 0.1 M NaOH. Unless otherwise mentioned, ultrapure water was used throughout the experiments. All the chemicals employed were of analytical reagent grade and were used without further purification.

Table S1. DNA sequence used in this work.

Oligonucleotide name	Sequences
DNA1	5’-AGCTTTCTTTTTTCTTCTTAACTCGTGTTCTCTTTTTTCTTCTTAACTCGG-3’
DNA2	5’-GATCCCGAGTTAAGAAGAAAAAAGAGAACACGAGTTAAGAAG AAAAAAGAA-3’
DNA3-CRO	5’- CGGTGTTCTGTATTCTTCTTTTTTCT-3’
DNA4	5’-TACAGAACACCGGGAGGATAGTTCGGTGGCTGTTCAGGGTCTCCTCCCGGTG-SH-3’

Construction of plasmid pEHISEGFPTEV-M

To construct plasmid pEHISEGFPTEV-M, the plasmid pEHISEGFPTEV was digested with BamHI/HindIII and the digested plasmid was isolated using gel extraction kid. Two DNA strands, DNA 1 and DNA2, were synthesized and hybridized to form double-stranded DNA. The duplex DNA was then ligated into the BamHI/HindIII-digested pEHISEGFPTEV to form pEHISEGFPTEV-M. The plasmid was sequenced to confirm its integrity.

Cancer Cell Culture

Ramos cells (target cells) and K562 cells (control cells) were cultured in cell flasks separately according to the instructions from the American Type Culture Collection. The cell line was grown to confluence in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 IU mL^{-1} of penicillin-streptomycin. The cells were maintained at 37 °C in a humidified atmosphere (95% air and 5% CO_2). The cancer cell densities were determined using a hemocytometer, and this was performed prior to each experiment. Then, a 1.0 mL suspension of 10^6 cells dispersed in RPMI 1640 cell media buffer was centrifuged at 1500 rpm for 10 min and washed with phosphate-buffered saline five times and resuspended in 1.0 mL cell media buffer.

Preparation of DNA4 modified Fe_3O_4@Au and hybridization with DNA3-CRO

The DNA modified Fe_3O_4@Au conjugates were synthesized by adding 50 µL of 10^{-6} M thiol-modified DNA4 to 70 µL of the aqueous Fe_3O_4@Au solution. DNA4 was activated with TCEP (10 mM) for 1 h before being attached to Fe_3O_4@Au. After 12 h, the phosphate concentration was increased from 0 to 10 mM by adding 1.0 M pH 7 PBS. Then, 2 M NaCl was added dropwise to increase the salt concentration to 0.05 M NaCl. The solution was allowed to stand for 8 h. This process was repeated to increase the salt concentration to 0.1, 0.2, and 0.3 M NaCl. The excess DNA4 was removed by magnetic separation. Following removal of the supernatant, the precipitate was washed with 10 mM PBS containing 0.3 M NaCl for three times. Then the precipitate was suspended in the solution of 20 µL of 10X Reaction Buffer for Klenow Fragment, 5 µL of 10^{-5} M DNA3-CRO, and 175 µL double distilled water, followed by heating to 90 °C and cooling to room temperature. Excess reagents were removed by magnetic field, the resulting precipitate was washed with 300 µL of 10 mM PBS containing 0.3 M NaCl, magnetic separated, and then redispersed in 200 µL 10 mM PBS containing 0.3 M NaCl for further use.

Fabrication of triplex DNA

For fabrication of triplex DNA, 400 μL extracted circular plasmid DNA and 160 μL 10^{-5} M CORA was added into the 200 μL DNA modified Fe_3O_4@Au solution. A solution of 0.1 M PBS containing 2 M NaCl was added dropwise to increase the NaCl concentration to 0.3 M. The mixed reaction solution was maintained at 30 ℃, 200 rpm vibration for 2 h. After fabrication, the precipitate was washed three times with and then redispersed in the solution of 10 mM PBS containing 0.3 M NaCl.

Detection of Cancer Cells

20 μL triplex DNA fabricated Fe_3O_4@Au solution was incubated with 150 μ L of PBS containing different numbers of cells at room temperature for 1 h. Then the triplex DNA was released from the Fe_3O_4@Au biocomplex, and separated with a magnetic field. The supernate was purified and concentrated by SanPrep Column PCR Product Purification Kit, and 25 μL DNA products were obtained. A mixed solution of 6 μL DNA, 10 μL S30 Premix Plus, and 9 μL T7 S30 Extract Circular was maintained at 37 °C, 320 rpm vibration for 1 h. S30 T7 High-Yield Protein Expression System simplifies the transcription and translation of eGFP cloned in the plasmid containing a T7 promoter by providing an extract that contains T7 RNA polymerase for transcription and all necessary components for translation, and thus the transcription and translation are performed in one reaction step. Then the product was put into ice-water bath to terminate the reaction. The concentration of the eGFP protein was determined by fluorescence spectrophotometer and TIRF with the excitation wavelength of 488 nm.

Supplementary Results

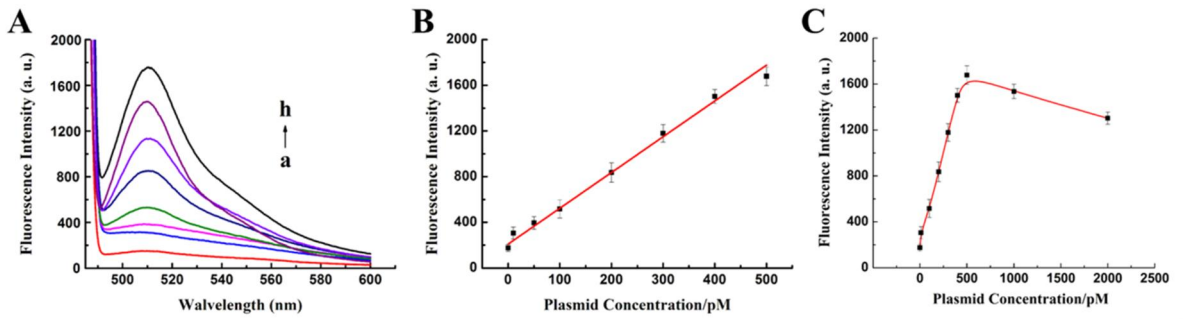

Fig. S1 Calibration curve of the determination of the plasmid pEHISEGFPTEV-M. From a to h: 0, 10, 50, 100, 200, 300, 400, 500 pM. The error bars in B and C are standard deviations of three repetitive measurements.

The plasmid DNA was analyzed quantitatively by determining the eGFP fluorescence intensity (excitation maximum = 488 nm, emission maximum = 509 nm) using Fluorescence spectrophotometer. Under optimum conditions, the dynamic range of the designed method for detection of the plasmid pEHISEGFPTEV-M was examined. As shown in Fig. S1A, the eGFP fluorescence signal was observed to increase with the increase of the concentration of the plasmid DNA, and reached a maximum at 500 pM. A linear dependence between the peak fluorescence and the DNA concentration was obtained in the range of 0 to 500 pM as shown in Fig. S1B. The regression equation could be expressed as y (a. u.) = 3.1204 x (pM) + 210.4015 (R = 0.9963). But introduction of the DNA at higher concentrations (> 500 pM) decreased the intensity (Fig. S1C). This might be attributed to the increase in the incidence of internal translational initiation or the number of prematurely arrested translation products caused by an increased amount of DNA.

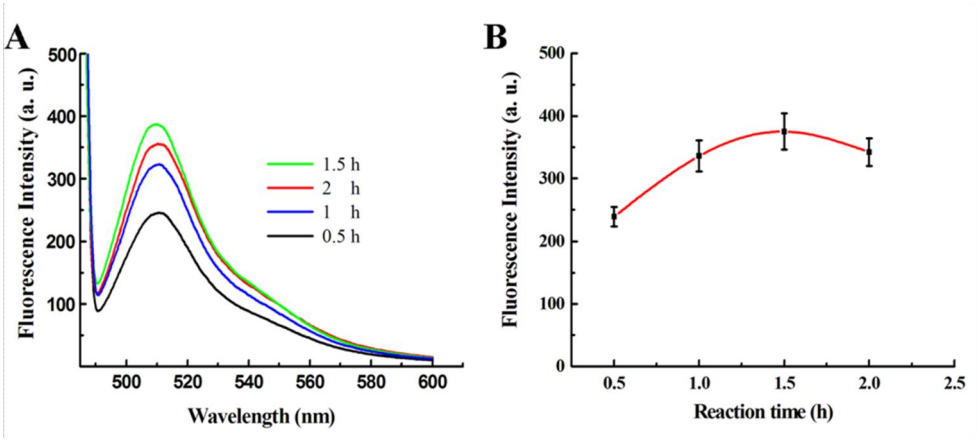

Fig. S2 Time-dependent eGFP production. The concentration of the Ramos cells was 1000 cells mL^{-1}. The time of transcription/translation in the protein synthesis system was 0.5 h, 1 h, 1.5 h and 2 h, respectively. The error bars in B are standard deviations of three repetitive measurements.

Transcription/translation time in the protein synthesis system is an important factor that will influence the eGFP protein levels. Thus, we first investigated the change in the fluorescence signals of the eGFP with reaction time in the presence of 1000 Ramos cells mL^{-1}. As shown in Fig. S2, the fluorescence intensity was observed to increase with the increase in reaction time from 0.5 h to 1.5 h, followed by a decrease after 2 h. The fluorescence intensity after 1 h was enough to meet the sensitivity requirements in the current assay. To meet the demand of fast reaction in analytical work, the reaction time of 1 h was selected for the following experiments.

Table S2. Comparison between the proposed strategy and other reported methods for cancer cell detection.

Cancer Cells	**Detection Method**	**Detection Limit**	**Ref.**
CCRF-CEM	fluorescence imaging	250 cells/mL	S1
Circulating Tumor Cells	fluorescence imaging of cells after being enriched	10 cells/mL	S2
Ramos	aptamer-nanoparticle strip biosensor	800 cells/mL	S3
MCF-7	fluorescence detection	500 cells/mL	S4
CCRF-CEM	fluorescence detection	4000 cells/mL	S5
Ramos	fluorescence imaging	398 cells/mL	[a]

[a] This method

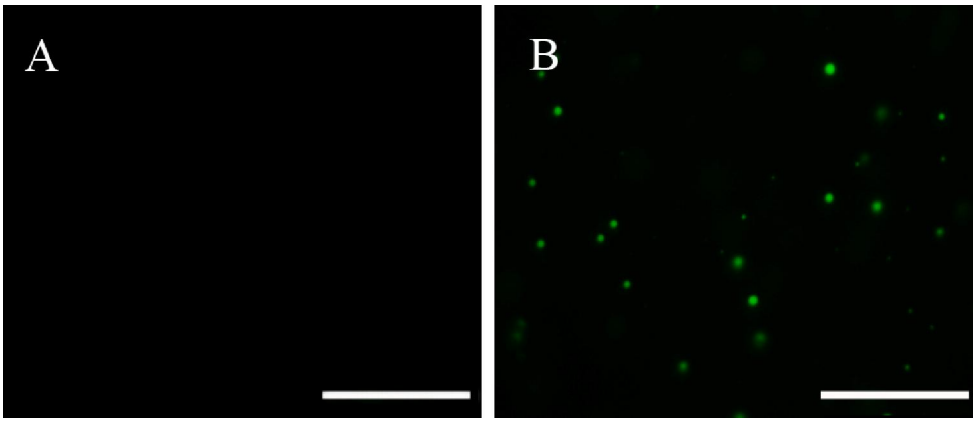

Fig. S3 eGFP fluorescence observations by TIRFM with (A) or without (B) addition of the triplex binder CORA. No target cell was introduced into the detection system.

Under optimum conditions, the insertions within pEHISEGFPTEV-M have the correct sequence to form a triplex with cDNA in scheme 1 through sequence-specific Hoogsteen base paring. The triplex structure is relatively unstable at room temperature and will only form in the presence of triplex DNA binding molecules. Thus, a triplex binder, coralyne (CORA), was used to increase the stability of the triplex.. This is proven by the TIRFM image as shown in Fig. S3. In the presence of CORA, almost no background fluorescent signal was observed without addition of Ramos cells (Fig.S3A). In the absence of CORA, however, the background fluorescence could be visualized (Fig.S3B). This was mainly ascribed to the unwinding of the triplex.

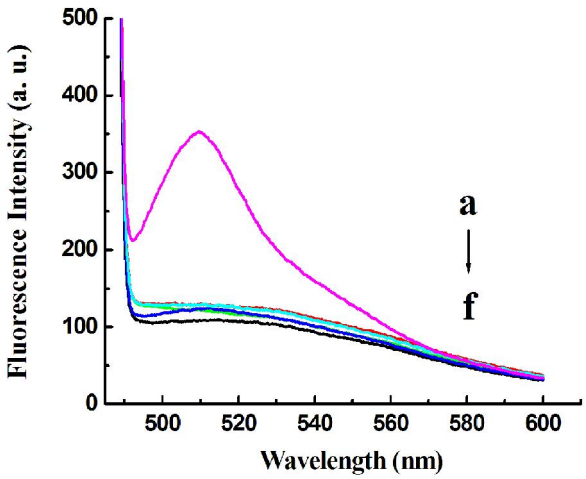

Fig. S4 Specificity of the assay for cancer cell detection.

The specificity of this assay was investigated by detecting Ramos cells and some other nontarget cells, respectively. From a to f: Ramos, K-562, MCF-7, Hela, A549, 0 cells. The

concentration of cells from a to e are 1000 cells mL^{-1}. As shown in Fig. S4, fluorescence signal was only observed when introducing Ramos cells, while no signal was observed in the absence of Ramos cells or in the presence of the other cells, illustrating the specificity of the detection system. It is proposed that the specificity is attributed to the specific recognition between the Ramos cells and aptamers.

Table S3. eGFP fluorescence intensity of Human Serum Samples spiked with Ramos Cells.

Sample	Added Ramos cells (cells/mL)	Detected Ramos cells (cells/mL)	Recovery (%)
1	500	476	95.2
2	5000	4517	90.3
3	50000	45801	91.6

References

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