Electronic Supplementary Information

Protein-responsive rolling circle amplification as tandem template to drive amplified transduction of fluorescence signal probes for highly sensitive immunoassay

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EXPERIMENTAL

Materials and reagents

Human mucin 1 (MUC1) and biotinylated-antibody (bio/Ab) of MUC1 were purchased from North Connaught Biotechnology (Shanghai, China), and Shanghai ZiYu Biotech Co., Ltd. (Shanghai, China), respectively. Biotin (bio), streptavidin (SA), T4 DNA ligase, phi29 DNA polymerase, dNTPs mixture and all DNA oligonucleotides used in this work were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Carcinoembryonic antigen (CEA), alpha fetoprotein (AFP) and thrombin (TB) were ordered from Sigma-Aldrich Chemical Co. (St. Louis, MO). Human serum samples were kindly provided by the Xinqiao Hospital, Army Medical University (Chongqing, China). Other chemicals used in this work were purchased from Kelong Chemical Company (Chengdu, China), all of which were of analytical grade and used without further purification. Deionized water (DI water) (≥18.2 MΩ, Milli-Q, Millipore) was used during the experiment process. Table S1 lists the detailed base sequences of all oligonucleotides used in this work. The bolded letters of the two specific biotinylated-ssDNAs (bio/S1 and bio/S2) are complementary each other. The italicized letters in 3'-terminus of bio/S1 and the underlined letters in 5'-terminus of bio/S2 as the primer sequence of RCA are complementary to those of a linear padlock DNA (PD), respectively. Especially, a recognition strand (RS) containing specific loop sequence complementary to those of RCA tandem repeats is flanked by two arm segments with 7-base pairs (double-underlined letters). The SP

labeled with FRET pair (FAM and TAMRA) in 5'- and 3'-terminus is initially designed as a hairpin-shape structure containing 5-base pair stems (bolded and italicized-letters) and a 10-base loop of $d(AG)_5$ (double-underlined letters) in 3'- and 5'-terminus, facilitating the assembly of THSS via Watson-Crick and Hoogsteen base pairings between the two arm segments of RS and the loop of SP.

Table S1 Base sequence of the oligonucleotides used in this work.

Name	Sequences
bio/S1	5'-biotin-TTTTTTTTTTTTTTTT**GTGAGG***GAACGGTCCTTG*-3'
bio/S2	5'-GATTAGACCGTC**CCTCAC**TTTTTTTTTTTTTTTT-biotin-3'
Padlock (PD)	5'-p-GACGGTCTAATCCAGCCTACCTACCCATCACCCAA*CAAGG ACCGTTC*-3'
RS	5'-CTCTCTCCAGCCTACCTACCCATCACCCAACTCTCTC-3'
SP	5'-FAM-***GAGGA***GAGAGAGAGA***TCCTC***-TAMRA-3'

Apparatus

All fluorescence measurements were carried out with a F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). UV-vis spectrophotometer (Shimadzu, Tokyo, Japan) were utilized. Gel Doc XR+ System(Bio-Rad, California, USA) was used to take images of gels.

Preparation of MUC1 affinity probes and triple-helix stem structure (THSS)

According to the previous method with slight modifications,[1,2] two affinity probes of MUC1 (bio/S1-SA-bio/Ab and bio/S2-SA-bio/Ab) were prepared. Briefly, bio/S1 and bio/S2 with the same concentration of 2 μM were mixed with

SA in 1:1 proportion and incubated for 0.5 h, followed by the addition of bio/Ab in a ratio of 4:1 for 0.5 h.[2] The obtained affinity probes were diluted to 200 nM with 40 mM phosphate buffer solution (PBS, pH 7.0) containing 25 mM KCl, 10 mM $MgCl_2$, 20 mM EDTA, and 1 mM biotin for the next use. The UV-vis spectra of different protein and DNA samples was conducted to investigate the coupling efficiency.

To obtain hairpin structure of SP, 10 μL of SP (1 μM) was initially annealed by heating to 95 ℃ for 5 min and cooling down to room temperature (RT) for 2 h. Next, the obtained hairpin-type SP was mixed with 10 μL RS (1.4 μM) and incubated for 1 h, resulting in the formation of a triple-helix stem structure (THSS).

FRET immunoassay of MUC1

Firstly, 10 μL bio/S1-SA-bio/Ab (200 nM), 10 μL bio/S2-SA-bio/Ab (200 nM) and 10 μL MUC1 with different concentrations were mixed at RT for 1 h, allowing for their proximity ligation through specific antigen-antibody recognition. This brought the complementary hybridization of bio/S1 and bio/S2, reserving two segments of 3'-terminus of S1 and 5'-terminus of S2 as the primer sequence of RCA. After that, 10 μL PD (100 nM), 1 μL T4 ligase (5 U) and 1×T4 ligation buffer were added and incubated for 1.5 h, forming a circular template by intramolecular ligation linking from 5' to 3' end of PD. When introducing 10 μL phi29 polymerase (10 U) and 10 μL dNTPs (0.5 mM) for 2 h, RCA reaction was initiated to generate a continuous, non-nicked ssDNA containing numerous

altered tandem repeats (~1400-15000 bases long).[3,4] Upon the final addition of the as-prepared THSS for keeping 1 h, thousands of RS in THSS is hybridized with the specific tandem repeats, disassembling THSS and releasing of a large amount of SP. The configuration switch of SP from opening into closure hairpin brought FRET pair FAM and TAMRA into spatial proximity, generating significantly amplified signal readout dependent on the concentration of target MUC1.

Fluorescence measurements

Fluorescent intensity of different samples was measured on a Hitachi F-7000 spectrophotometer (Tokyo, Japan) by utilizing excitation and emission slits of 10 nm, and the PMT voltage was set as 950 V. The fluorescence spectra were collected from 500 nm to 620 nm by exciting the samples at 475 nm with a 150 W Xenon lamp (Ushio Inc, Japan) as the excitation source at RT. The fluorescent emissions of FAM and TAMRA were measured at 518 nm and 580 nm, respectively.

Polyacrylamide gel electrophoresis characterization

In the polyacrylamide gel electrophoresis (PAGE) assay, a tested sample containing 10 μL of each oligonucleotide and 2 μL 6×loading buffer was subjected to 16% gel electrophoresis in a Model DYCP-31E electrophoretic device (Beijing, Wo De Life Sciences Instrument Co., Ltd., China). All the gels were prepared by using a 1×TBE buffer and run at 100 V for 90 min. After staining with ethidium bromide (EB) for 20 min, the photographing was scanned with the FR-980A gel image analysis system (Shanghai, China).

RESULTS AND DISCUSSION

UV-Vis characterizations

Fig. S1 displayed the UV-vis spectra of different protein and DNA samples, including SA, biotin, bio/Ab, bio/S1, bio/S2 and their mixtures. Obviously, the absorption spectrum of bio/S1-SA-bio/Ab and bio/S2-SA-bio/Ab showed a merged peak at 260 nm (curve e) from a typical peak of bio/S1 and bio/S2 at 260 nm (curve d) and bio/Ab at 278 nm (curve c), indicating that bio/S1 and bio/S2 were respectively tagged with Ab through streptavidin (SA)-biotin interaction.

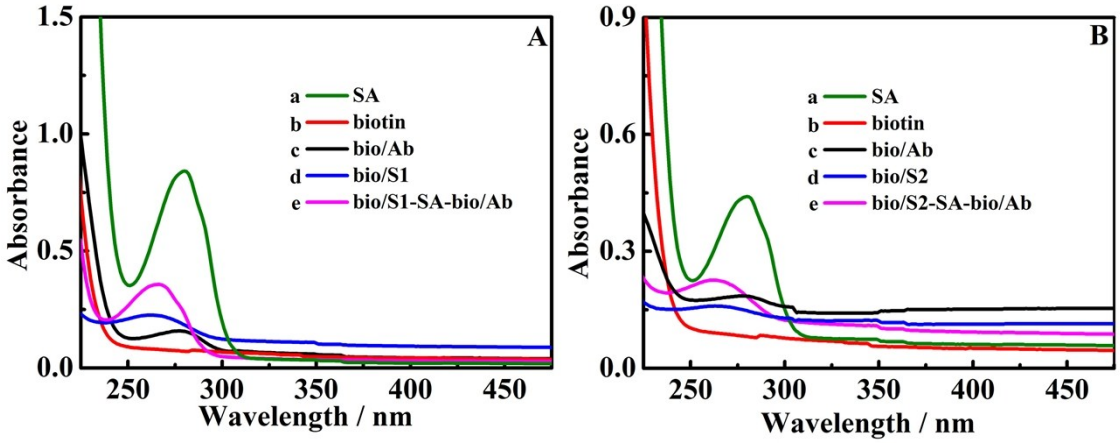

Fig. S1 (A) UV-Vis absorption spectra of (a) SA (2 μM), (b) biotin (1 μM), (c) bio/Ab (2 μM), (d) bio/S1 (2 μM), (e) SA+bio/S1+bio/Ab; (B) UV-Vis absorption spectra of (a) SA (2 μM), (b) biotin (1 μM), (c) bio/Ab (2 μM), (d) bio/S2 (2 μM), (e) SA+bio/S2+bio/Ab.

Experimental optimization.

To achieve the optimal analytical performances, experimental parameters were optimized, involving in RS concentration and different interaction time, such as the proximity binding of MUC1 with two affinity probes, RCA reaction, and

THSS incubation. Herein, the concentration of RS relative to that of SP is a key parameter to affect the stability of THSS maintained by Watson-Crick and Hoogsteen base pairing. As shown in Fig. S1A, when the concentration of SP was set to 1 μM, the FRET signal ratio of F_A/F_D gradually decreased with the rising of the RS concentration in the range of 0.4 to 2.0 μM, and reached to the minimum at 1.4 μM. This suggested that more RS in the solution allowed for the assembly of more THSS. As such, more SP adopted an "open" configuration within the triplex-helix stem region of THSS, so more FRET pair FAM and TAMRA in the opposite site generated less FRET signal. Thus, 1.4 μM RS relative to 1 μM SP was chosen for the formation of THSS.

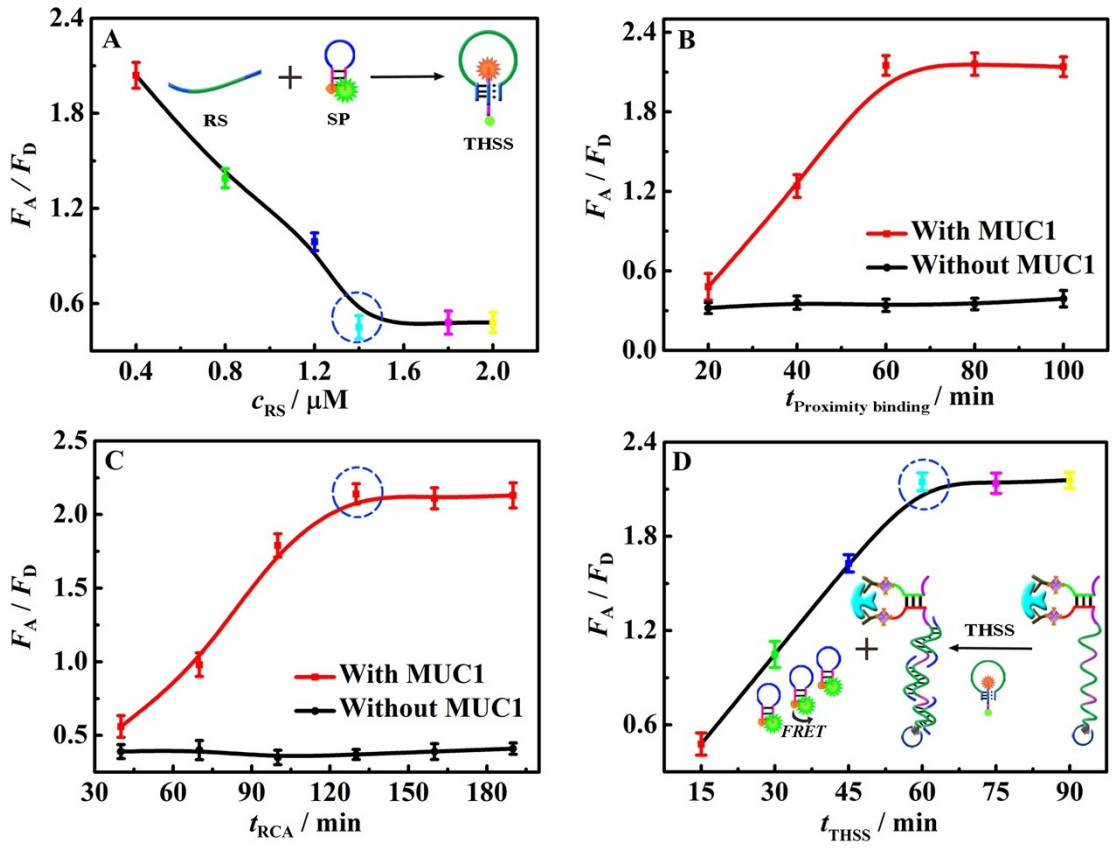

Fig. S2 Effects of (A) different concentrations of RS, (B) PLA reaction time, (C) RCA reaction time, (D) incubation time for THSS on the developed sensing

method. Error bars: *s*, *n*=3.

From Fig. S1B, S1C and S1D, we could see that the FRET signal ratio of F_A/F_D gradually increased with the extending of interaction time of MUC1 proximity ligation, RCA reaction and THSS incubation, and leveled off at 60 min (Fig. S1B), 130 min (Fig. S1C), and 60 min (Fig. S1D), respectively. Meanwhile, the background signal all kept at a lower steady value. These observations indicated that 60 and 130 min were sufficient to the MUC1-triggered proximity binding and subsequent RCA reaction. Correspondingly, the conformation conversion of THSS was completed within 60 min through the attachment of RS in the RCA product with continuous tandem repeats and resultant releasing of SP. This may be attributed to the acceleration of hybridization reaction of RS due to the increasing local concentration from the proximity ligation.

Table S2 Comparison of different methods for MUC1 determination.

Methods	Strategies	Linear range	LOD	Ref.
ELISA and SPR	Aptamer-antibody sandwich	8-100 μg·mL^{-1}	1 μg·mL^{-1}	5
DPV	HO switch, AuNPs, and enzyme signal amplification	8.8-353.3 nM	2.2 nM	6
EIS	ZrHCF@mFe_3O_4@mC nanocomposite and aptamer	0.01 ng·mL^{-1}-1.0 μg·mL^{-1}	0.90 pg·mL^{-1}	7
ECL	Aptamer and ERET from Ru1 to GO	64.9-1036.8 nM	40 nM	8
Fluorescence	Aptamer and GO as quencher	0.04-10 μM	28 nM	9

Fluorescence	MoS_2 QDs as quencher	0-10 μM	0.5 nM	10
FRET	Proximity binding-initiated RCA inducing amplified transduction of SP	0.001-20 ng·mL^{-1}	0.23 pg·mL^{-1} (0.76 nM)	This work

ELISA: enzyme-linked immunosorbent assay; SPR: surface plasmon resonance; DPV: differential pulse voltammograms; EIS: electrochemical impedance spectroscopy; ECL: electrochemiluminescence; HO: hairpin oligonucleotide; ZrHCF: zirconium hexacyanoferrate; QDs: quantum dots; TiO_2NTs: TiO_2 nanotube arrays; ERET: electrochemiluminescence resonance energy transfer; Ru1: bis(2,2′-bipyridine)-(5-aminophenanthroline)ruthenium(II); GO: graphene oxide.

Table S3 Recovery of MUC1 in serum samples by using the proposed FRET immunosensor (*n*=6).

Samples	Added (pg·mL^{-1})	Found (pg·mL^{-1})	Recovery (%)	RSD (%)
1	10.0	10.3	103	1.4
2	100	98.7	98.7	1.7
3	500	495	99.0	3.4
4	1000	965	96.5	2.2

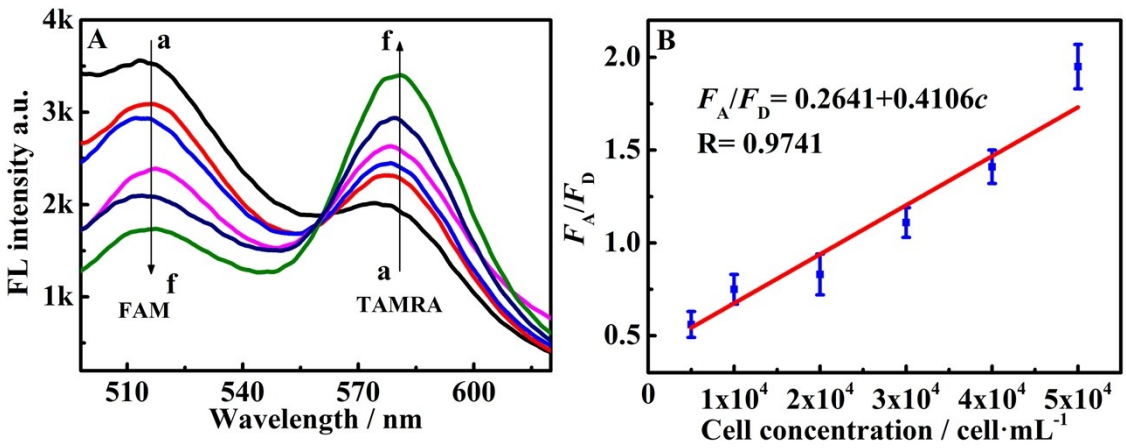

Fig. S3 (A) Fluorescence spectra upon addition of MDA-MB-231 cancer-cell lysates with different concentrations: (a) 5×10^3, (b) 1×10^4, (c) 2×10^4, (d) 3×10^4, (e) 4×10^4 and (f) 5×10^4 cells·mL^{-1}. (B) The resultant linear calibration curve plotted by the F_A/F_D ratio of the fluorescence intensity of FAM and TAMRA vs low concentrations of MDA-MB-231 cancer-cell lysates from 5×10^3 to 5×10^4 cells·mL^{-1}. Error bars: standard deviation (s), n=3.

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