Y-Shaped Backbone-Rigidified Polyvalent Aptamer Network-Enabled Catalytic Self-Assembled Quantum Dot FRET System for Sensitive Detection of Circulating Tumor Cell

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EXPERIMENTAL SECTION

Reagents and Materials

All DNA oligonucleotides (**Table S1**) were obtained from Sangon Biotech (Shanghai, China) Co., Ltd. T4 DNA Ligase, deoxyribonucleotides mixture (dNTPs) (10 mM) and Phi29 DNA polymerase were purchased from New England Biolabs Inc. (USA). Exonuclease I (Exo I) and Exonuclease III (Exo III) were obtained from Thermo Fisher Scientific (USA). Streptavidin-modified MBs (2.8 μm, 10 mg/mL) were obtained from Beaver biomedical engineering Co., Ltd. (Suzhou, China). The streptavidin-conjugated CdSe/ZnS QDs with a maximum emission of 605 nm (Qdot 605 ITK) were obtained from Invitrogen Corporation (California, USA). All other reagents were of analytical grade and used as received without further purification. Ultrapure water obtained from a Millipore filtration system (Temecula, CA, USA) was used throughout all experiments.

Table S1. The sequences of the oligonucleotides

Name	Sequence (5'−3')
*b*DNA	Biotin -A*A*T*TCTGGCT TTT CAGAT TGGGG CCTTG
*c*DNA	GAGTT CCACC CCCAT TTT AGCCAGAATT
*lc*DNA	ATGGGGGTGG AACTCATG ATG GA *AAA AAA AAA AAA*C CAA GGG AAT *AAA AAA AAA AAA* GCT CTC TTT *AAA AAA AAA AAA* AAT ACA G CAAGGCCCCAATCTG
primer	*TTT TTT TTT TTT TTT* TGCGTCTGTCCCACGTTGTCATGG
linear DNA	PO_4^{3-}GGGACAGACGCAACCTCTGTAGTGAAAAAAAAAAAAAAAAAAAACAGGCC AACCCCCCATGACAACGT
Padlock	GTCTGTCCCACGTTGTCA
apt-cDNA	GAC AGA CGC AAC CTC TGT AGT G
H1	Cy5-C*A*C* T ACA GAG GTT GCG TCT GTC GAT GTT GAA ACC GAC AGA CGC AAC* C*T*
H2	C*G*T* CTG TCG GTT TCA ACA TC GAC AGA CGC AAC CTC TGT AGT GAA CCT A*G*C*-biotin

Monovalent SYL3C aptamer	CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTG

Cell Culture

All cell lines were maintained in a humidified incubator at 37 °C with 5% CO_2 for exponential phase by using Dulbecco's modified Eagle's medium containing fetal bovine serum (10%) and streptomycin/penicillin (1%). Thereafter, the cells were collected by centrifugation (1000 rpm, 5 min) and then resuspended in phosphate-buffered saline (PBS) with different concentrations to be captured.

Preparation of a Circular DNA Template

A linear DNA template and the ligation DNA (padlock) with equal molar concentrations (5 μM) were first annealed together in 20 μL of 1×T4 DNA ligase buffer by heating up to 90 °C for 5 min and cooled down to 25 °C at 1 °C min^{-1}. Then, T4 DNA ligase (25 U) was added to the abovementioned solution and incubated at 16 °C overnight to ligate the gap of the DNA template. Subsequently, the mixture was heated to 75 °C for 10 min to denature ligase. After that, Exo I (20 U) and Exo III (100 U) were introduced to digest the residual linear DNAs at 37 °C for 30 min, then the solution was heated to 80 °C for 10 min to inactivate the exonucleases. Finally, the products of circular DNA template were stored at 4 °C for further use.

Preparation of on-particle Y-polyvalent aptamer-functionalized 3D network (MB@Y-PANs)

A biotin-modified DNA strand (*b*DNA) and another complementary DNA strand (*c*DNA) with partial sequences were dissolved in hybridization buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 M NaCl, and 0.05% Tween-20), with a final concentration of 1 μM. Then, the mixture was heated at 95 °C for 5 min and gradually cooled to room temperature to form a Y-shaped probe. Whereafter, 100 μL of 10 mg/mL of MBs was washed three times with washing buffer and then resuspended with 100 μL of binding buffer. Later, to 50 μL of 1 μM Y-shaped probes were added MB suspension followed by the incubation at room temperature for 1 h with the aid of oscillation. After that, the

MBs were separated, and the supernatant was discarded to remove the uncombined Y-shaped probes and then washed three times with the washing buffer. The resulting MBs with a modified Y-shaped probe (Y-MB) was resuspended in 100 μL 2×SSPE (0.3 M NaCl, 0.02 M NaH_2PO_4, 0.0025 M EDTA, pH=7.4). Whereafter, 10 μL of 2 μM long carrier DNA (*lc*DNA) and 10 μL of 10 μM primer probes were added, and the mixture was shaken at 37 °C for 40 min, allowing the Y-shaped probe to bind to the *lc*DNA with binding primers. The unbound *lc*DNA and primers were subsequently removed by magnetic separation and washed three more times with washing buffer. Following, 10 μL of 10 × phi29 DNA polymerase reaction buffer, 8 μL of DEPC water, 5 μL of dNTP (10 μM), 5 μL of 2 μM circular DNA templates, 2 μL of Phi29 DNA polymerase were added, the mixture was incubated at 37 °C for 90 min to perform the RCA reaction, after which the reaction was stopped by heating at 65 °C for 10 min. After magnetic separation, the Y-MBs covered with RCA products were resuspended in a binding buffer (50 μL) and stored at 4 °C for the subsequent experiment. For the aptamer-complementary DNA strand (*H*) binding process, 20 μL of 10 μM apt-cDNA strands was added to the aforementioned solution to hybridize with the on-beads RCA product. The mixture was heated to 55 °C for 10 min, 45 °C for 5 min, and then incubated at 25 °C for 1 h to achieve full hybridization, forming on-particle Y- polyvalent aptamer networks (MB@Y-PANs) with octopus arm morphology. The resulting MB@Y-PANs was washed three times and resuspended in the 20 μL of binding buffer.

Detection of Cancer Cells with MB@Y-PANs-QD FRET System

The cells were added to 40 μL of DPBS containing 0.5 mg/mL MB@Y-PANs probe, and incubated at room temperature for 60 min. Then, the supernatant was collected via magnetic separation. The supernatant was mixed with 50 μL of solution containing 10 μL of H1 (10 μM), 10 μL of H2 (10 μM), 10 μL of 10× reaction buffer (200 mM Tris-HCl, 1.4 M NaCl, 50 mM KCl, pH 7.5), 2 μL of 1 mg/mL BSA followed by the incubation at 37 °C for 2 h. Subsequently, 20 μL of QDs (0.5 μM) were added, and the mixture was incubated at 37 °C for 30 min to form the QD/H1-H2/Cy5 FRET nanostructures. Subsequently, the reaction solution was introduced to fluorescence spectroscopy to detect the cancer cells. The emission spectra were collected from 550

and 750 nm with excitation wavelengths of 488 nm. The maximum fluorescence emission was at 605nm (QDs) and 667 nm (Cy5), respectively.

All experiments involving human blood samples were performed in compliance with the institutional guidelines and relevant national regulations in China. The study was approved by the Ethics Committee of Liaocheng University. Informed consent was obtained from all individual participants prior to sample collection.

Gel Electrophoresis Analysis

To verify the assembly of H1-H2 duplex and Y-PANs nanostructures, the reaction products were analyzed by 12% nondenaturating polyacrylamide gel electrophoresis (PAGE) analysis in 1× TBE (9 mM Tris−HCl, pH 7.9, 9 mM boric acid, 0.2 mM EDTA) at a 120 V constant voltage for 35 min at room temperature. After electrophoresis, the gel was analyzed by a ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

RESULTS AND DISCUSSION

Feasibility of the MB@Y-PANs-enabled QD-FRET system for CTC detection

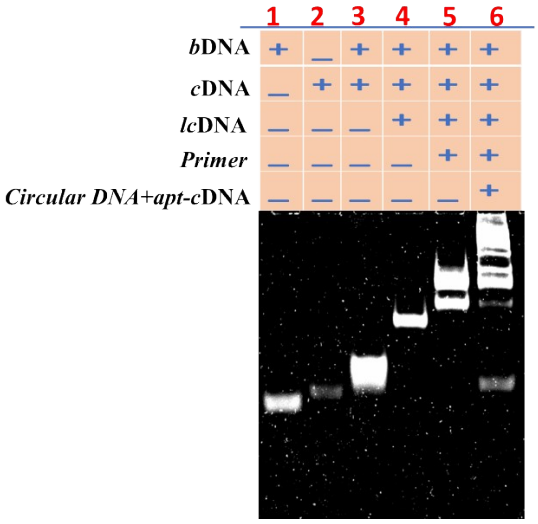

Figure S1 Nondenaturing PAGE analysis of the stepwise assembly of Y-PANs.

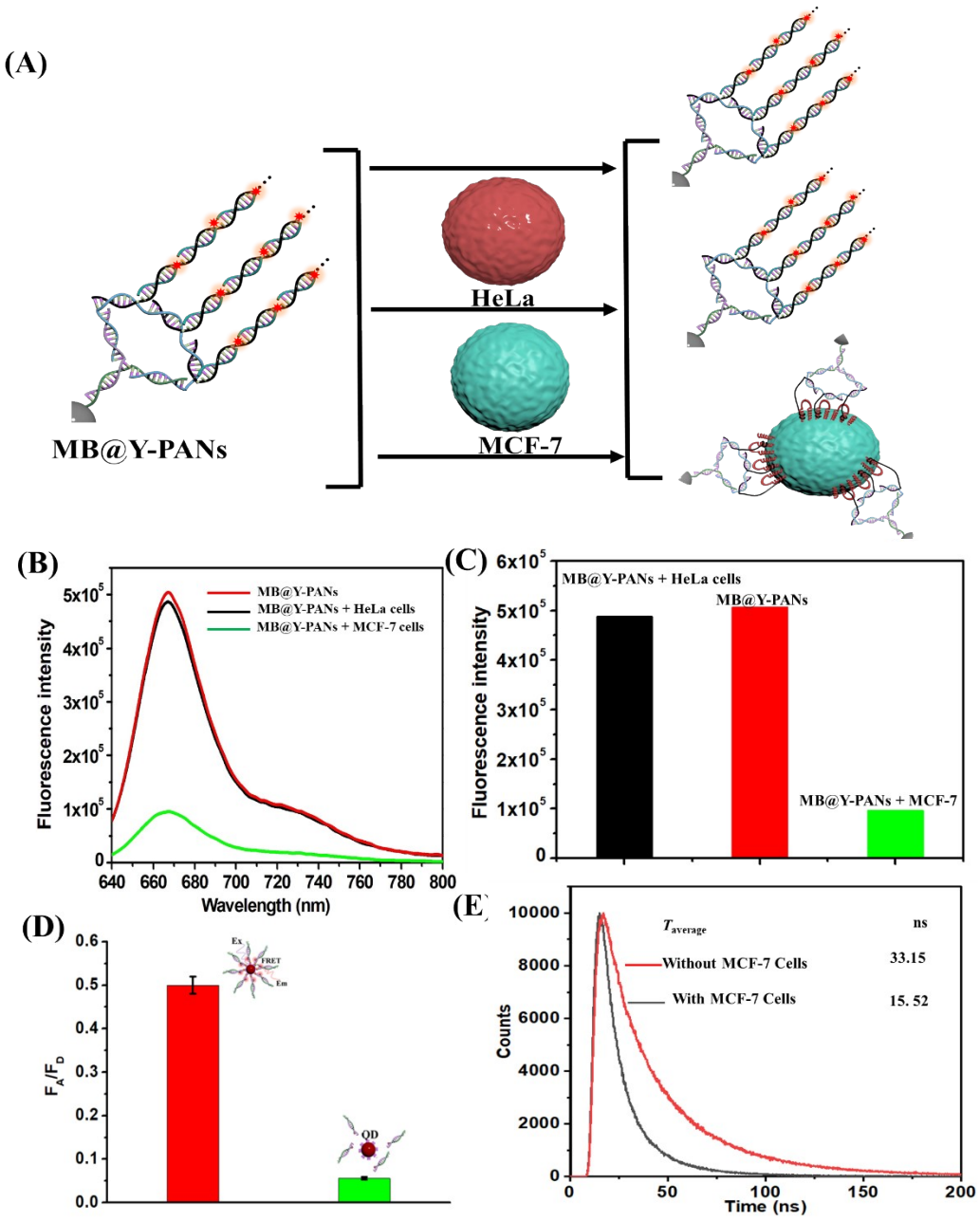

Figure S2 (A) Fluorescence response of MB@Y-PANs to HeLa and MCF-7 cells. (B) Kinetics comparison between MB@Y-PANs and MB@PANs different capture probes. (**C**) Feasibility of the catalytic self-assembled QD FRET amplifier for MCF-7 cells (1 × 10^2 cells/mL) detection. (D) A comparison of F_A/F_D ratios between QD-confined CHA and free-solution CHA (prepared without biotin modification) at identical H1/H2 concentrations. (E) Fluorescence lifetime curves of QD in absence (red curve) and presence (black curve) of target CTCs, respectively.

Characterizations and Optimization of MB@Y-PANs-enabled QD-FRET system

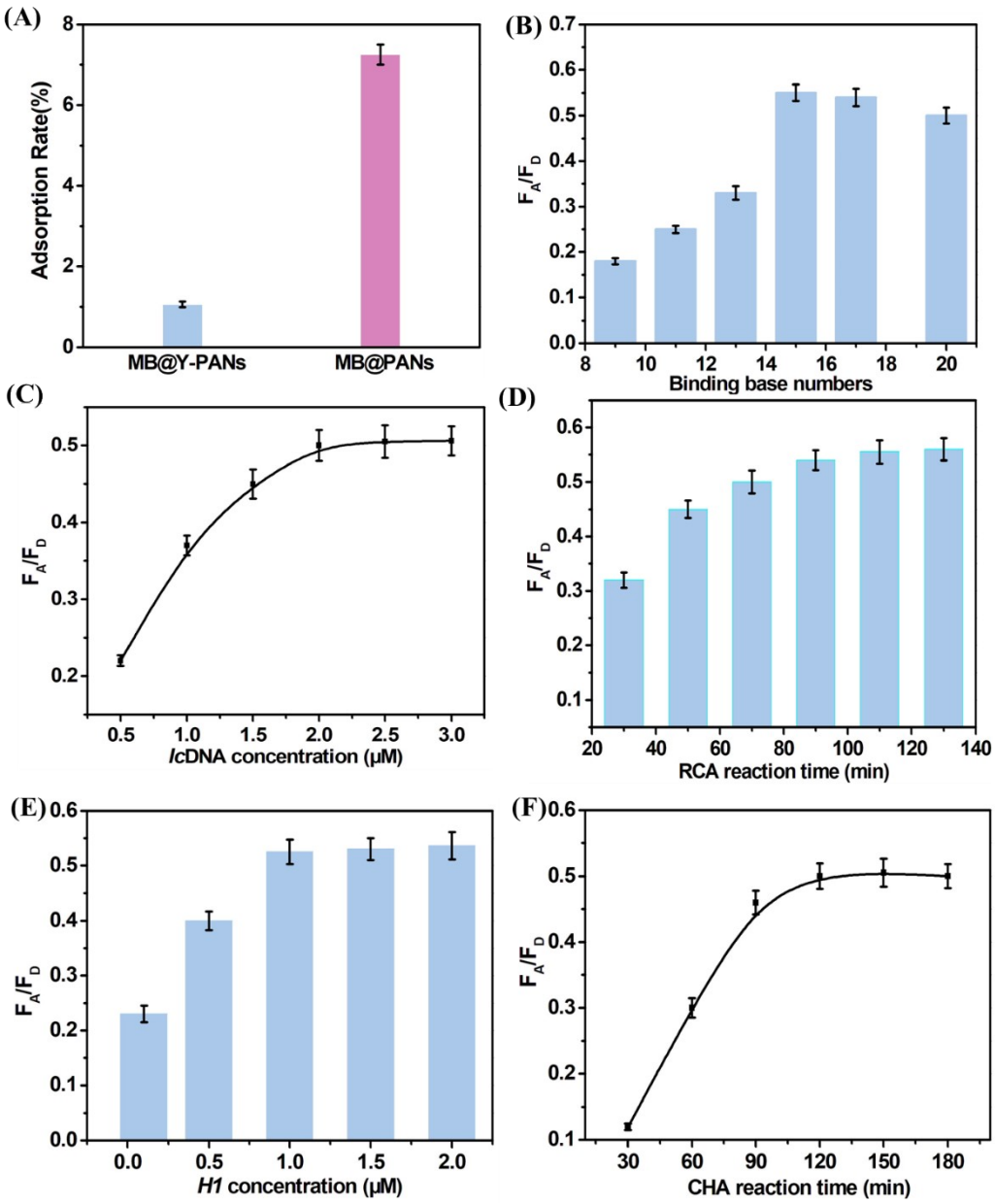

Figure S3. (A) Adsorption rates of BSA (70 mg/mL) using MB@Y-PANs and MB@PANs. **(B)** Optimization of different binding bases between Y-shaped probe and *lc*DNA, **(C)** Optimization of the *lc*DNA concentration, **(D)** Optimization of RCA reaction time, **(E)** Optimization of H1concentration, **(F)** Optimization of CHA reaction time.

Analytical performance

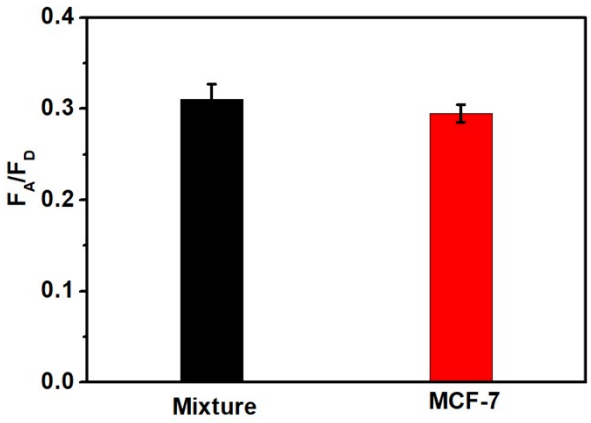

Figure S4A. Selectivity evaluation of the proposed sensor under physiological cell ratio (~10^5:1, non-target cells: MCF-7 CTCs). 1 × 10^3 cells mL^{-1} MCF-7 cells were mixed with 1 × 10^8 cells mL^{-1} pooled non-target cells (LO2, HeLa, HepG-2, MDA-MB-231) to simulate the cellular environment of human peripheral blood.

Analysis of practical application capability

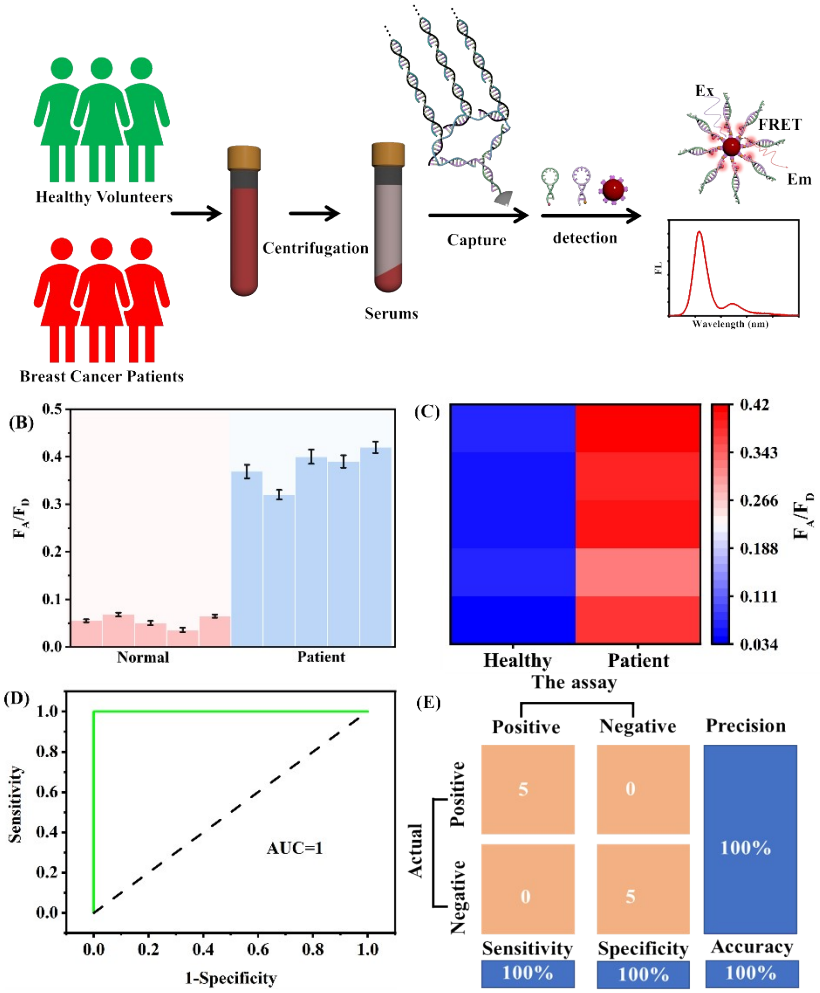

Figure S5 (A) Schematic of MCF-7 cell detection in clinical peripheral blood samples using the MB@Y-PANs-enabled QD-FRET sensing platform. (B) Histogram and (C) Heatmap of fluorescence responses in healthy (n = 5) and breast cancer patient sera (n = 5) (***p ≤ 0.001). (D) ROC curve for discriminating healthy and cancer patient samples. (E) Confusion matrix summarizing diagnostic system's ability to differentiate between healthy and breast cancer patient.

Table S2. Comparison of previous reports for the detection CTCs.

Strategy	Method	Linear range (cells/mL)	LOD (cells/mL)	Ref.
$CoFe_2O_4$@Ag magnetic nanohybrids	Electrochemistry	10^2 ~1×10^6	47	1
DAN/AAO hybrid	Electrochemistry	3×10^2 ~1×10^3	80	2
ITO/rGO-Au/MOF@Pt@MOF	Electrochemistry/ colorimetry	5 ~5×10^5	5	3
Conducting polymer hydrogel (CPH)	Electrochemilumi nescence	100-6500	80	4
MNP@QD	Florescence	10^2-10^7	100	5
Polyvalent Aptamer Network	Electrochemistry	10^2-5×10^4	23	6
Au@COF-LZU1@Ru	Electrochemilumi nescence	8-1×10^5	2	7
TDNE-c/MVMNPs-CHA biomimetic sensor	Fluorescence/colo rimetric	10-10^4	3/6	8
DNA network	Electrochemistry	50-2×10^4	6	9
MDANs-Cas12a strategy	Fluorescence	50-1×10^6	26	10
MW-LMMBs@DNA walker	Fluorescence	10-5×10^6	2	11
AMNPs-Apt_{AS1411}@HCR	absorbance/colori metric/photother mal	10-10^6	5	12
DNA network	Fluorescence	10-4×10^5	10	13

Y-PANs@CHA FRET sensor	Fluorescence	5-5×10^4	2	This work

Table S3 Quantitative Analysis of MCF-7 Cells in Spiked Healthy Human Peripheral Blood.

Sample	Added (cells)	Found	RSD (%)	Recovery (%)
1	100	98.2	4.2	98.2
2	1000	1015	2.5	101.5
3	10000	10535	4.5	105.4

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