Electronic Supplementary Material (ESI) for Analytical Methods.
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Supplementary Materials for

Rational design of nonlinear hybridization immunosensor chain reactions for simultaneous ultrasensitive detection of two tumor marker proteins

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1. Oligonucleotides used in the experiment

Name	Sequence (5′-3′)
Trigger	Biotin-AAAAATGACGAACTAGTTGATGAAGCTG
F-A	6-FAM-GTGTGCCTATTATGTCTCCTCCTGTGTGCCTATTATGTCTCCT CCTCAGCTTCATCAACTAGTTCGTCA
Q-A	AACTAGTTGATGAAGCTGGACATAATAGGCACACGACATAATAGGCACAC-BHQ1
F-B	AGGAGGAGACATAATAGGCACACTGACGAACTAGTTGATGAAGCTG-6-FAM
Q-B	BHQ1-CAGCTTCATCAACTAGGTGTGCCTATTATGTCTC
Assistant A	GTGCCTATTATGTCGTGTGCCTATTATGTCCAGCTT
Assistant B	ACACCTAGTTGATGAAGC

Table S1. Composition and volume of different NHCR mixtures

NHCR mixture	Composition and volume
(1)	27 μL NHCR buffer + 10 μL 0.1 μM DNA + 10 μL NHCR A + 20 μL NHCR B
(2)	27 μL NHCR buffer + 10 μL 0.01 μM DNA + 10 μL NHCR A + 20 μL NHCR B
(3)	27 μL NHCR buffer + 10 μL 0.001 μM DNA + 10 μL NHCR A + 20 μL NHCR B
(4)	37 μL NHCR buffer + 10 μL NHCR A + 20 μL NHCR B
(5)	47 μL NHCR buffer + 10 μL 0.001 μM DNA + 10 μL NHCR A
(6)	37 μL NHCR buffer + 10 μL 0.001 μM DNA + 20 μL NHCR B

Table S2. Comparison of the proposed method with other analytical methods for AFP detection

Detection method	Signal output	LOD	Reference
Aptamer nanoprobe based FRET	Fluorescence	1.38 ng/mL	[1]
Phage-mediated sandwich immunoassay of double nanoantibody	Chemiluminescence	0.24 ng/mL	[2]
Photoelectrochemical electrode based on reduced graphene oxide honeycomb structure	Photoelectrochemical	0.05 ng/mL	[3]
Fluorescence immunoassay based on polymer device	Fluorescence	13.00 pg/mL	[4]
Sandwich temperature-responsive polymicrogel based on ELISA	Colorimetric	8.40 pg/mL	[5]
NHCR based immunoassay	**Fluorescence**	**1.74 pg/mL**	**This method**

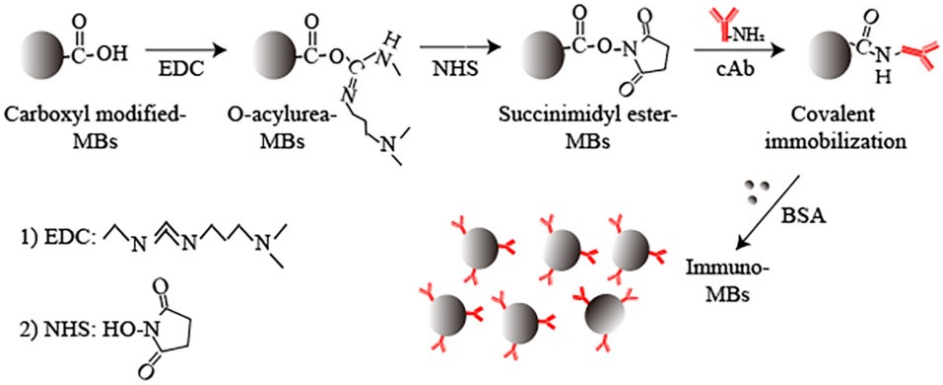

Fig. S1 Schematic diagram of preparation process of immunomagnetic beads. Carboxyl groups on MBs react with EDC to form unstable O-acylurea on MBs. NHS replaces EDC to form stable succinyl ester on MBs. The -NH2 group on the capture antibody replaces NHS and forms a stable amide bond on the MBs. BSA is added to block the redundant binding sites on the MBs.

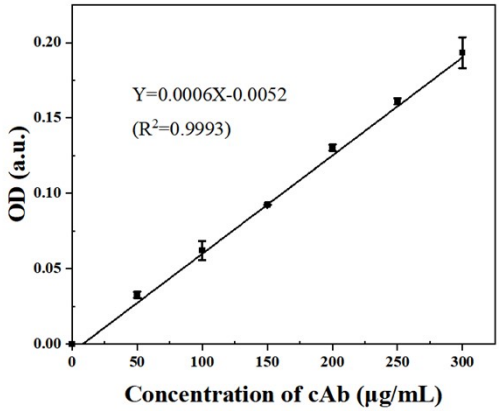

Fig. S2 The relationship between MBs conjugated cAbAFP concentration and OD.

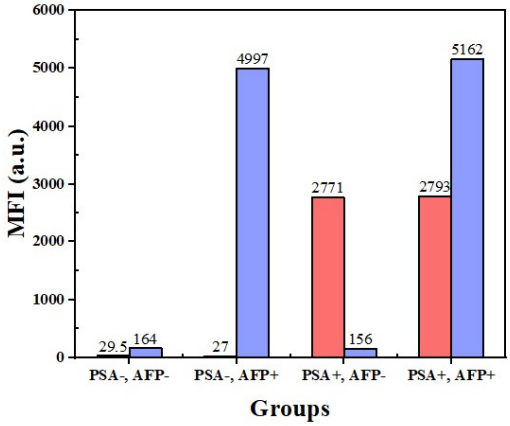

Fig. S3 Fluorescence signal diagram of simultaneous detection of AFP and PSA by

immunoassay based on NHCR. The concentrations of PSA and AFP are 4 ng/mL and 10 ng/mL respectively.

Reference

[1] Li G, J Zeng, H Liu, et al. A fluorometric aptamer nanoprobe for alpha-fetoprotein by exploiting the FRET between 5-carboxyfluorescein and palladium nanoparticles[J]. Mikrochimica Acta, 2019, 186(5): 314.

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[4] Chang J, N Gao, P Dai, et al. Facile engineered polymeric microdevice via co-coupling of phenylboronic acid and Protein A for oriented antibody immobilization enables substantial signal enhancement for an enhanced fluorescence immunoassay[J]. Sensors and Actuators B: Chemical, 2021, 346: 130444.

[5] Su R, L Xia, G Li, et al. A controlled recognizing and releasing glycoprotein based on temperature-responsive phenylboronic microgels for colorimetric analysis of complex samples[J]. Talanta, 2022, 241: 123260.