Supporting Information

Emission Wavelength Switchable Carbon Dots Combined with Biomimetic Inorganic Nanozymes for Two-Photon Fluorescence Immunoassay

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TABLE OF CONTENTS

EXPERIMENTAL SECTION

Reagents and materials. *o*-phenylenediamine (*o*PD), triethanolamine (TEOA), sodium salicylate, (3-aminopropyl)triethoxysilane (APTES), and tetraethyl orthosilicate (TEOS) were purchased from Aladdin Chemical Industries, Ltd (Shanghai, China). $FeSO_4$, $FeCl_3$, H_2O_2, glucose, hexadecyl trimethyl ammonium bromide (CTAB), Na_2CO_3, NaCl, NaN_3, ethanol, hydrochloric acid (HCl), chloroauric acid ($HAuCl_4$), sodium borohydride ($NaBH_4$), tris(hydroxymethyl)aminomethane (Tris), glutaraldehyde solution (50%), and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Sinopharm Group Co., Ltd (Shanghai, China). Bovine serum albumin (BSA) was achieved from Sangon Biotech Group Co., Ltd. (Shanghai, China). TWEEN-20 was acquired from ABCONE Biotech Group Co., Ltd. (Shanghai, China). Human carcinoembryonic antigen (CEA, cat#: 11077-H08H), monoclonal anti-human CEA capture antibody (Ab_1, cat#:11077-MM02), polyclonal anti-human CEA detection antibody (Ab_2, cat#: 11077-MM04), prostate specific antigen (PSA, cat#: 10771-H08H), and Alpha-fetoprotein (AFP, cat#: 12177-H08H) were purchased from Sino Biological Technology Co., Ltd (Beijing, China). Squamous cell carcinoma antigen (SCCA) and neuron-specific enolase (NSE) were purchased from Abcam Biological Technology Co., Ltd (Hong Kong). The commercially available human CEA ELISA kit (AMEKO, No. AE94830Hu) was purchased from Shanghai Lianshuo Biological Technology Co.,Ltd. High-binding polystyrene ELISA plate 12-well strips were obtained from Jet Bio-Filtration Co., Ltd (ref. FEP-100-012, Guangzhou, China). All reagents were of analytical grade and used as received. Ultrapure water was obtained from a Millipore purification system (18.2 MΩ•cm, Millipore, USA) and used for the project.

Instruments. Fluorescence spectra were recorded on a F-4600 fluorescence spectrophotometer (Hitachi, Japan) equipped with a xenon lamp. UV-vis absorption spectra were obtained with a TU-19 UV-visible absorption spectrophotometer (Purkinje, Beijing) and microplate reader (Thermo scientific, USA). Fourier transform infrared spectra (FT-IR) were scanned by using a Nicolet iS50 FT-IR system (Thermo scientific, USA). Field emission transmission electron microscope (FETEM) and field emission scanning electron microscope (FESEM) spectra were conducted by using FEI Talos F200S G2 instrument (FEI, USA) and Nova NanoSEM 230 (FEI, Czech), respectively. Atomic force microscope (AFM) image was obtained by using Veeco-Multimode-V AFM system

(Bruker, USA). Size distribution was measured by nanoparticle size and zeta potential analyzer (Malvern, UK). Fluorescence lifetime was measured with a F900 fluorescence spectrometer (Edinburgh Instruments Ltd., U.K.). X-ray powder diffractometer was processed by RIGAKU Ultima IV system (Rigaku, Japan). X-ray photoelectron spectroscopic (XPS) was detected by using ESCALAB 250 system (VG, USA). Brunauer-Emmett-Teller (BET) nitrogen adsorption-desorption isotherms were recorded using a ASAP 2020 surface area and porosity analyzer (Micromeritics Instrument Corp, USA).

Preparation of *o*-CDs. *o*PD (0.60 g) was dissolved in absolute ethanol (60 mL) and sonicated for 5 min to make it homogeneous. The mixed solution was transferred to a 100 mL Teflon-lined autoclave and reacted for 12 h under 180 °C, then naturally cooled to room temperature to obtain a brown clear solution, which was further purified through a silica gel column chromatography and the fluorescent component was collected as a pure carbon dots.

Determination of Fe^{2+} and H_2O_2. Different concentrations of Fe^{2+} solution (50 μL) was added into *o*-CDs (100 μL, 20 μg/mL), after reacting for 5 min, the above reaction solution was removed to a fluorescent cuvette and measured by a F4600 fluorescence spectrometer. Following that, 50 μL of H_2O_2 with various concentrations were added into the above system (*o*-CDs+Fe^{2+}) and continued to react for 5 min. Then, the fluorescence intensity of *o*-CDs was quantitatively determined.

Synthesis of the NH_2-functionalized DMSN. DMSN was prepared according to the previous report with minor modifications.[1] Briefly, TEOA (68 mg) was diluted with 25 mL of ultrapure water and stirred at 80 °C for 30 min, followed by adding sodium salicylate (168 mg) and CTAB (380 mg) to the reaction solution. After keep stirring for 1 h, TEOS (4.0 mL) was slowly added dropwise to the above mixed solution, and gently stirring for at least 12 h to obtain a milky white slurry. The suspension was centrifuged at 8000 rpm for 15 min, and the precipitate was repeatedly washed with water and ethanol to remove unreacted precursor. Finally, the obtained product was continuously stirred for 6 h in a mixed solution of HCl and ethanol (HCl: ethanol = 1:9) at 60 °C to remove the template, centrifuged and repeated three times to obtain DMSN, which was dried at 80 °C in a vacuum drying oven.

100 μL APTES was added into the DMSN dispersion solution (10 mL, 10 mg/mL) under magnetically stirring for 12 h to obtain NH_2-functionalized DMSN. The suspension was centrifuged

at 8000 rpm for 15 min, and the precipitate was repeatedly washed with water and ethanol and was dried at 80 °C in vacuum.

Preparation of DMSN-Au NPs-Ab_2 conjugates. NH_2-functionalized DMSN-Au NPs colloids (1.0 mL, 10 mg/mL) were added to a sodium carbonate buffer solution (0.1 mol/L, pH = 8.0), followed by the addition of 1.0 mL of 5.0% glutaraldehyde solution and slowly stirred at room temperature for 1 h. The mixed solution was centrifuged at 8000 rpm for 15 min to remove unbound glutaraldehyde, and the precipitate was redispersed in PBS (10 mM, pH = 7.4). 50 μL of Ab_2 (1.0 mg/ml) was added and incubate for 2 h at room temperature and centrifuge at 8000 rpm for 15 min, the precipitate was redispersed in PBS (10 mM, pH=7.4, containing 2.0% BSA) and incubated at 37 °C for 2 h to block unbonded sites. The above solution was again centrifuged to obtain DMSN-Au NPs-Ab_2 conjugates, and the precipitate was redispersed in PBS (10 mM, pH = 7.4) and stored at 4 ° C before use.[2]

Preparation of Ab_1-coated microplate. A high-binding polystyrene ELISA mocroplates was incubated overnight at 4 ºC with 100 μL per well of Ab_1 at a concentration of 5.0 μg/mL in sodium carbonate buffer solution (pH = 9.6, 0.1 M). The microplate was covered with adhesive plastics plate sealing film to prevent evaporation. Aspirate each well and wash three times with at least 300 μL wash buffer (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH = 7.4), and then incubated with 300 μL per well of blocking buffer (5.0% BSA in wash buffer) for 2 h at 37 °C with shaking. The microplate was then washed as before, and used for the detection of target CEA.[3]

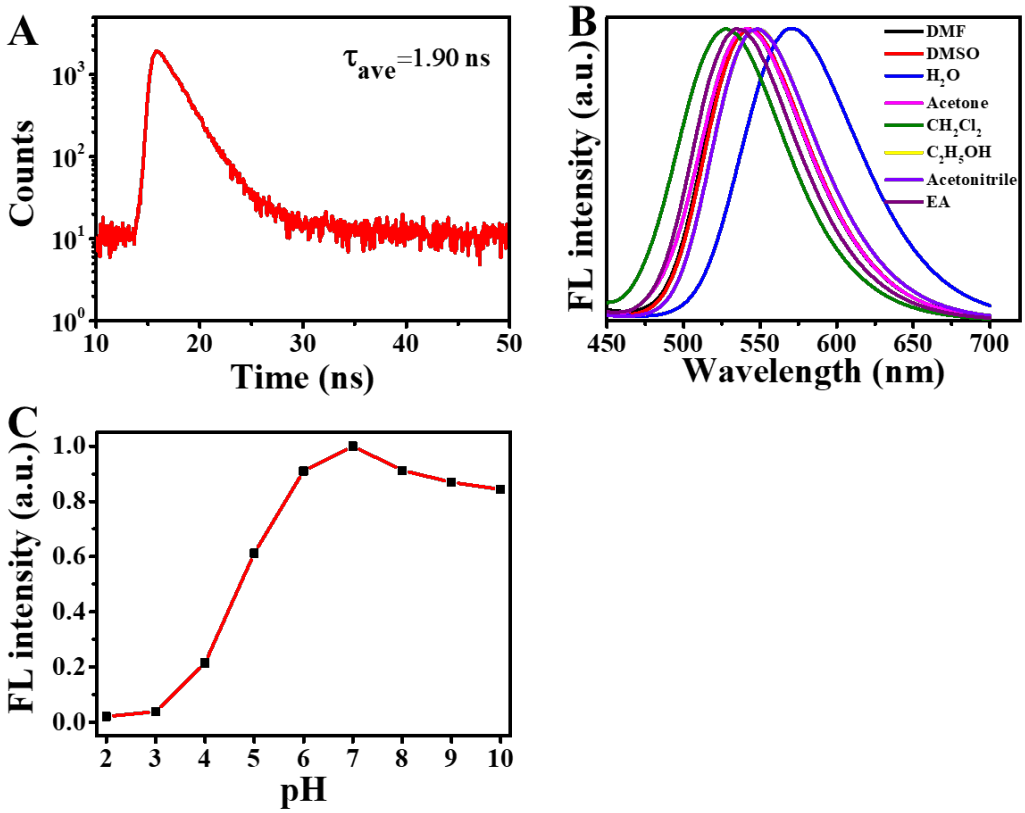

Figure S1. Fluorescence properties of *o*-CDs. (A) Time-resolved fluorescence decay curves. (B) Solvent effect. (C) The fluorescence intensity at different pH.

o-CDs exhibit solvent effect and different color fluorescence in different polar solvents. As the polarity of the solvent increases, the fluorescence emission wavelength gradually bathochromic shift, and the fluorescence gradually transitions from green to yellow (Figure S1B). Besides, as indicated in Figure S1C, the fluorescence intensity of *o*-CDs varies greatly at different pH. As the pH increases, the fluorescence intensity increases significantly and the fluorescence intensity reaches maximum at pH = 7.0, when the pH is greater than 7.0, the fluorescence intensity gradually decreases.

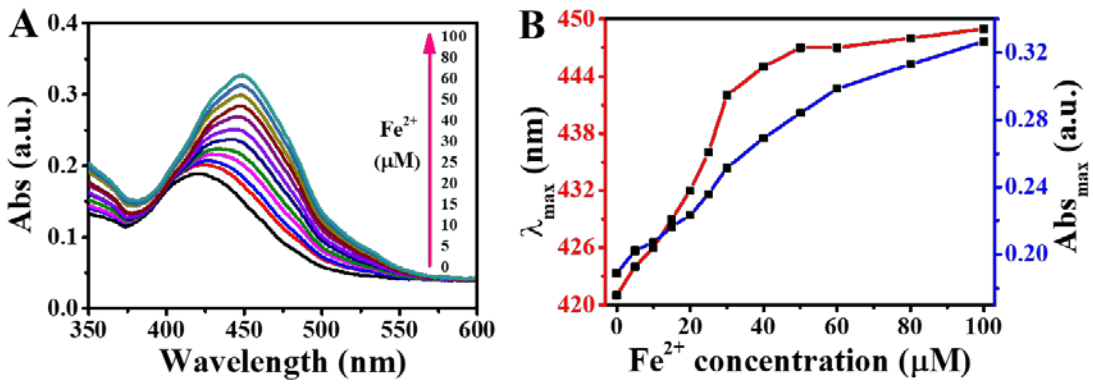

Figure S2. The change of the UV-vis absorption spectrum (A) and absorption wavelength (B) of *o*-CDs in response to Fe^{2+}.

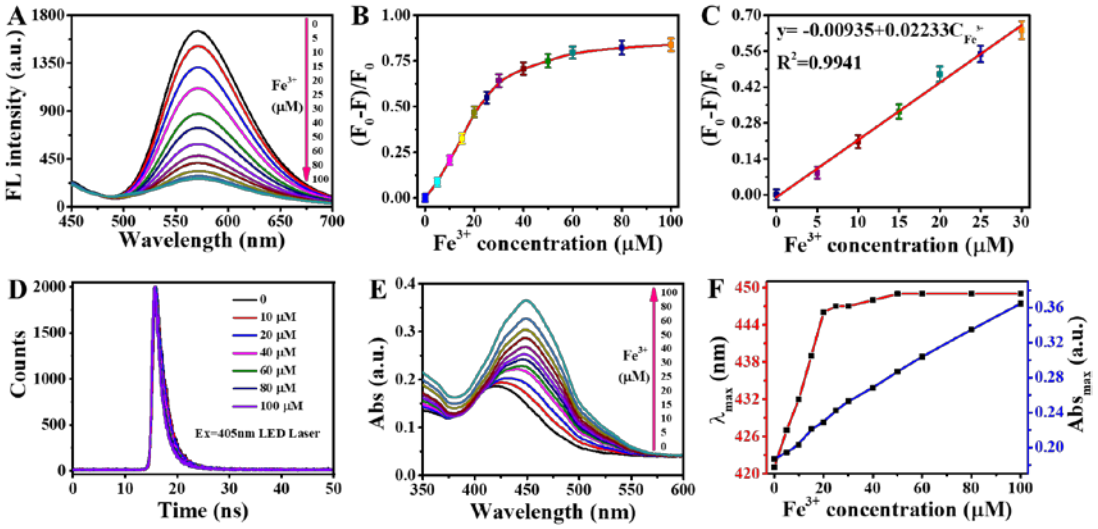

Figure S3. (A) Fluorescence spectra of *o*-CDs adding different concentrations of Fe^{3+} (0 – 100 μM). (B-C) Plot of the fluorescence intensity of *o*-CDs against Fe^{3+} concentration. Time-resolved fluorescence decay curves (D), UV-vis absorption spectrum (E), and absorption wavelength (F) of *o*-CDs versus different concentrations of Fe^{3+}.

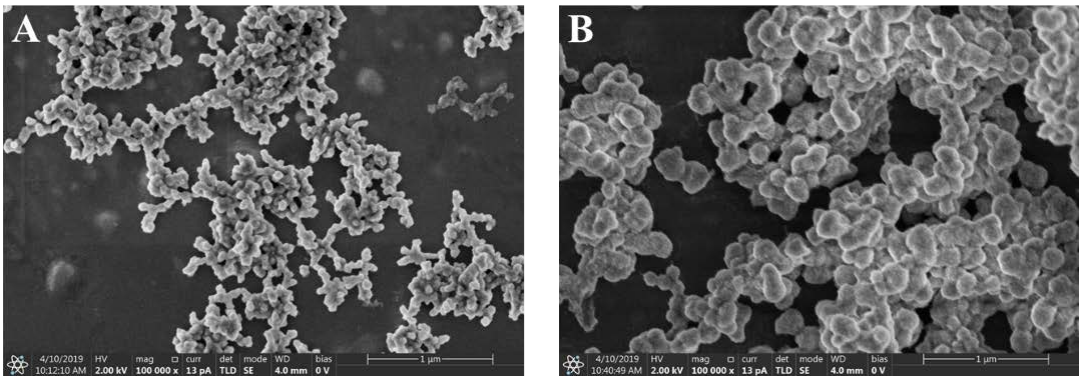

Figure S4. SEM of nanoparticles were formed by the interaction of *o*-CDs and Fe^{2+} (A) and (*o*-CDs + Fe^{2+}) + H_2O_2 (B).

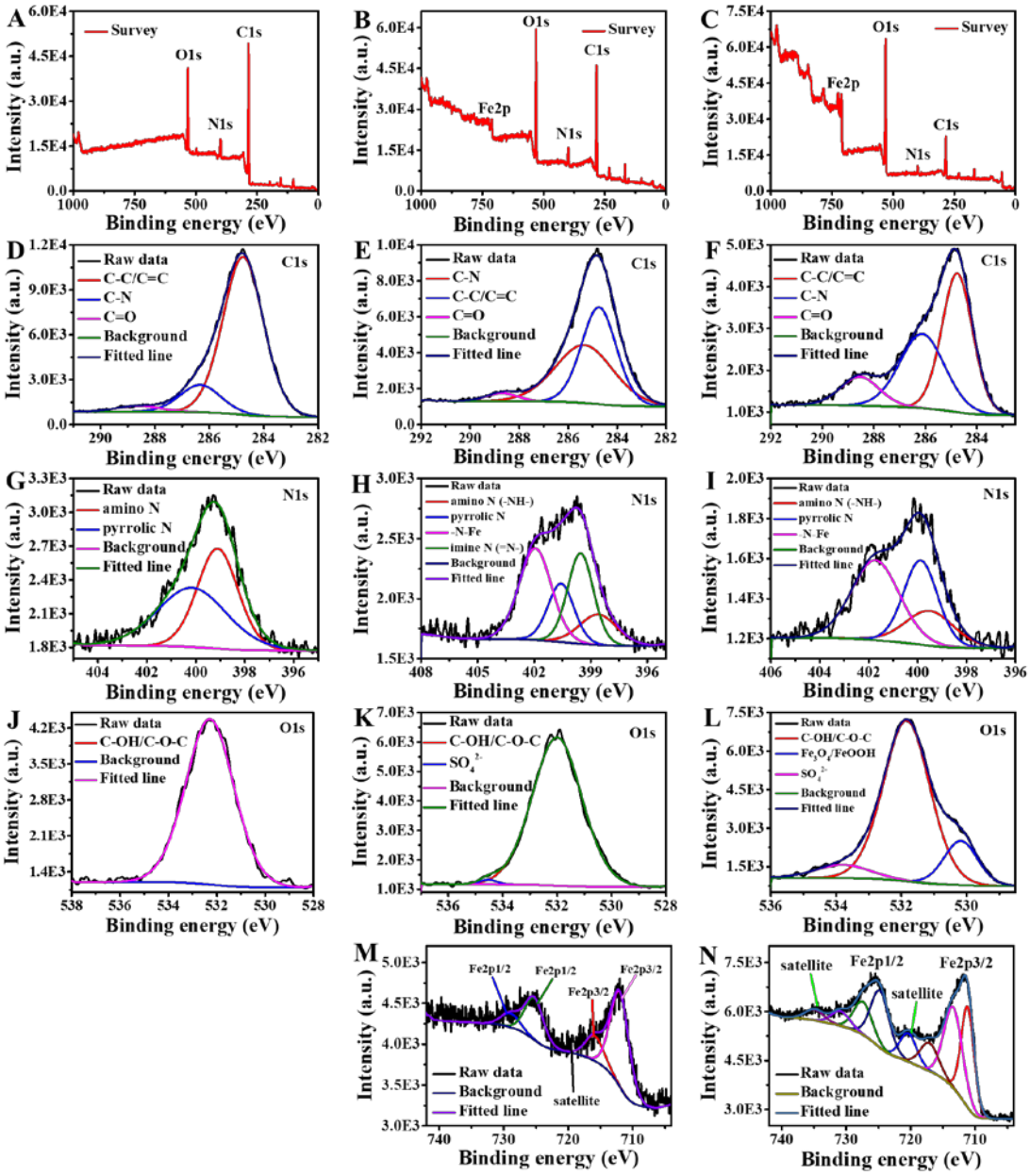

Figure S5. The XPS spectra of *o*-CDs responding to Fe^{2+} and H_2O_2. The survey XPS spectra (A-C), narrow scan spectra of C 1s (D-F), N 1s (G-I), O 1s (J-L), and Fe 2p (M, N) of *o*-CDs.

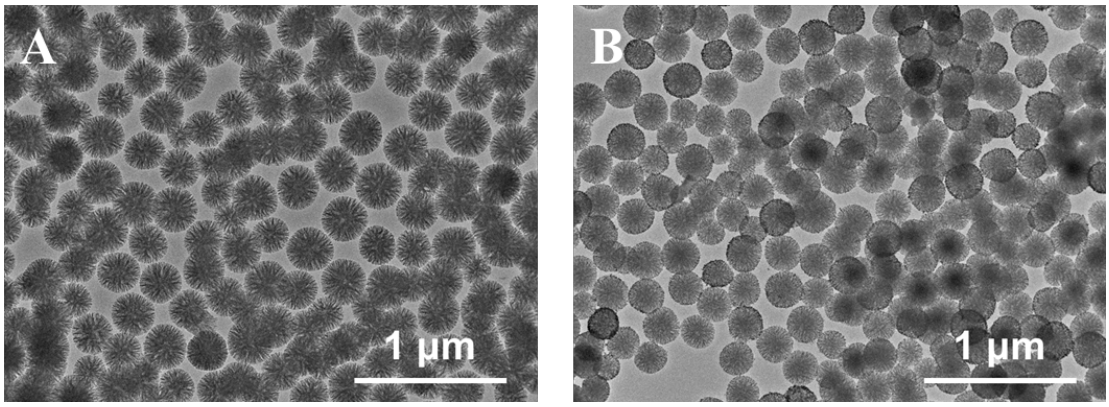

Figure S6. TEM of DMSN (A) and DMSN-Au NPs (B).

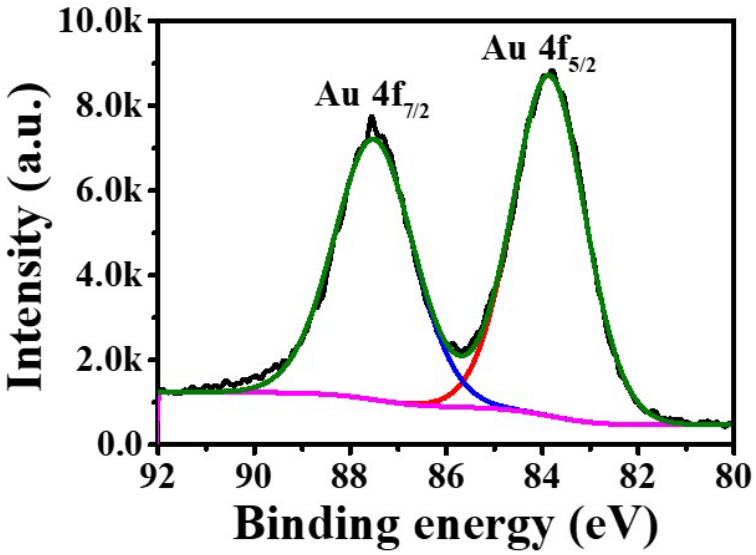

Figure S7. The XPS spectra of Au 4f in DMSN-Au NPs.

Table S1. Comparison of the sensing performance of different methods for detecting CEA.

Method	Linear range (ng/mL)	LOD (ng/mL)	Reference
SERS	1-1000	1.0	[4]
Electrochemical	100-10^5	0.14	[5]
Colorimetric	0.05-100	0.0211	[6]
Chemiluminescence	0.1-64	0.085	[7]
Fluorescence	0.1-80	0.0745	**This work**

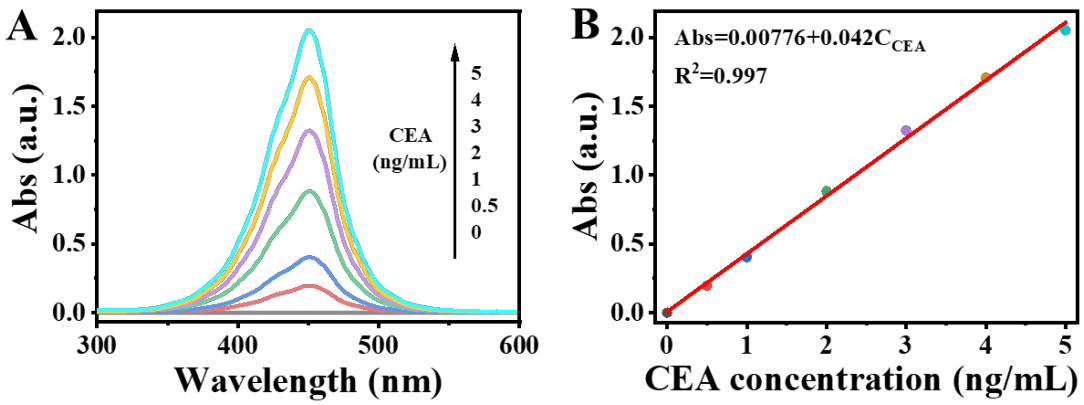

Figure S8. The commercially available human CEA ELISA kit was applied for the detection of different concentrations of CEA. (A) The UV-vis absorption spectrum of TMB adding different concentrations of CEA (0–5.0 ng/mL). (B) Plot of the absorption of TMB against CEA concentration. The maximum absorption wavelength of TMB is located at 450 nm.

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