Supporting Information

Carbon Dots-Based Fluorescent Nanoprobe for Associated Detection of Iron Ion and Determination the Fluctuation of Ascorbic Acid Induced by Hypoxia in cells and *in Vivo*

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1. General Experimental Section

1.1 Instruments.

Fluorescence spectra were obtained by a HORIBA Scientific Fluoromax-4 spectro fluorometer with a Xenon lamp and 1.0-cm quartz cells. Absorption spectra were measured on Thermo Scientific NanoDrop 2000/2000C spectrophotometer. All pH measurements were performed with a basic pH-Meter PH-3C digital pH-meter (Lei Ci Device Works, Shanghai) with a combined glass-calomel electrode. MTT Assay was carried out by a microplate reader (Tecan, Austria). The fluorescence images of cells were taken using a confocal laser scanning microscope (Japan Olympus Co., Ltd) with an objective lens (×60). The fluorescence images of zebrafish were taken using a confocal laser scanning microscope with an objective lens (×20). The fluorescence images of mice liver slices were taken using a confocal laser scanning microscope with an objective lens (×20). Flow cytometry and intracellular fluorescence detection was carried out on flow cytometry (Aria, BD) with excitation at 405nm and emission at 420-500 nm. The mean particle size was determined by DLS Zetasizer Nano ZS90 (Malvern Instruments Ltd, UK) and by TEM (JEOL, model JEM-1230, Japan). Citric acid was heated by a 1600 °C box type muffle furnace (SGM, M15/16, Zhengzhou).

1.2 Materials

Ascorbic acid (AA), vitamin A, vitamin B_1, vitamin B_2, vitamin B_6, biotin, folic acid, vitamin B_{12}, inositol, vitamin D, tocopherol, vitamin K, glutathione, L-cysteine, tryptophan, glycine, alanine, cystine, leucine, L-serine, histidine and desferrioxamine B were purchased from Sigma-Aldrich (USA). All the solutions were prepared using reagent analytical grade and deionized-distilled water was used throughout. 2-methylimidazole, Mercaptopropionic acid (MPA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxy succinimide (NHS) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore, Bedford, MA, USA). The stock solutions of CDs-DB, CDs-DB@Fe were solute in ultrapure water and maintained in refrigerator at 4 °C. Other chemicals were purchased from Sigma-Aldrich unless otherwise stated and straightforward used without further purification, unless otherwise stated. HEPES was obtained from Aladdin. All reactions were performed under argon protection and dark. Human hepatocarcinoma cell line (HepG2 cells) was obtained from the Committee on Type Culture Collection of the Chinese Academy of Sciences. The antibody of hypoxia inducible factor 1-1α (HIF-1α) and β-actin were obtained from Cell Signaling Technology (Beverly, MA. USA).

1.3 Assay procedure

The CDs-DB powder was dissolved in HEPES buffer (10 mM, pH 7.4) with a concentration of 0.1 mg/mL. Metal ions aqueous solutions containing Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+}, Fe^{2+}, Cu^{2+}, Ca^{2+}, Fe^{3+}, Ag^{+}, Mg^{2+}, Mn^{2+} and Hg^{2+} were prepared, respectively, with a concentration of 5 mM. In a typical assay, 10 μL of metal ion solution was mixed with 90 μL of the CDs-DB solution, and equilibrated for 5 min at room temperature before the fluorescence spectral measurements. The sensitivity for Fe^{3+} was confirmed by adding of serial concentrations of Fe^{3+} in a similar way. The control sample was prepared by mixing 10 μL of pure water with 90 μL of the CDs-DB solution.

For the AA detection, 90 μL of the CDs-DB solution (0.1 mg/mL) was mixed with 10 μL Fe^{3+} solution (1 mM) for 5 min, and then 10 μL of serial concentration of AA were added. After equilibrated for 5 min, the fluorescence spectra were recorded. The selectivity for AA was confirmed by adding other proteins stock solutions (vitamin A, vitamin B_1, vitamin B_2, vitamin B_6, biotin, folic acid, vitamin B_{12}, inositol, vitamin D, tocopherol, vitamin K, glutathione, L-cysteine, tryptophan, glycine, alanine, cystine, leucine, L-serine, histidine) instead of AA in a similar way. The fluorescence spectra were recorded under excitation at 405 nm and all

experiments were performed at room temperature.

1.4 Spectroscopic Methods.

UV-visible spectra were obtained with 1.0-cm glass cells. The nanoprobe CDs-DB (0.1 mg/mL) was dissolved in HEPES buffer (10 mM, pH 7.4), and added to a 10.0-mL color comparison tube. Different concentrations of Fe^{3+} were added to 10.0-mL color comparison tube. CDs-DB@Fe was also performed as above and different concentrations of AA were added to 10.0-mL color comparison tube. Fluorescence spectra were obtained with a Xenon lamp and 1.0-cm quartz cells. The CDs-DB (0.1 mg/mL) was dissolved in HEPES buffer (10 mM, pH 7.4), and added to a 5.0-mL color comparison tube. Different concentrations of Fe^{3+} were added. After the CDs-DB@Fe was also performed as above and different concentrations of AA were added to 10.0-mL color comparison tube. The mixture was incubated for 20 min before measurement. Then the fluorescence emission spectra were integrated from 420 to 650 nm with excitation at 405 nm.

1.5 Cell Lines and Culture.

Human hepatocarcinoma cell line (HepG2) was obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). HepG2 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 ^{o}C, incubated in a humidified incubator (Thermo Scientific 3111, USA) in a humidified atmosphere containing 5% CO_2. Fluorescent images were acquired on an Olympus FluoView FV1000 confocal laser-scanning microscope (Japan) with an objective lens (×60). The excitation wavelength was 635 nm. Cell imaging was carried out after being washed with PBS for three times.

1.6 Cell Staining Procedures

HepG2 cells were seeded in a flatbottom 6-well plate with glass coverslips in 2 mL culture medium. After overnight incubation, the cells were treated with CDs-DB@Fe for 1 h at 37 °C and then washed with PBS buffer (pH = 7.4) to remove excess CDs-DB@Fe. The treated cells were then incubated with AA (50 μM) for another hour. Then the cells on the plate were washed with PBS buffer. After being washed three times with PBS buffer, the cells were imaged under a fluorescence microscope. Cells incubated with CDs-DB@Fe for 1 h served as a control. Excitation wavelength of HepG2 cells was 405nm, and the emission was collected from 450nm to 550nm.

1.7 Flow cytometry.

FCM assay was carried out for the detection of the intracellular AA by CDs-DB@Fe. The HepG2 cells were cultured at 2.0×10^5 cells/well in 6-well plates, and treated with 0.1 mg/mL CDs-DB@Fe for 15 min at 37 °C. After harvest, cells were washed, and resuspended in PBS and analyzed by flow cytometry. And then the cells were treated with AA for 1 h. After harvest, cells were washed, and resuspended in PBS and analyzed by flow cytometry. Excitation wavelength was 405 nm. The collected wavelengths were 450 - 550 nm.

1.8 Cytotoxicity of CDs-DB and CDs-DB@Fe

The cytotoxicity of CDs-DB was assessed by the MTT assay. HepG2 cells were cultured in DMEM supplemented with 10% FBS in an atmosphere of 5% CO_2 and 95% air at 37 ^{o}C. The cells (8000/cell) were plated into 96-well plates and allowed to adhere for 24 hours. Subsequently, the cells were incubated with 0, 40, 80, 120, 160 and 200 μg/mL (final concentration) of CDs-DB at 37 ^{o}C in an atmosphere of 5% CO_2 and 95% air for 24 h. An untreated assay with DMEM was also performed under the same conditions. MTT solution (5.0 mg/mL in PBS, 20 μL) was added to each well, and 4 h later, the remaining MTT solution was carefully removed. In addition, DMSO (150 μL) was added to each well to dissolve the formazan crystals. The plate was shaken for 10 min and the absorbance was measured at 570 nm and 630 nm using a microplate reader (TECAN infinite M200pro).

1.9 Western Blot.

1×10^6 HepG2 cells were seeded in 6-well plate and incubated overnight. They were treated with different O_2 concentration by an AnaeroPackTM and multi gas incubator (Sanyo). For comparison, the control cells were treated in an atmosphere of 5% CO_2 and 95% air at 37 ^oC. After 4 h, all cells were washed with PBS, protein extracts were prepared by suspending the cells in 200 μL RIPA lysis buffer containing 1% PMSF (Solarbio, China) and 20% PhosSTOP (Roche, Germany). Then the extracts were quantified with BCA protein assay kit (Biogot, China). After denatured, the equal amounts of protein were electrophoresed on 10% SDS-polyacrylamide gels (Bio-Rad, USA) and transferred to PVDF membranes. The membrane was incubated with 5% BSA (Sigma-Aldrich, USA) and incubated with primary antibodies overnight at 4 °C with gentle shake. A horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology, USA) was used to mirror the quantity of proteins and signals were detected with an enhanced chemiluminescence (ECL) detection system. The results were analyzed by Image J to acquire the grey value of every bond. The primary antibodies (dilution) were incubated, as follows: hypoxia inducible factor 1-1α (HIF-1α) (abcam, 1/1000), β-actin (Mouse, Sigma) (1/1000), followed by secondary antibody incubation.

1.10 Imaging of Zebrafish and Toxicity Analysis.

Four to five pairs of zebrafish were placed in crossing tanks for spawning overnight. Embryos were settled to the bottom of the tank, and were collected using a sieve and transferred to petri dishes for embryo culture. They were screened, incubated at 27 °C, 0.4% CO_2 and grown in egg water (10% NaCl; 1.63% $MgSO_4\cdot7H_2O$; 0.4% $CaCl_2$; 0.3% KCl). After 22 h postfertilization, PTU was added to prevent melanin formation to yield optically transparent fish. 3 d postfertilization the embryos were seeded to into 96 well plates at 1 embryo per well. The embryos were soaked in 0.1 mg/mL of CDs-DB-Fe at various concentrations for 24 h and were imaged for uptake using confocal laser scanning microscope imaging (Japan Olympus Co., Ltd) using 405 nm. Before imaging, 1 mM AA was added to zebrafish.

1.11 H&E staining.

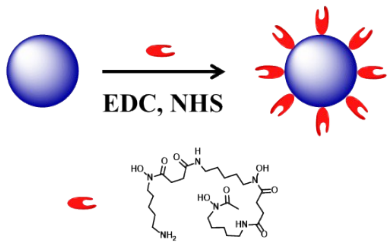
Heart, liver, spleen, lung and kidney of tumor-bearing mice and normal mice in each group, tumor tissue of tumor-bearing mice in each group were all excised and fixed in 10% formaldehyde and embedded in paraffin and stained with hematoxylin and eosin (H&E) to confirm histology. Then the treated liver tissue of liver ischemia mice model were prepared to frozen sections and stained with CDs-DB@Fe to confirm the amount of AA.

2. The Comparison of Such Nanoprobe of Recent Reports

Table S1. Comparison of several features of recent reports.

Reports	LOD (Fe^{3+})	LOD (AA)	Ligand	In cells	In tissue	Model
Raveendran et al.[1]	374 nM	79 nM	No	No	No	No
Shamsipur et al.[2]	13.7 nM	82 nM	No	Yes	No	No
Shi et al.[3]	27.8 nM	1.8 mM	No	Yes	No	No
Xu et al.[4]	NG	80 nM	No	No	No	No
Anjali Devi et al.[5]	25.5 zM	18.4 pM	No	No	No	No
Guo et al.[6]	18 nM	86 nM	No	No	No	No
This work	45 nM	80 nM	Yes	Yes	Yes	Yes

3. Synthetic Procedures and Characterization Details of CDs-DB



Scheme S1. Synthetic Approaches of CDs-DB.

4. Effect of pH Values on CDs-DB and CDs-DB@Fe

As a starting point, it is necessary to understand the pH effect on the potential fluorescence behaviour of our nanoprobe CDs-DB and CDs-DB@Fe. The results demonstrate that fluorescence intensity of CDs-DB and CDs-DB@Fe shows pH-dependent property over the pH range from 3.0-10.0 (Figure. S1). The fluorescence intensity of CDs-DB shows almost no effect with the changed pH value. As shown in Figure S2, after the CDs-DB@Fe was incubated with AA for 20 min, the fluorescence intensity increased dramatically and stayed at high level before pH 7.0, and then decreased gradually with the increase of pH values. When pH ranges from 7.0 to 8.0, the fluorescence intensity of CDs-DB@Fe + AA is still about 10 fold higher than that of only CDs-DB@Fe, which indicates that CDs-DB@Fe +AA has an ideal pH stability in pH ranges from 5.0 to 8.0.

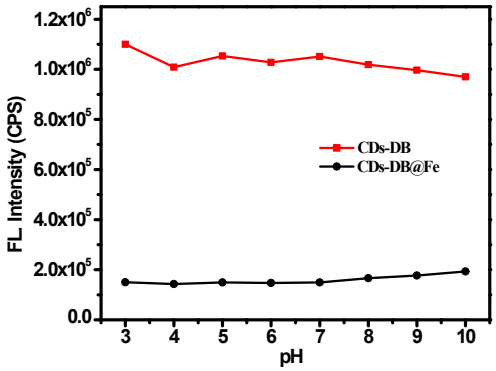


Figure S1. Effect of pH values on CDs-DB and CDs-DB@Fe. pH ranges from 3.0 to 10.0.

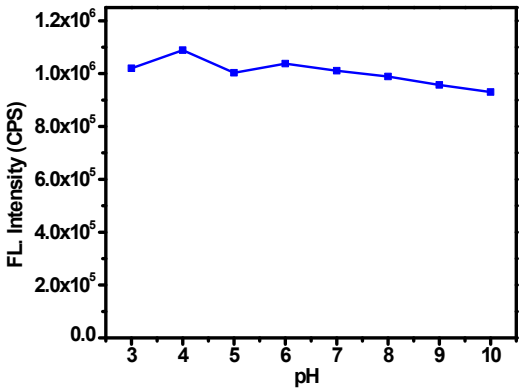


Figure S2. Effect of pH values on CDs-DB@Fe + AA. pH ranges from 3.0 to 10.0.

5. Selectivity of CDs-DB to Fe^{3+}

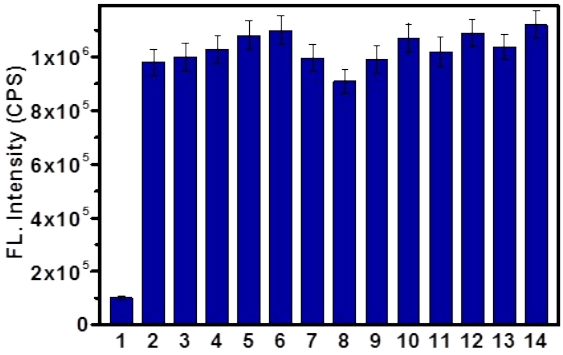


Figure S3. Fluorescence response of CDs-DB (10 μg/mL) to Fe^{3+} and various metal ions. 1. Fe^{3+} (50 μM), 2. Cd^{2+} (100 μM), 3. Zn^{2+} (100 μM), 4. Co^{2+} (100 μM), 5. Ni^{2+} (100 μM), 6. Pb^{2+} (100 μM), 7. Fe^{2+} (100 μM), 8. Cu^{2+} (100 μM), 9. Ca^{2+} (100 μM), 10. Ag^{+} (100 μM), 11. Mg^{2+} (100 μM), 12. Mn^{2+} (100 μM), 13. Hg^{2+} (100 μM), 14. Blank. All data were acquired in 10 mM HEPES (pH 7.4) at 37 °C after maintained 10 min (λ_{ex} = 405 nm, λ_{em} = 470 nm.).

6. Selectivity of CDs-DB@Fe towards Common Anion

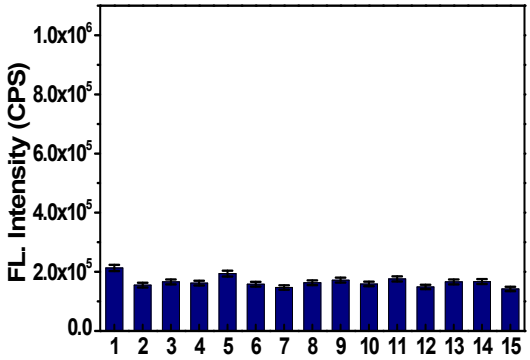


Figure S4. Fluorescence response of CDs-DB@Fe (10 μg/mL) towards various some common anion (100 μM). 1. OH^-, 2. NO_3^-, 3. SO_4^{2-}, 4. HSO_4^-, 5. CO_3^{2-}, 6. HCO_3^-, 7. SO_3^{2-}, 8. HSO_3^-, 9. ClO^-, 10. PO_4^{3-}, 11. F^-, 12. Cl^-, 13. Br^-, 14. I^-, 15. Blank. All data were acquired in 10 mM HEPES (pH 7.4) at 37 °C after maintained 10 min (λ_{ex} = 405 nm, λ_{em} = 470 nm.).

7. Cytotoxicity of CDs-DB

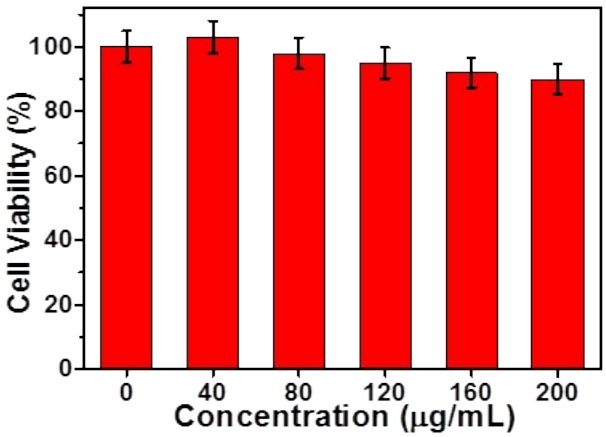


Figure S5. The 24 h cell viability for CDs-DB, the concentration of CDs-DB was 0, 40, 80, 120, 160 and 200 μg/mL. Data are presented as mean ± SD (n = 5).

8. Cytotoxicity of CDs-DB@Fe

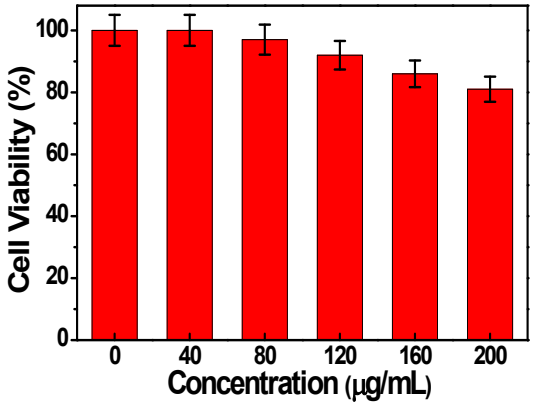


Figure S6. The 24 h cell viability for CDs-DB@Fe, the concentration of CDs-DB@Fe was 0, 40, 80, 120, 160 and 200 μg/mL. Data are presented as mean ± SD (n = 5).

9. The Quantification of Fluorescence Intensity of Fig. 3a, 3c and Fig. 6b

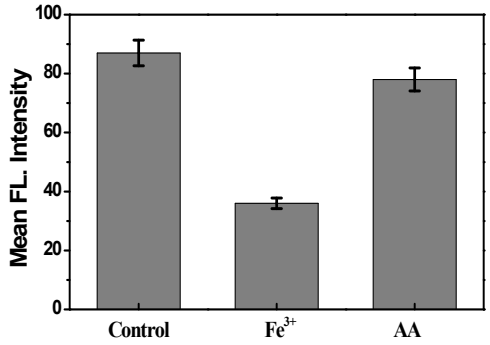


Figure S7. The quantification of fluorescence intensity of Fig. 3a. Data are presented as mean ± SD (n = 5).

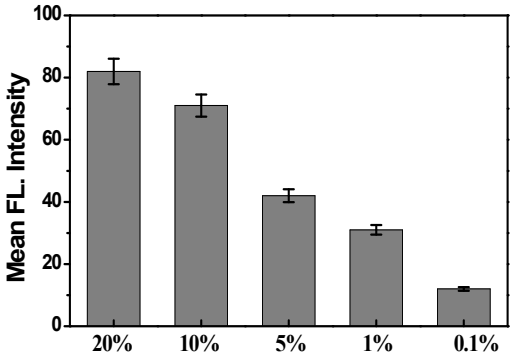


Figure S8. The quantification of fluorescence intensity of Fig. 3c. Data are presented as mean ± SD (n = 5).

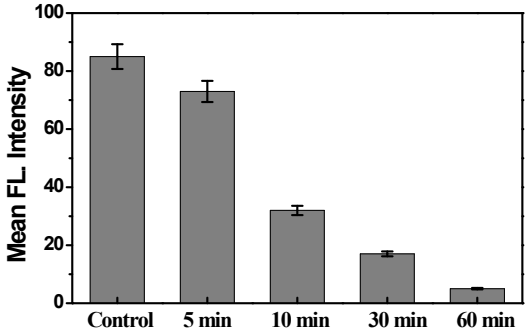


Figure S9. The quantification of fluorescence intensity of Fig. 6b. Data are presented as mean ± SD (n = 5).

10. References

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