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Electronic Supplementary Information

An Aggregation-Induced Emission Based pH-sensitive Fluorescent Probe for Intracellular Acidity Sensing

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Experimental section

Chemicals and materials

4-formyl-3-hydroxybenzoic acid and hydrazine monohydrate were purchased from Bide pharmatech and Aladdin, respectively. All other reagents were of analytical grade and used as received. Doubly distilled water was used throughout the experiments. Phosphate buffered solutions (PBS) were prepared by titrating 0.1 M phosphate solutions with a concentrated NaOH (5M) to the required pH values. The aqueous mixtures of HDBB for pH-responsive UV and PL tests were prepared by adding an aliquot of DMSO stock solution into aqueous buffers with specific pH values.

The sample for in-situ NMR was prepared by following process: firstly, HDBB (10 mg) was added in 0.5 mL of DMSO (A solution, for neutral medium study). And then KOH in D_2O (2M, 20μL) was added into A solution.

Cell culture medium Dulbecco's modified Eagle's medium (DMEM) from Thermo (Rockford, IL). LysoTracker Red RB-EDA (#C0104) from Bioluminor. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4,6-Diamidino-2-phenylindole (DAPI) from Sigma were used in this study. HepG2 liver cancer cells (ATCC HB-806) or human embryonic kidney cells (HEK293, ATCC CRL 11554) used in this study were from American Type Culture Collection (ATCC).

Apparatus

UV−vis absorption was characterized by a UV/vis/NIR spectrophotometer (Shimadzu, Japan) equipped. Fluorescence spectra were recorded on a Hitachi High-Technologies Corporation Tokyo Japan 5J2-0004 model F-7000 FL spectrofluorometer. Emission lifetimes were determined on an Edinburgh Analytical Instrument (FLS900 fluorescence spectrometer) with a light-emitting diode lamp (405 nm) and analyzed by the use of a program for exponential fits. All NMR spectra were recorded using a AVANCE DRX 500 NMR spectrometer (Bruker, Germany) operated at 500 MHz. Electrospray ionization mass spectra were obtained with a High Performance 1100 Liquid Chromatography-Mass Spectrometer

(Agilent Technologies, USA). The morphology of aggregations was characterized by Scanning Electron Microscopy (SEM) (Quanta, FEI). All pH measurements were made with a Model pHS-3C pH meter (Shanghai Precision& Scientific Instrument Co. Ltd, Shanghai, China). The treated cells were visualized under a Zeiss LSM5 EXCITER Confocal Laser Scanning Microscope equipped with a PlanApo □40/1.3 objective lens (Zeiss).

Synthesis of HDBB (Scheme 1a)

4-formyl-3-hydroxybenzoic acid (166.2 mg, 1.0 mmol) was dissolved in methanol (10 mL). Then hydrazine hydrate (24 μL, 0.5 mmol; 80%) was added and refluxed at 75 °C. After refluxing overnight, the resulting precipitate was filtered and washed with 30 mL methanol. After drying, HDBB was obtained as a yellow solid (120.5 mg, 73.5% yield). ^{1}H NMR (500 MHz, DMSO-d6), δ (TMS, ppm): 8.93(s, 2H), 7.60(d, 2H), 7.39(d, 2H), 7.38(s, 2H). ^{13}C NMR(125 MHz, DMSO-d6), δ (TMS, ppm):170.2, 163.5, 158.6, 144.0, 131.1, 121.2, 119.7, 117.6. Mass spectrometry: calculated for $C_{16}H_{12}N_2O_6$ $[M + H]^+$ 329.0729, found 329.0767 (high resolution). ^{1}H NMR (500 MHz, DMSO-d6), δ (TMS, ppm): 8.86(s, 2H), 7.49(d, 2H), 6.89(s, 2H), 6.69(d, 2H). ^{13}C NMR(125 MHz, DMSO-d6), δ (TMS, ppm):174.0, 171.6, 159.9, 142.9, 126.1, 126.1, 135.5, 123.0, 113.5.

Fluorescence quantum yield

The fluorescence quantum yield was calculated from the relation:

$$\phi_x = \phi_s \left(\frac{n_x}{n_s}\right)^2 \left(\frac{A_s}{A_x}\right)\left(\frac{F_x}{F_s}\right)$$

Where the subscripts s and x indicate the standard and text respectively, ϕ is the fluorescence quantum yield, A corresponds to the absorbance of the solution, F is the fluorescence intensity, and n is the refractive index of the solvent. The quantum yields of orange and green emission of this probe are 5.32% and 1.56%, respectively.

Cell culture experiment

HepG2 hepatocellular carcinoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Wel Gene), penicillin (100 units/mL), and streptomycin (100 μg/mL). The cell line was incubated at 37 °C in a humidified atmosphere consisting of 5/95 (v/v) CO2/air.

MTT Assays

A number of different cancer cell lines were seeded in 96-well plates. Untreated and pH-sensitive fluorescent probe (HDBB)-treated cells were stained with 0.05 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and measured at 570 nm with an automated microplate reader (Thermo).

Confocal microscopy

To assess uptake of the pH-sensitive fluorescent probe (HDBB), HepG2 hepatocellular carcinoma cells were seeded on culture plate and grew approximately 24 h, before incubating with HDBB (20 μM) for 2 h. Furthermore, LysoTracker was added in the medium with a dilution ratio of 1000: 1 for 1 h. The treated cells were washed with PBS pH 7.4 for three times, and were further visualized under a Zeiss LSM5 EXCITER Confocal Laser Scanning Microscope equipped with a Plan Apo □40/1.3 objective lens (Zeiss). The excitation wavelength of 405 nm and emission filter of 530-600 nm were applied for HDBB, while excitation wavelength of 543 nm and emission filter of 560-615 nm were applied for Lyso Tracker.

The determination of intracellular pH value

The pH value of the lysosome in Fig.4 has been calculated by the equation (J. Phys. Org. Chem. 2013, 26 858–862):

$$\log\left[\frac{I_{F,\,max}-I_F}{I_F-I_{F,\,min}}\right]=pH-pKa$$

Here, $I_{F,\,max}$, I_F and $I_{F,\,min}$ are the fluorescence intensity of maximum, unknown and minimum value, respectively. In our experiment, $I_{F,\,max}$ and $I_{F,\,min}$ were gotten by determining fluorescence intensity at 590 nm of HepG2 cells treated in pH 4.0 and pH 7.4 buffer medium, respectively. We have selected 22 different locations to determine the $I_{F,\,max}$, I_F and $I_{F,\,min}$ value. The detail data is shown as follow:

	Cytoplasm(pH=7.4, Fmin)	Lysosome(pH=?)	pH=4.0
	420	1754	1985
	452	1583	1928
	691	1734	2122
	542	1609	2062
	581	2160	2162
	379	1793	2147
	406	1996	2152
	265	1445	2162
	513	2063	2002
	364	1771	2072
	417	1721	1958
	600	1835	2162
	415	1550	1891
	479	2046	1709
	476	1008	1886
	453	1282	2160
	404	1043	2060
	569	2027	1943
	837	1404	2162
	616	1808	2156
	630	2181	2162
	422	1553	2200
	--	--	--
Mean	496.86	1698	2056.5
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Standard Deviation	127.61	326.67	127.82

The pKa value is 5.61. $I_{F,\,max}$, I_F and $I_{F,\,min}$ are 2056, 1698 and 496.86. Substitute these data into the equation, and then get the pH= 5.09 .

Development of HepG2 hepatocellular carcinoma tumor model in nude mice and in-vivo imaging

All animal experiments were carried out in accordance with guidelines approved by the Animal Care and Ethics Committee of Xiamen University. HepG2 hepatocellular carcinoma cells (1×10^6 tumor cells suspended in 200 μL PBS) were inoculated into subcutaneous of BALB/c nude mice with body weight of 18~21 g. When the tumor volume reached ~350 mm3 (~2 weeks), pH-sensitive fluorescent probe (HDBB) (50 μM in 200 μL normal saline) was injected through the tail vein into tumor-harboring nude mice. In Vivo Imaging System IVIS ® Lumina II (Caliper Life Sciences, Hopkinton, MA) was applied to collect the fluorescent images, which were performed

using a Cy5.5 channel. Spectral unmixing software was utilized to remove the auto-fluorescence. After 12 h, the mice were sacrificed. Furthermore, the tumor and major organs of nude mice were carefully isolated and imaged. The average Cy5.5 fluorescence intensities in the organs were quantified.

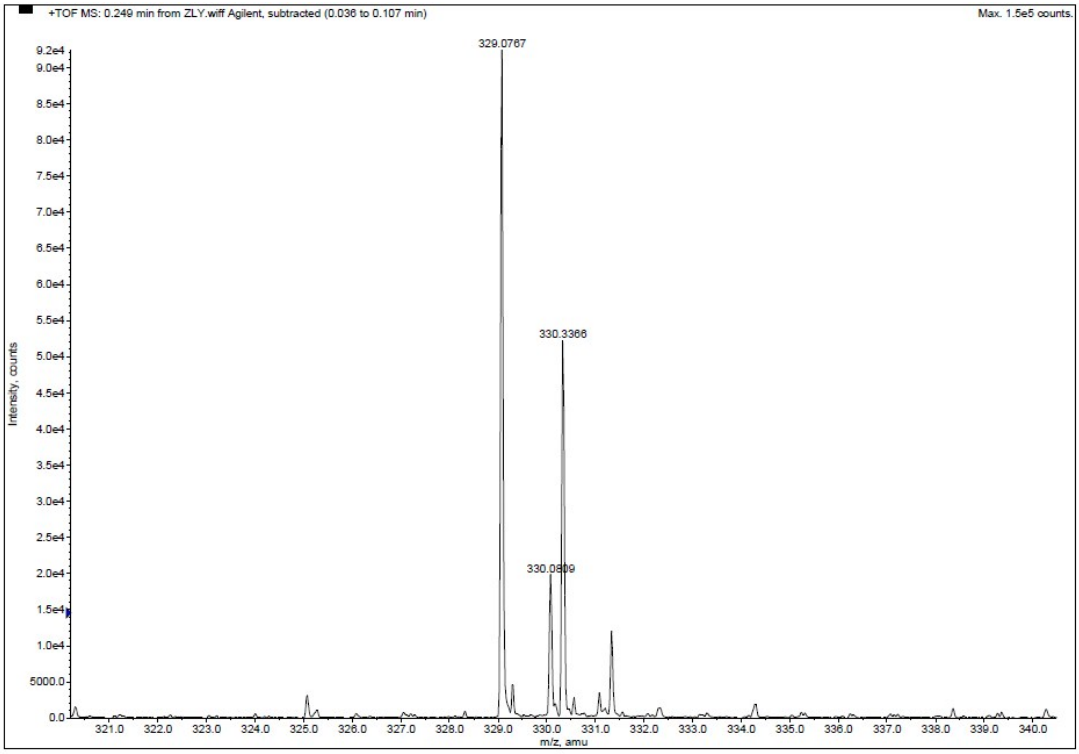

Fig. S1. High-resolution mass spectrum of HDBB.

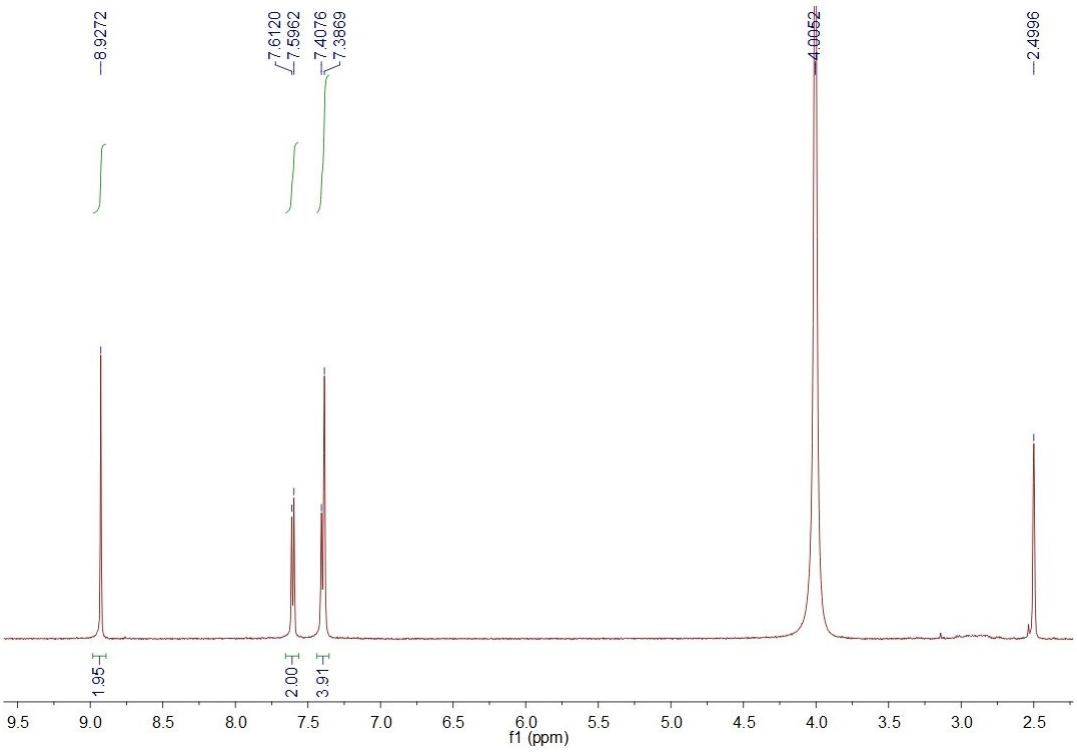

Fig. S2. ^{1}H NMR spectrum of HDBB in DMSO-$d_{6.}$

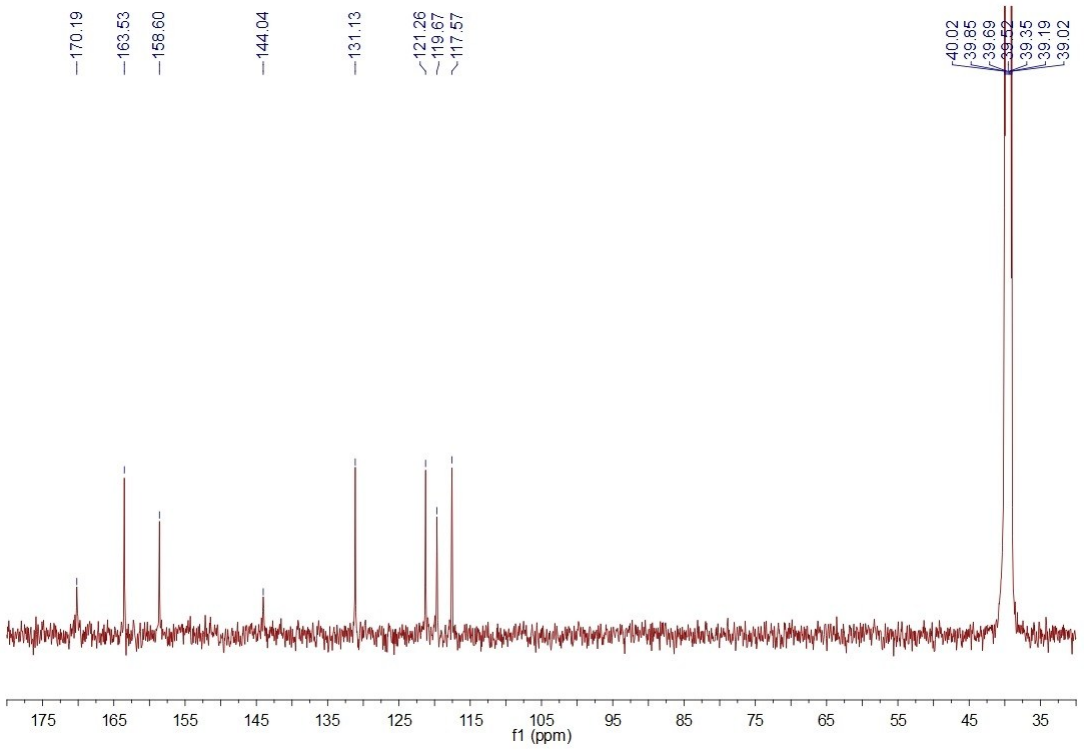

Fig. S3. ^{13}C NMR spectrum of HDBB in DMSO-d_6.

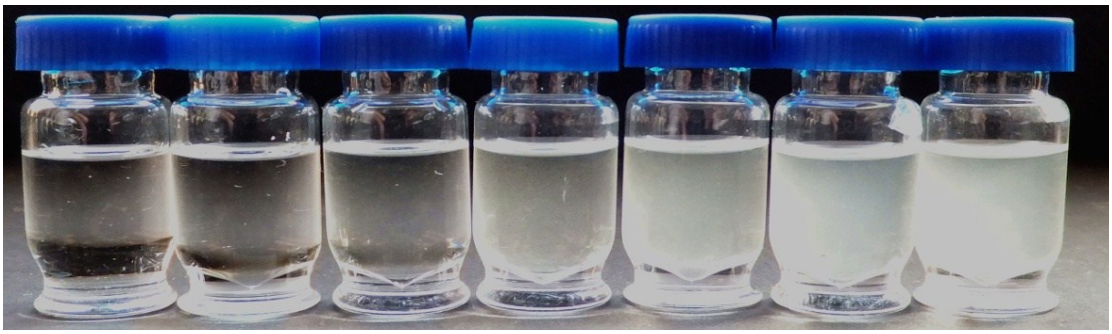

Fig. S4. The photographs of HDBB in different ratio mixtures of DMSO/methanol under day lamp. From left to right, the fm value of these solution are 0%, 30%, 50%, 60%, 70%, 80% and 90%, respectively.

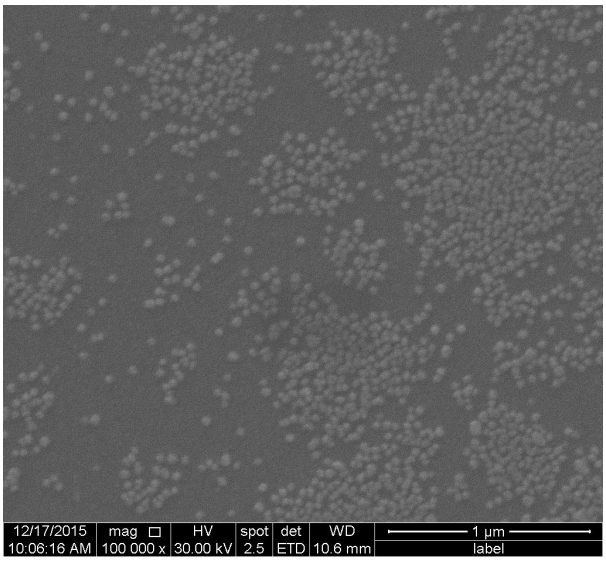

Fig. S5. SEM image of the aggregations of HDBB in the dimethyl sulfoxide /methanol solvent mixtures with 90% methanol.

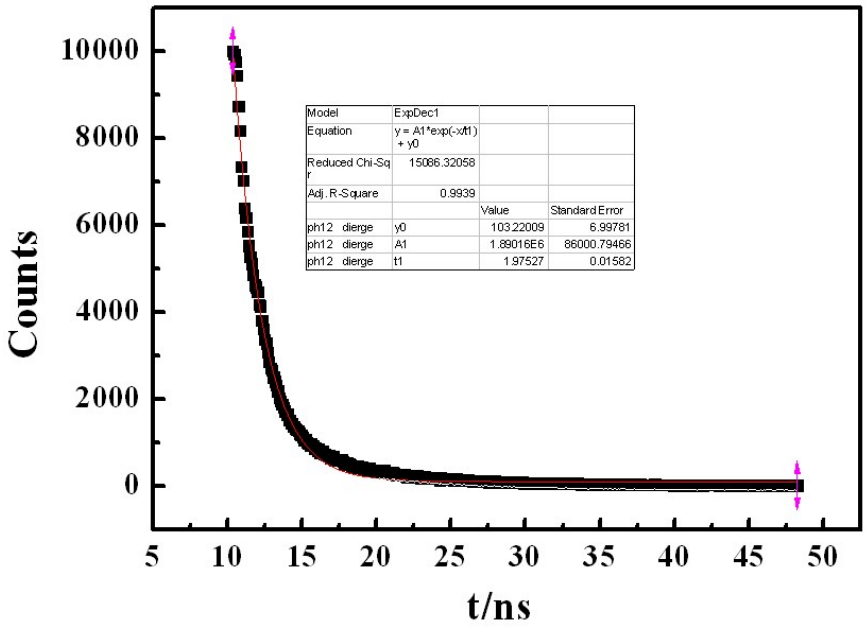

Fig. S6. Emission lifetime spectrum of HDBB in pH=5 PBS.

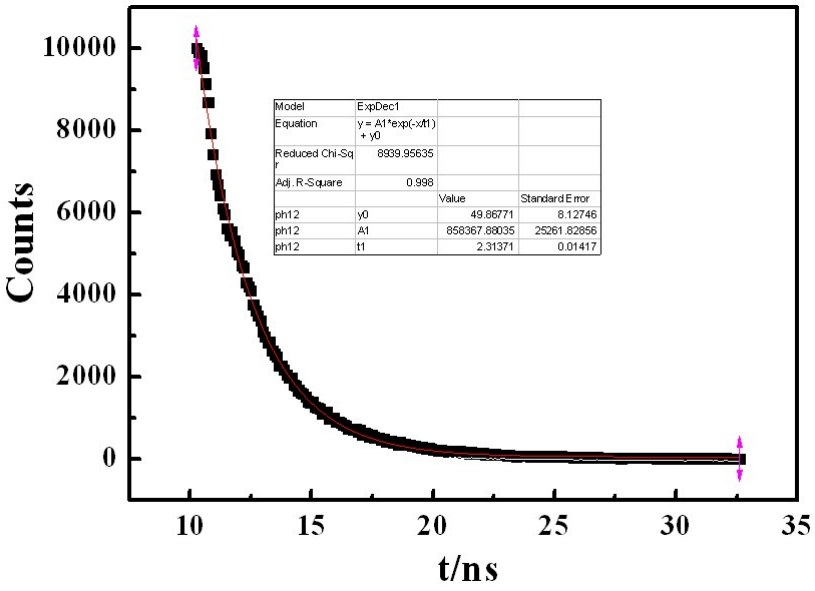

Fig. S7. Emission lifetime spectrum of HDBB in pH=12 PBS.

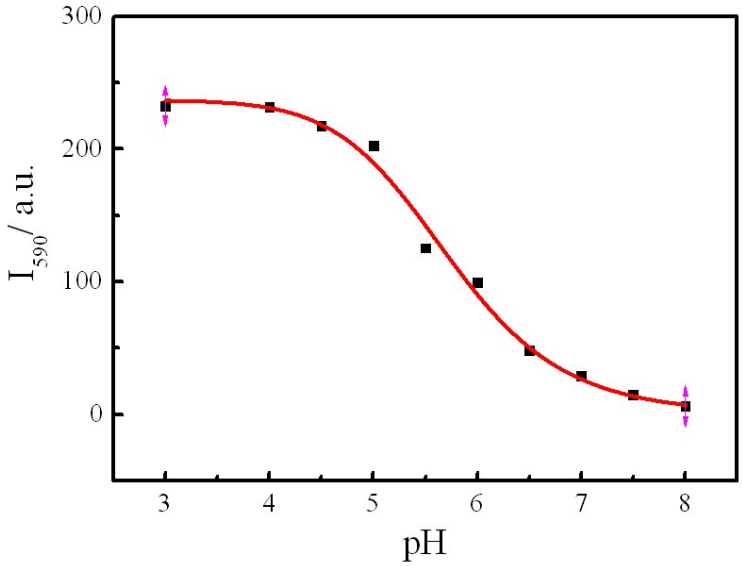

Fig.S8. The fluorescence emission changes at 590 nm with the pH titration curve of 25 μM HDBB in 0.1M PBS buffer solution (pH 3.0~8.0). Excitation was performed at 365 nm.

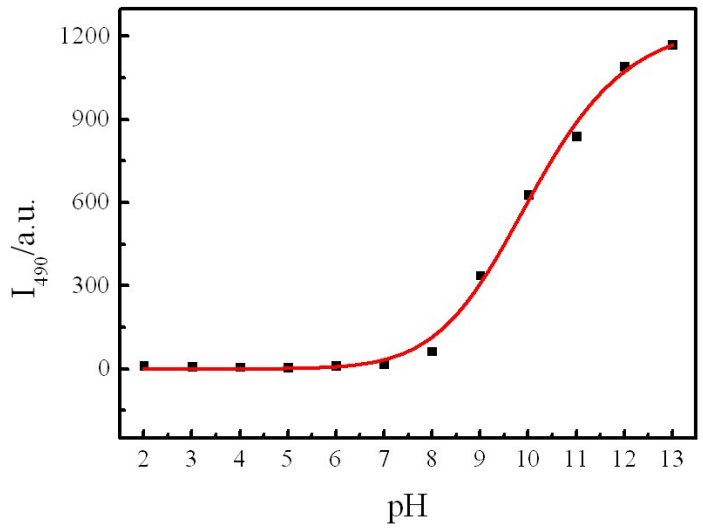

Fig.S9. The fluorescence emission changes at 490 nm with the pH titration curve of 25 μM HDBB in 0.1M PBS buffer solution (pH 2.0~13.0). Excitation was performed at 365 nm.

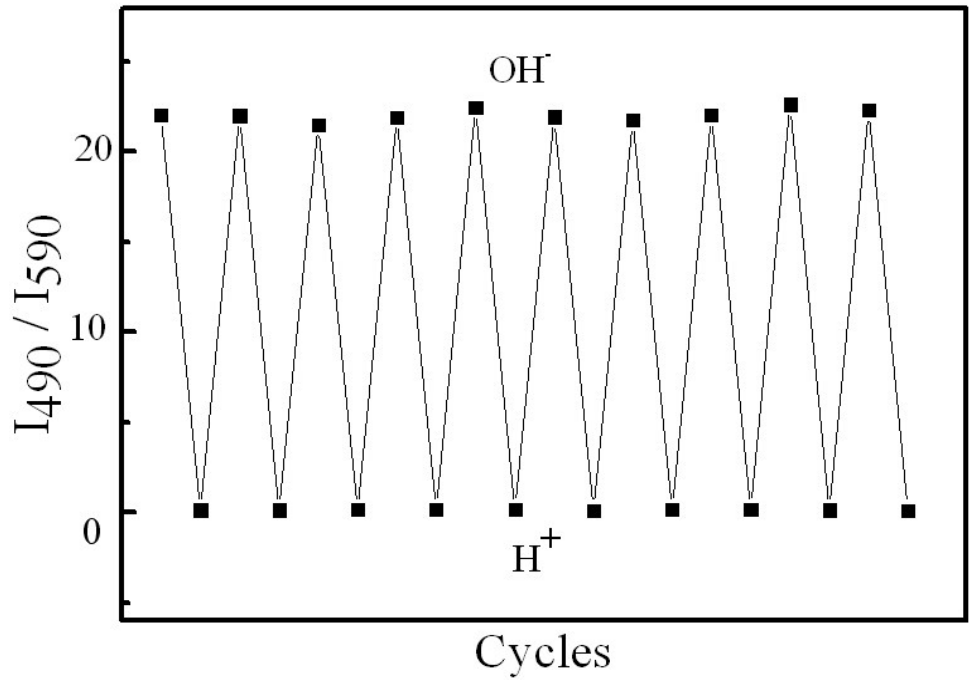

Fig. S10. Emission maximum intensity versus switching cycles.

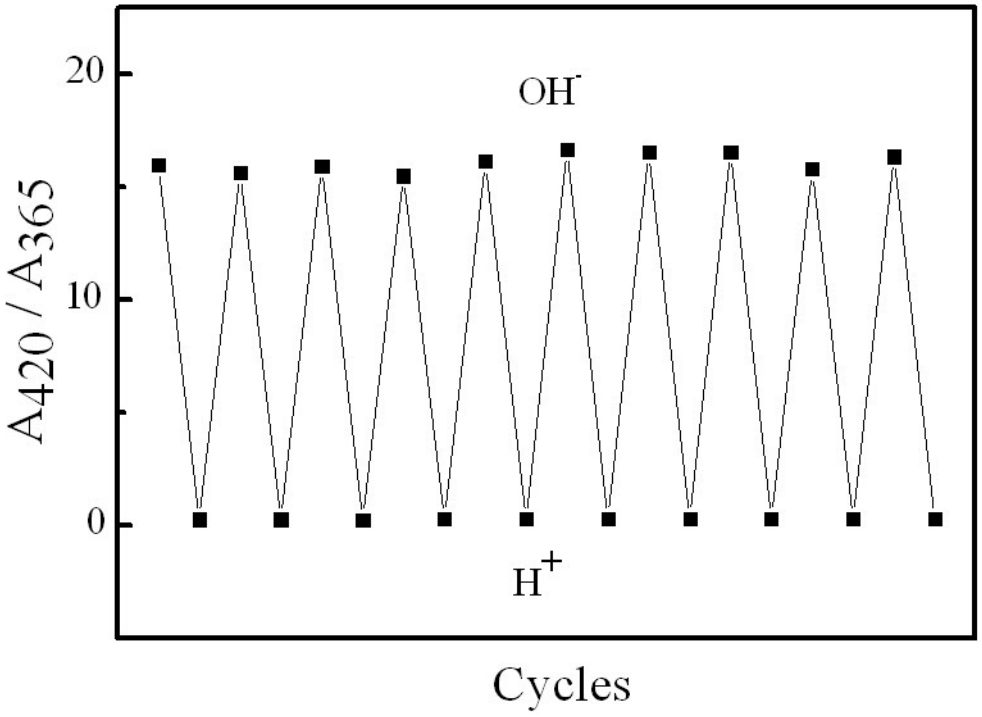

Fig. S11. UV-Visible peak intensity versus switching cycles.

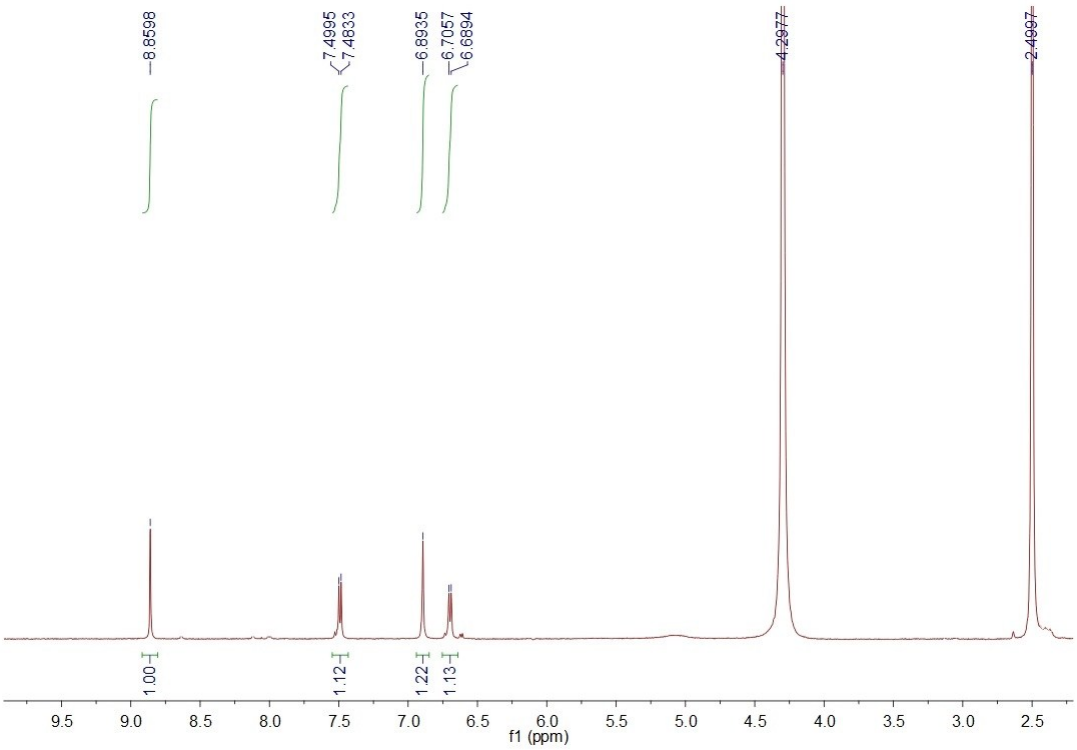

Fig. S12. ^{1}H NMR spectrum of HDBB in DMSO-d_6/KOH.

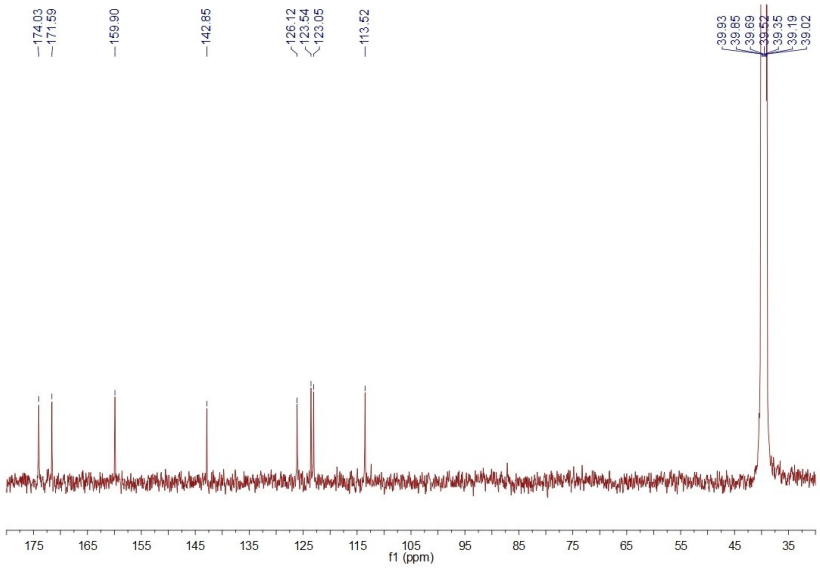

Fig. S13. ^{13}C NMR spectrum of HDBB in DMSO-d_6/KOH

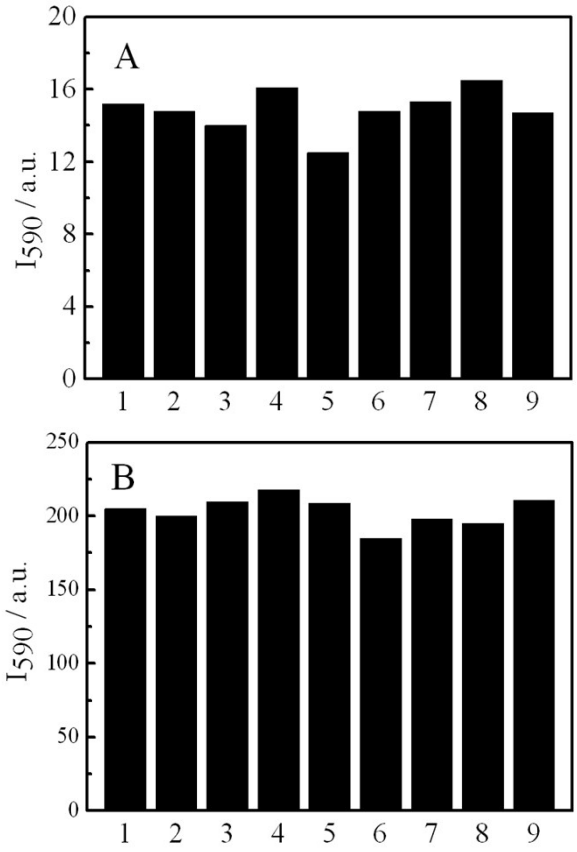

Fig. S14. Fluorescence intensity at 590 nm of HDBB (25 μM) in the absence or presence of 0.3 mM Zn^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, Cd^{2+}, 5 mM Mg^{2+}, Ca^{2+}, and 10 mM K^{+} ions in phosphate buffer solution at pH 7.40 (A) and 5.00 (B). 1: HDBB, 2: HDBB + Mg^{2+}, 3: HDBB + K^{+}, 4: HDBB + Zn^{2+}, 5: HDBB + Cu^{2+}, 6: HDBB + Fe^{3+},7: HDBB + Ca^{2+}, 8: HDBB + Mn^{2+}, 9: HDBB + Cd^{2+}. Excitation was performed at 365 nm.

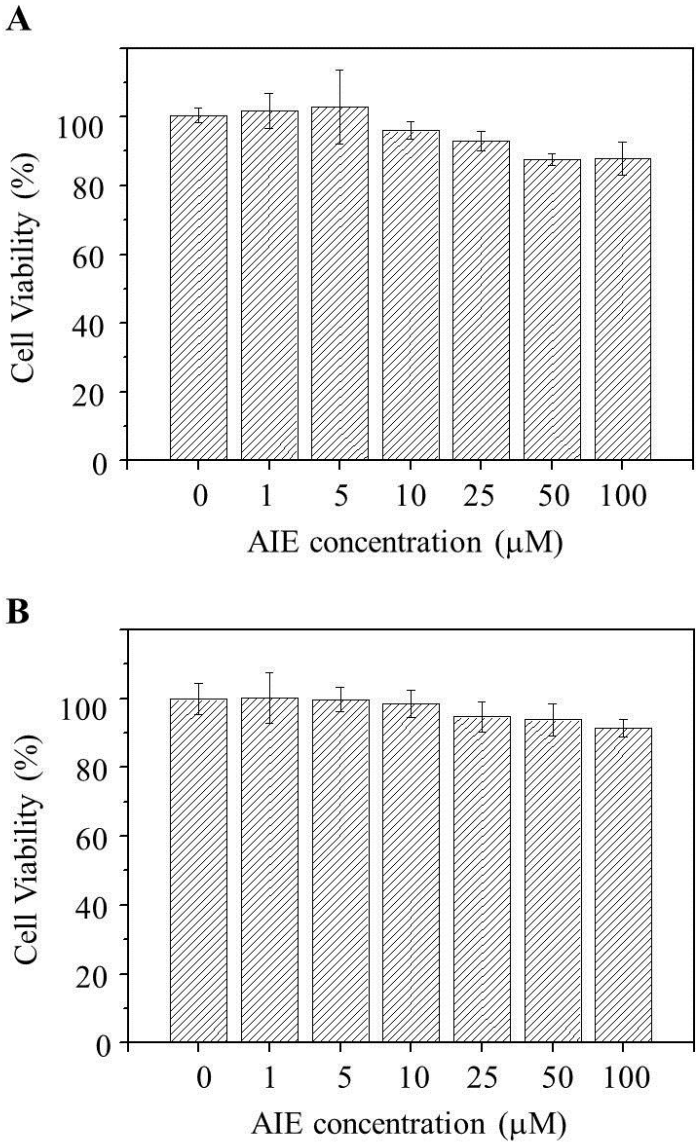

Fig. S15. Cell viability of HepG2 (A) and HEK293 (B) cells with incubation of HDBB with varying concentrations for 24 h.

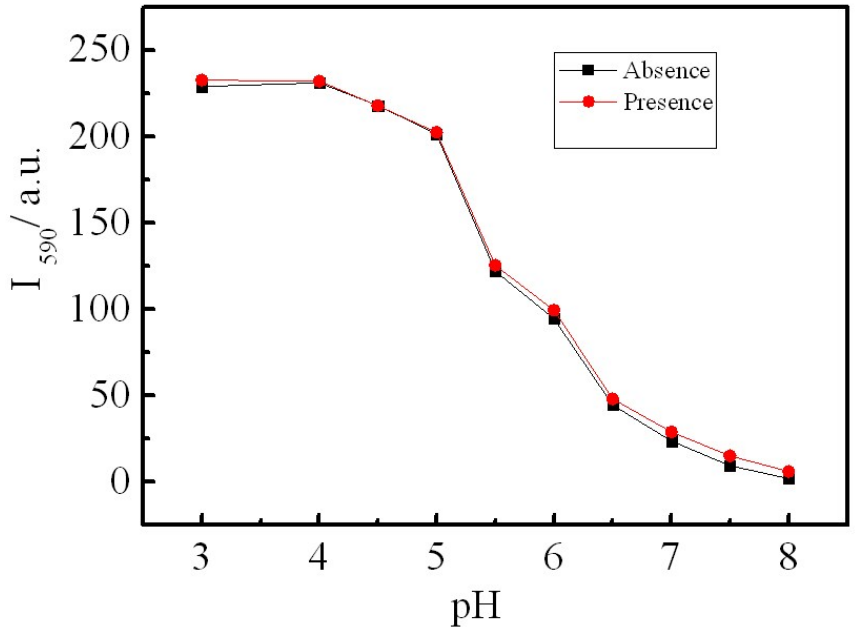

Fig. S16. The plots of fluorescence intensity at 590 nm of HDBB (25μM) against pH in the absence (black curve) and presence (red curve) of intracellular species (150 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM glucose, 10 mM $NaHCO_3$, 0.1mM H_2O_2 ,1mM glutathione and 1 mg/mL bovine serum albumin). Excitation was performed at 365 nm.