

## **A novel luminescent Ir (III) complex for dual mode imaging: synergistic response to hypoxia and acidity of tumor microenvironment**

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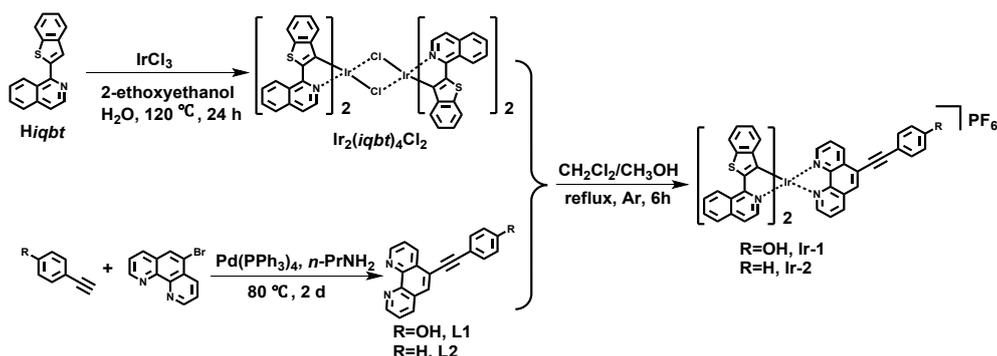
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## **Experimental section**

**General procedures:** All operations were carried out under a pure argon atmosphere using standard Schlenk techniques. All reagents were purchased from commercial sources and used without further purification. All other chemical reagents of analytical grade were used directly without further purification.

The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker DRX-400 at 298 K using deuterated solvents. Chemical shifts are given in ppm, and are referenced against internal TMS. High resolution mass spectrometric data were determined using an Agilent LTQ Orbitrap XL mass spectrometer. Fluorescence spectra were determined using a FluoroMax-4 spectrofluorometer (HORIBA) with a 5 nm slit for both excitation and emission. Lifetime was acquired through a time-correlated single photon counting (TCSPC) technique by using a HORIBA Jobin Yvon Fluorolog-3 modular spectrofluorometer. Absorption spectra were recorded using a Shimadzu UV-3100 spectrophotometer. All pH measurements of media were accomplished using a Model PHS-3C meter. Flow counters (HORIBA, STEC. SEC-E40JS. 60 SCCM) were used to control the oxygen concentration during the spectra. Confocal luminescence images were captured in the confocal laser scanning microscopy (CLSM, Zeiss LSM-710 microscope, Germany). The PLIM setup is integrated with Olympus FV1000 laser scanning confocal microscope. The lifetime values were calculated with professional software provided by PicoQuant Company.

## Synthetic procedures



**Synthesis and characterization of L1:** 4-Ethynylphenol was prepared according to the reported procedure.<sup>[1]</sup> In a pressure tube were added 5-bromo-1,10-phenanthroline (300 mg, 1.16 mmol), 4-ethynylphenol (274 mg, 2.32 mmol), Pd(PPh<sub>3</sub>)<sub>4</sub> (134 mg, 0.12 mmol), and n-propylamine (25 mL). After heating at 80 °C for 2 days, the reaction mixture was concentrated under reduced pressure. Chromatography on silica and recrystallization from methanol afforded **L1** as an off-white solid (C<sub>20</sub>H<sub>12</sub>N<sub>2</sub>O, 192 mg, 56%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 10.07 (s, 1H), 9.22 (dd, *J* = 4.3, 1.7 Hz, 1H), 9.11 (dd, *J* = 4.3, 1.7 Hz, 1H), 8.84 (dd, *J* = 8.2, 1.7 Hz, 1H), 8.50 (dd, *J* = 8.2, 1.6 Hz, 1H), 8.31 (s, 1H), 7.91 (dd, *J* = 8.2, 4.3 Hz, 1H), 7.80 (dd, *J* = 8.1, 4.3 Hz, 1H), 7.60 (d, *J* = 8.6 Hz, 2H), 6.89 (d, *J* = 8.7 Hz, 2H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>): δ 158.60, 150.58, 150.41, 145.34, 145.21, 136.06, 134.29, 133.43, 130.21, 127.96, 127.58, 123.86, 123.77, 119.22, 115.88, 111.87, 96.01, 84.01.

**Synthesis of Ir<sub>2</sub>(iqbt)<sub>4</sub>Cl<sub>2</sub>:** 1-(Benzo[b]thiophen-2-yl) isoquinoline (*Hiqbt*) was prepared following a literature protocol.<sup>[2]</sup> Iridium dimer was synthesized according to the method by Nonoyama.<sup>[3]</sup> IrCl<sub>3</sub> (191 mg, 0.64 mmol) and *Hiqbt* (364 mg, 1.39 mmol) were heated for 24 h at 120 °C in a 3:1 mixture of 2-ethoxyethanol (12 mL) and water (4 mL). A dark-brown suspension was formed. After cooling to room temperature, water (20 mL) was added and the suspension was filtered. The solid was thoroughly washed with water, diethyl ether and hexane. After that, the solid was dried under high vacuum to yield Ir<sub>2</sub>(iqbt)<sub>4</sub>Cl<sub>2</sub>, which was used in the next step without further purification.

**Synthesis and characterization of Ir-1:** Under argon atmosphere, a mixture of Ir<sub>2</sub>(iqbt)<sub>4</sub>Cl<sub>2</sub> (154 mg, 0.1 mmol) and **L1** (59 mg, 0.2 mmol) in CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (12 mL, 2:1, v/v) was heated to reflux for 6 h. After the completion of the reaction, the mixture was cooled to room temperature, and 10-fold excess of NH<sub>4</sub>PF<sub>6</sub> was added. The suspension was stirred for 15 min and then filtered

to remove the insoluble inorganic salts. The solution was concentrated to dryness under reduced pressure. The obtained solid was further purified by column chromatography on silica gel to yield **Ir-1** as a crimson solid (175 mg, 76%). <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>CN-*d*<sub>3</sub>) δ 9.08 (d, *J*=8.4, 3H), 8.60 (d, *J*=8.4, 1H), 8.40 (s, 1H), 8.35 (dd, *J*=5.1, 1.4, 1H), 8.27 (dd, *J*=5.1, 1.4, 1H), 7.91-7.95 (m, 6H), 7.89-7.82 (m, 3H), 7.77 (dd, *J*=8.3, 5.1, 1H), 7.60 (d, *J*=8.7, 2H), 7.50 (s, 1H), 7.38 (d, *J*= 6.6, 1H), 7.34 (d, *J*= 6.6, 1H), 7.26 – 7.13 (m, 4H), 6.91 (d, *J*=8.8, 2H), 6.73 (t, *J*=7.7, 2H), 6.19 (d, *J*=8.3, 2H). <sup>13</sup>C NMR (101 MHz, CD<sub>3</sub>CN-*d*<sub>3</sub>) δ 166.14, 159.58, 155.75, 155.55, 153.25, 152.93, 147.59, 146.99, 145.41, 144.46, 143.10, 139.69, 138.82, 138.22, 137.21, 137.17, 134.84, 133.66, 132.30, 132.16, 131.77, 130.30, 128.72, 128.66, 127.77, 127.43, 126.74, 125.89, 125.54, 123.57, 120.89, 116.88, 113.60, 99.58, 83.43. HR-MS (C<sub>54</sub>H<sub>32</sub>F<sub>6</sub>IrN<sub>4</sub>OPS<sub>2</sub>, positive mode, *m/z*): Calcd. 1009.1647, found 1009.1646 for [(M-PF<sub>6</sub>)<sup>+</sup>].

**Synthesis and characterization of Ir-2:** 5-(Phenylethynyl)-1,10-phenanthroline (**L2**) was synthesized as described in the literature.<sup>[4]</sup> The complex was prepared in a manner similar to that for **Ir-1**, with **L2** instead of **L1** (crimson solid, 62%). <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>CN-*d*<sub>3</sub>) δ 9.07 (d, *J* = 8.4 Hz, 3H), 8.57 (dd, *J* = 8.3, 1.4 Hz, 1H), 8.43 (s, 1H), 8.33 (dd, *J* = 5.0, 1.4 Hz, 1H), 8.27 (dd, *J* = 5.1, 1.4 Hz, 1H), 7.96 – 7.87 (m, 6H), 7.87 – 7.79 (m, 3H), 7.78 – 7.64 (m, 3H), 7.49 (d, *J* = 6.9 Hz, 3H), 7.36 (dd, *J* = 10.5, 6.6 Hz, 2H), 7.22 – 7.10 (m, 4H), 6.70 (t, *J* = 7.6 Hz, 2H), 6.20 (d, *J* = 8.2 Hz, 2H). <sup>13</sup>C NMR (101 MHz, CD<sub>3</sub>CN-*d*<sub>3</sub>) δ 166.17, 155.73, 155.55, 153.34, 153.21, 147.60, 147.21, 145.45, 144.50, 143.16, 139.87, 138.78, 138.27, 137.24, 133.71, 132.98, 132.62, 132.30, 132.07, 130.97, 130.35, 129.94, 128.81, 128.72, 127.80, 127.47, 126.78, 125.93, 125.59, 123.60, 123.05, 122.57, 120.95, 98.79, 84.74. HR-MS (C<sub>54</sub>H<sub>32</sub>F<sub>6</sub>IrN<sub>4</sub>PS<sub>2</sub>, positive mode, *m/z*): Calcd. 993.1698, found 993.1691 for [(M-PF<sub>6</sub>)<sup>+</sup>].

### Quantum yield determination

Phosphorescence quantum yields of **Ir-1** and **Ir-2** were determined in pure acetonitrile with Ru(bpy)<sub>3</sub>Cl<sub>2</sub> ( $\Phi=0.018$ ,  $\lambda=450$  nm, air-saturated) as a reference. The quantum yield was calculated using the following equation:

$$\Phi_S = \Phi_R \frac{F_S A_R n_S^2}{F_R A_S n_R^2}$$

Where  $\Phi_S$  and  $\Phi_R$  are the photoluminescence quantum yield of the sample and that of the reference.  $F_S$  and  $F_R$  are the corresponding integrated fluorescence intensity (areas) of sample and reference spectra, respectively.  $A_S$  and  $A_R$  are the absorbance of the sample and reference solution at the reference excitation wavelength.  $n_S$  and  $n_R$  are the solvent refractive indexes of the sample and reference. Absorbance of sample and reference at their respective excitation wavelength was controlled to be lower than 0.05.

### Cell lines and culture conditions

MCF-7 cells were cultured in RPMI (Roswell Park Memorial Institute)-1640 medium supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO<sub>2</sub> and 95% air at 37 °C. In each experiment, cells treated with vehicle control (1% DMSO) were kept as the reference group.

### Dark cytotoxicity assay

The *in vitro* dark cytotoxicity of **Ir-1** towards MCF-7 cell lines was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Cells were seeded into a 96-well cell culture plate at  $1 \times 10^4$  per well, under 100% humidity, and were cultured at 37 °C with 5% CO<sub>2</sub> for 24h. **Ir-1** in different concentrations (1, 2, 4, 6, 8, 10, 20  $\mu$ M diluted in RPMI-1640 medium) were added into the wells. The cells were subsequently incubated for 24h at 37 °C under 5% CO<sub>2</sub>. After that, MTT (50  $\mu$ L/well, 5 mg/mL) was added to each well and the plate was incubated for an additional 4 h at 37 °C under 5% CO<sub>2</sub>. The medium was then replaced with 200  $\mu$ L DMSO per well, and OD570 was monitored by an enzyme-linked immunosorbent assay reader (Thermo Scientific, Varioskan Flash).

### Photo-cytotoxicity test

MCF-7 cells were used to determine the photo-cytotoxicity of **Ir-1**. After 4 h incubation with **Ir-1** (1, 2, 4, 6, 8, 10, 20  $\mu$ M diluted in RPMI-1640 medium), the cells were exposed to a 450 nm laser irradiation for 300 s (diode laser, Lasever, 30  $\text{J}\cdot\text{cm}^{-2}$ ). After the irradiation, the cells were incubated for an additional 20 h at 37  $^{\circ}\text{C}$  under 5%  $\text{CO}_2$ . MTT assay was conducted to measure the cell cytotoxicity.

### Annexin V-PE apoptosis assay

The assay was performed according to the manufacturer's (KeyGEN, China) protocol. MCF-7 cells were seeded into 6-well plates and were then exposed to **Ir-1** at the indicated concentrations (5 or 10  $\mu$ M) for 4h. After that, the cells were exposed to a 450 nm laser irradiation for 300 s (diode laser, Lasever, 30  $\text{J}\cdot\text{cm}^{-2}$ ). After the irradiation, the cells were incubated for an additional 20 h at 37  $^{\circ}\text{C}$  under 5%  $\text{CO}_2$ . Then the cells were collected and washed twice with PBS.

The cells were re-suspended in 500  $\mu$ L annexin binding buffer. The cell suspension was stained with 1  $\mu$ L annexin V-PE at room temperature for 15 min in the dark and then analyzed immediately by flow cytometry (BD LSRFortessa).

### Quantification of singlet oxygen generation ( $\Phi_{\Delta}({}^1\text{O}_2)$ )

A singlet oxygen sensor (DPBF, 1,3-diphenylisobenzofuran), which is highly selective for singlet oxygen, was used to evaluate the singlet oxygen quantum yields ( $\Phi_{\Delta}({}^1\text{O}_2)$ ) of **Ir-1**. The air-saturated acetonitrile solution of photosensitizer containing DPBF was prepared in the dark and irradiated with 450 nm laser beam in an interval of 10s. DPBF oxidation was monitored by UV-Vis spectrophotometer. The following equation was used to calculate the singlet oxygen quantum yield of the sensitizer with respect to the reference. [5]

$$\Phi_{\Delta}({}^1\text{O}_2)^S = \Phi_{\Delta}({}^1\text{O}_2)^R \frac{S^S F^R}{S^R F^S}$$

where  $\Phi_{\Delta}({}^1\text{O}_2)$  is the quantum yield of singlet oxygen, superscripts "S" and "R" represent **Ir-1** and methylene blue (MB) ( ${}^1\text{O}_2$  quantum yield of 0.52 in acetonitrile), respectively. "S" is the slope of a plot of difference in change in absorbance of DPBF (at 411 nm) with the irradiation time, and "F" is the absorption correction factor, which is given by  $F = 1 - 10^{-\text{OD}}$  (OD at the irradiation wavelength).

### **Detection of ROS generation**

MCF-7 cells were planted on confocal petri dish and incubated with **Ir-1** (10  $\mu\text{M}$ ) for 2 h at 37  $^{\circ}\text{C}$ . Then the cells were incubated with 10  $\mu\text{M}$  DCFH-DA for 20 min. After that, the cells were irradiated by the 450 nm diode laser (30  $\text{J}\cdot\text{cm}^{-2}$ ). The ROS generation was observed by the fluorescence signal of DCF with CLSM (band path, 500-550 nm).

### **Cellular uptake mechanism**

MCF-7 cells were planted on confocal petri dish and allowed to adhere for 24h. Then the cells were treated under different conditions. After that, the cells were imaged by CLSM.

37 °C: the cells were incubated with **Ir-1** or **Ir-2** (10 µM) at 37 °C for 2 h.

4 °C: the cells were incubated with **Ir-1** or **Ir-2** (10 µM) at 4 °C for 2 h.

MI (Metabolic inhibitors): the cells were pretreated with 2-deoxy-D-glucose (50 mM) and oligomycin (5 µM) at 37 °C for 1 h and then incubated with **Ir-1** or **Ir-2** (10 µM) at 37 °C for 2 h.

NH<sub>4</sub>Cl: the cells were pretreated with NH<sub>4</sub>Cl (50 mM) at 37 °C for 1 h and then incubated with **Ir-1** or **Ir-2** (10 µM) at 37 °C for 2 h.

TEA (tetraethylammonium chloride): the cells were pretreated with TEA (1 mM) at 37 °C for 1 h and then incubated with **Ir-1** or **Ir-2** (10 µM) at 37 °C for 2 h.

### **Synergistic hypoxia and acidity imaging on monolayer cells**

MCF-7 cells were planted on confocal petri dish and allowed to adhere for 24 h. For pH imaging, the cells were stained with RPMI-1640 medium containing **Ir-1** or **Ir-2** (10 µM) and 0.1% DMSO for 2h at 37 °C, then the cells were further incubated (30 min) with high K<sup>+</sup> buffers (137 mM NaCl, 120 mM KCl, 10.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>) of different pH values (5.4, 6.4, 7.4, 8.4) in the presence of 10 µM nigericin sodium salt. For hypoxia imaging, after staining the cells with **Ir-1** or **Ir-2** (10 µM), the cells were further incubated under three different oxygen conditions (0, 10, 21% O<sub>2</sub>) for another 1h at 37 °C. Anaero Pack-Anaero and Anaero Pack-Micro aero (Mitsubishi Gas Chemical Co., Inc., Japan) were used to create cell culture environments with oxygen levels of 0% and 10%. For synergistic hypoxia and acidity imaging, the **Ir-1** or **Ir-2** stained cells were incubated under hypoxia conditions (0% O<sub>2</sub>) for 1h after incubating with high K<sup>+</sup> buffer of pH 6.4 (containing 10 µM nigericin sodium salt). After that, confocal imaging was conducted with the band path of 650-750 nm upon excitation at 488 nm.

### **Generation of 3D multicellular spheroids**<sup>[6]</sup>

MCSs were cultured using the liquid overlay method. MCF-7 cells in the exponential growth phase were dissociated by a trypsin solution to gain single-cell suspensions. A number of  $6 \times 10^3$  diluted MCF-7 cells were transferred to 1.5% agarose-coated transparent 96-well plates with 200  $\mu\text{L}$  of Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum. The single cells would generate MCSs approximately 500  $\mu\text{m}$  in diameter at day 4 with 5 %  $\text{CO}_2$  in air at 37  $^\circ\text{C}$ .

### **Synergistic hypoxia and acidity imaging on 3D MCSs**

MCF-7 MCSs of diameters about 500  $\mu\text{m}$  were chosen for synergistic imaging of **Ir-1**. The medium was carefully replaced with probe supplemented standard medium (**Ir-1**, 10  $\mu\text{M}$ ). After that, the MCSs were incubated for 6 h with 5 %  $\text{CO}_2$  in air at 37  $^\circ\text{C}$  to make sure the complex penetrated throughout the spheroids. Then the MCSs were used for hypoxia imaging directly. For synergistic imaging, the medium was removed and the MCSs were washed three times with PBS (pH=7.4). Then the MCSs were further incubated (30 min) with high  $\text{K}^+$  buffers (137 mM NaCl, 120 mM KCl, 10.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.8 mM  $\text{KH}_2\text{PO}_4$ ) of pH 6.4 in the presence of 10  $\mu\text{M}$  nigericin sodium salt. All the images were recorded under a  $10\times$  objective. Confocal imaging and PLIM were conducted with the band path of 650-750 nm upon excitation at 488 nm (intensity mode) or 405 nm (lifetime mode).

## Supporting figures and tables

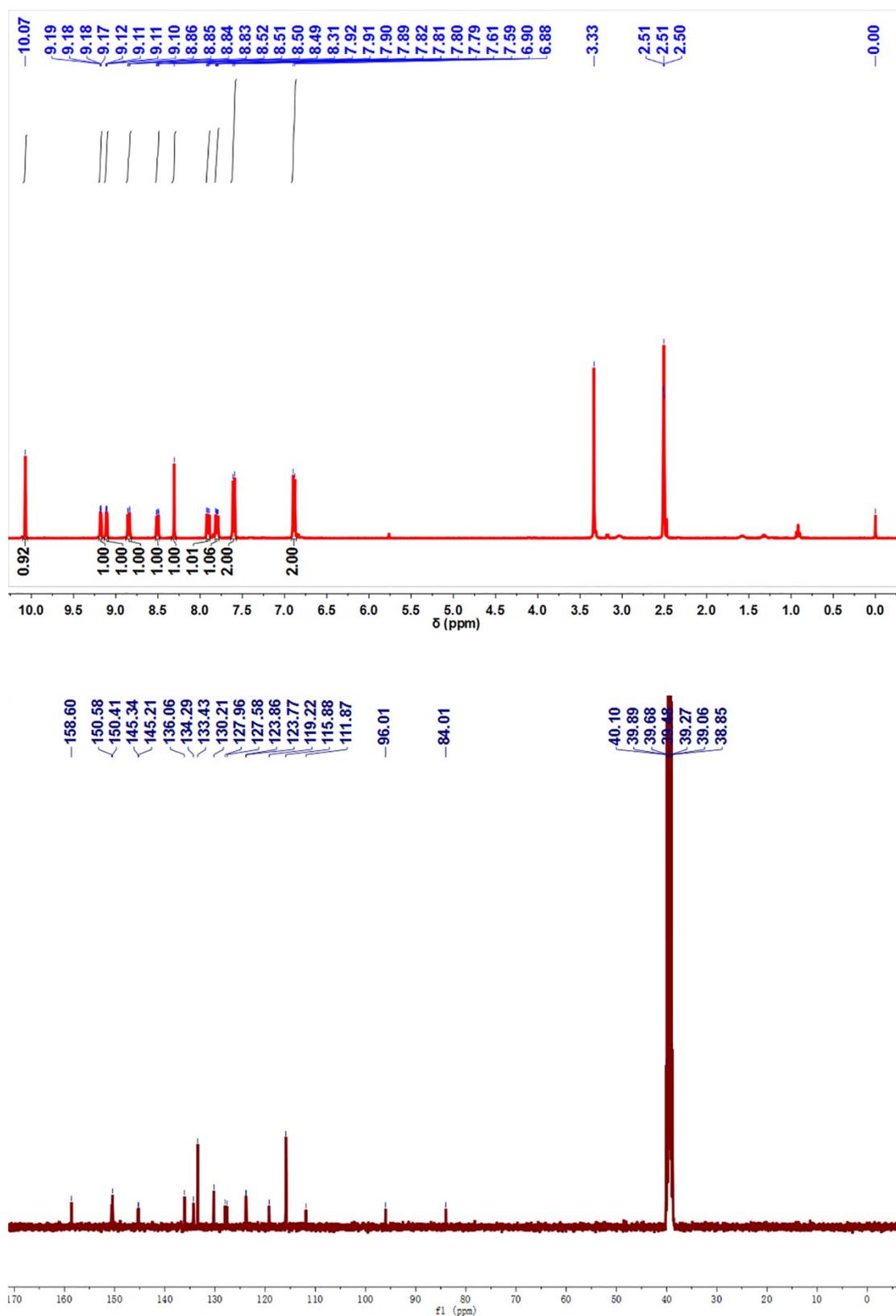
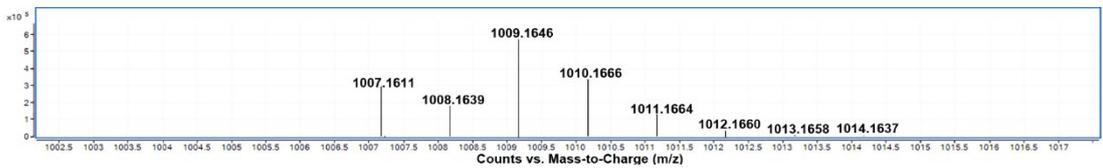
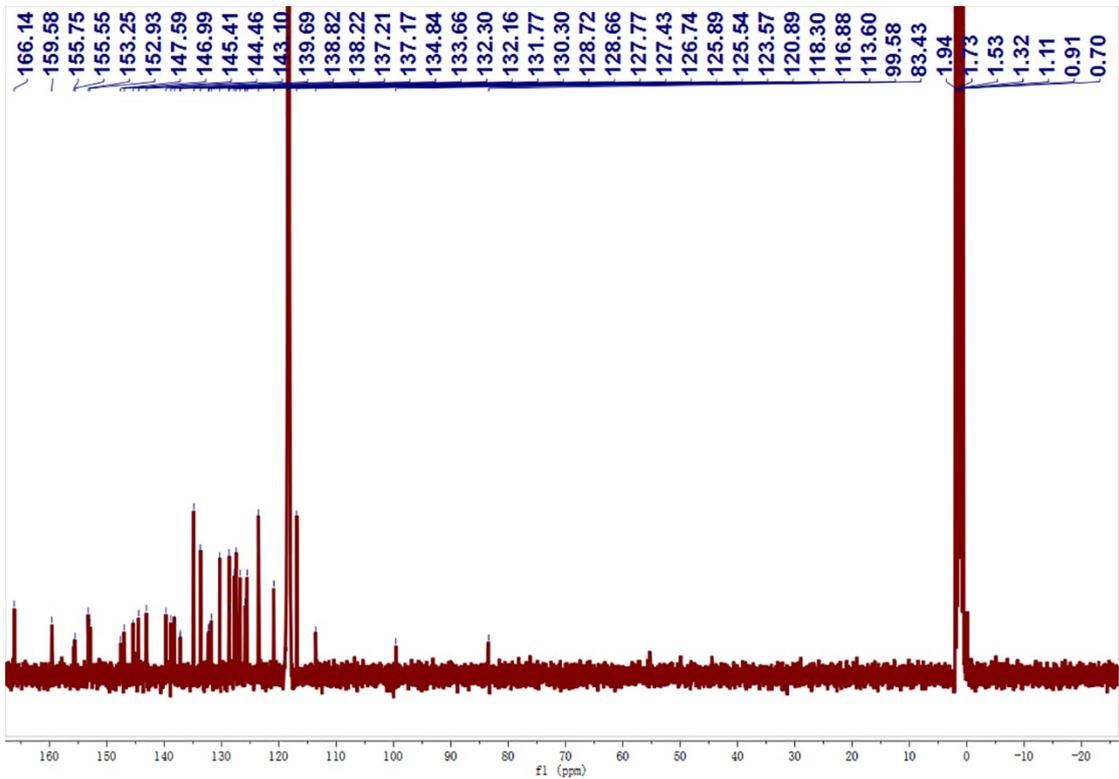
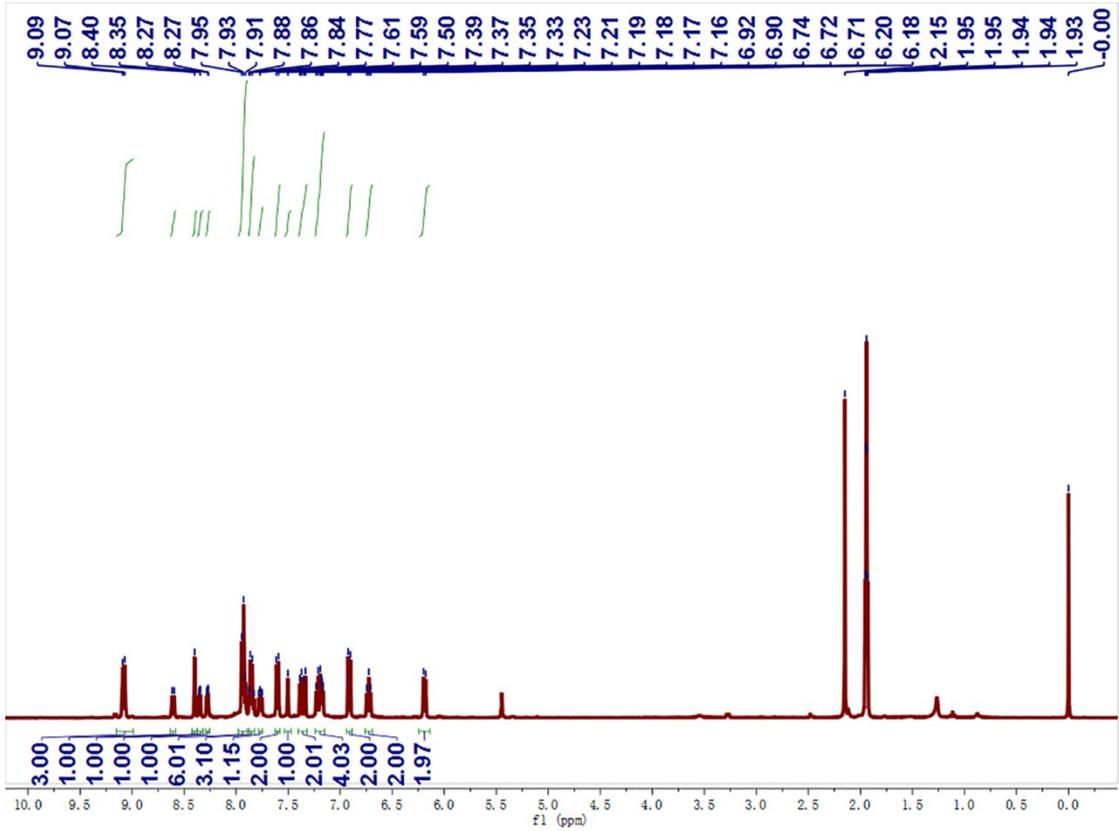
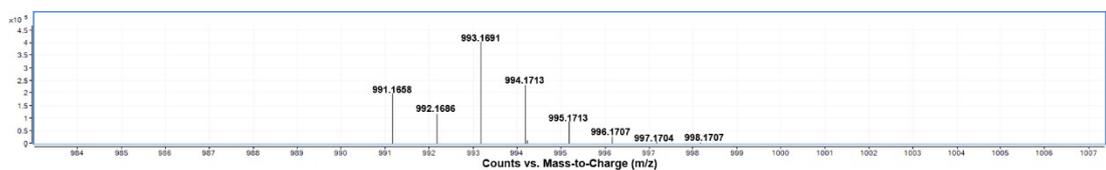
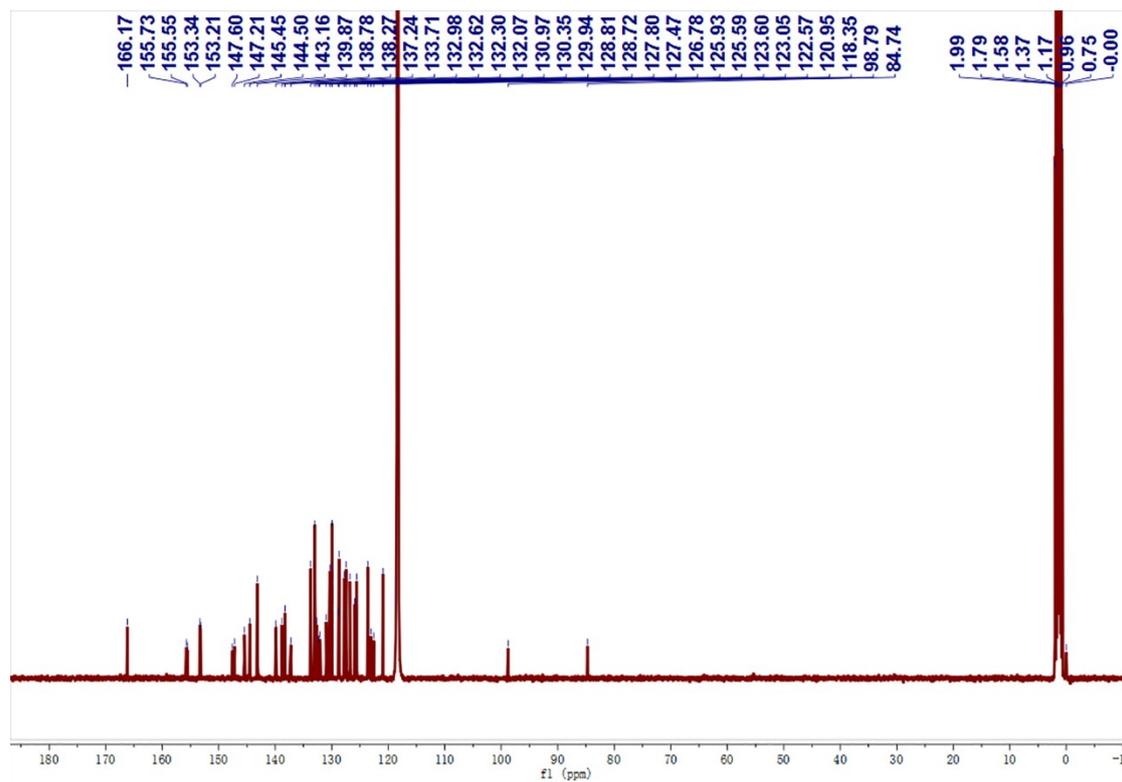
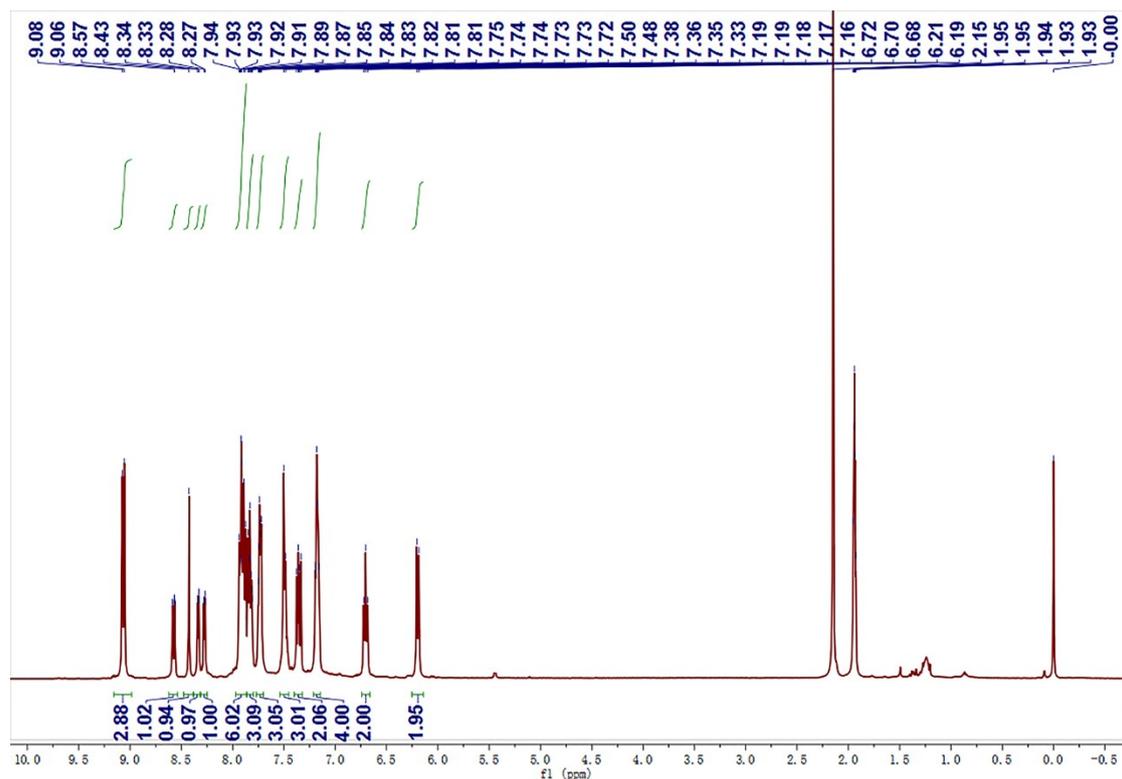


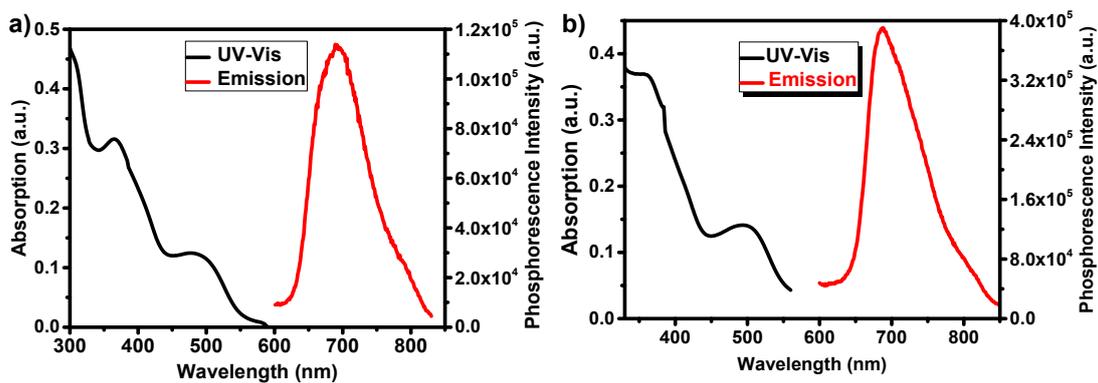
Figure S1. <sup>1</sup>H and <sup>13</sup>C NMR spectra of L1 in DMSO-*d*<sub>6</sub>.



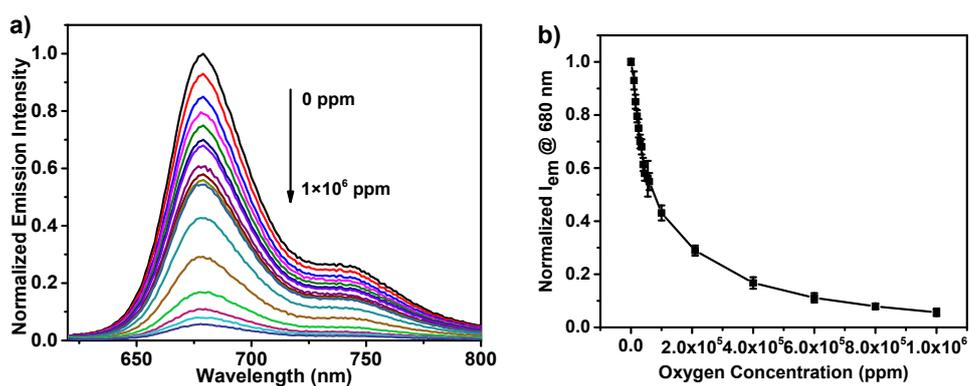
**Figure S2.**  $^1\text{H}$ ,  $^{13}\text{C}$  NMR in  $\text{CD}_3\text{CN-}d_3$  and HR-MS spectra of **Ir-1**.



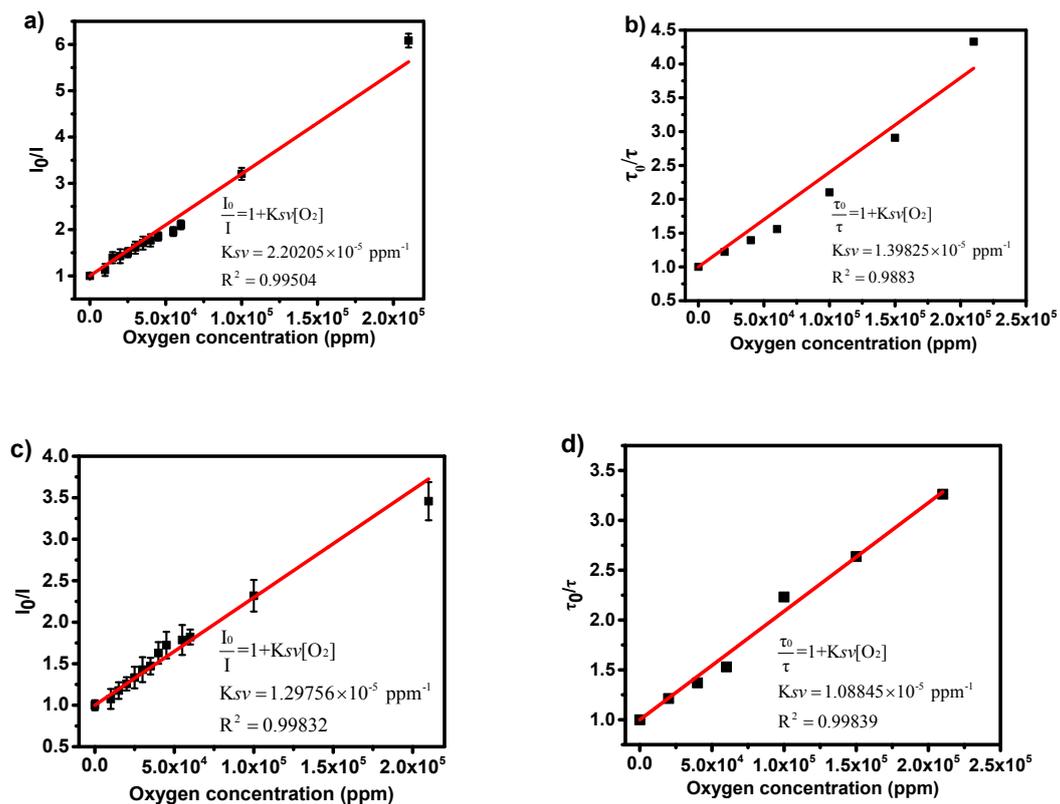
**Figure S3.** <sup>1</sup>H, <sup>13</sup>C NMR in CD<sub>3</sub>CN-*d*<sub>3</sub> and HR-MS spectra of Ir-2.



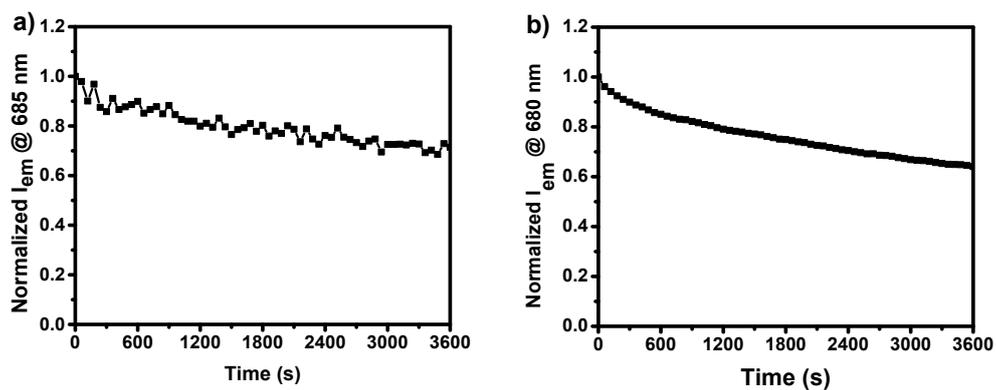
**Figure S4.** UV-Vis (black) and phosphorescence emission (red) spectra of **Ir-1** (a, 10  $\mu$ M) and **Ir-2** (b, 10  $\mu$ M) in PBS buffer (pH 7.4, containing 10% DMSO, v/v).



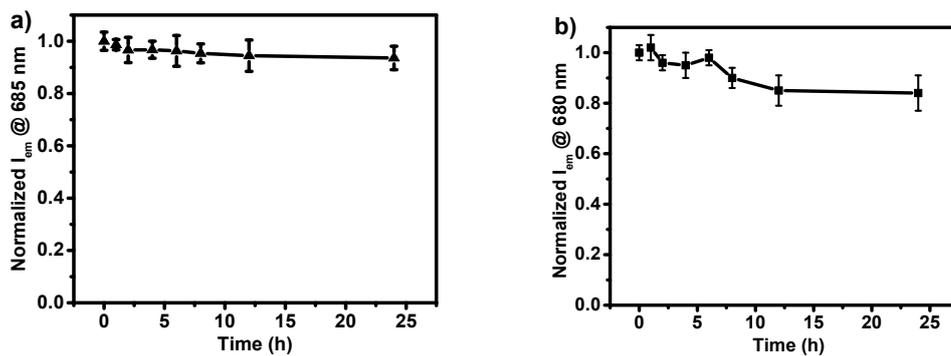
**Figure S5.** a) Phosphorescence emission spectra of **Ir-2** in toluene at different oxygen concentrations. Excitation: 488nm. b) Normalized phosphorescence emission intensities of **Ir-2** at various oxygen concentrations.



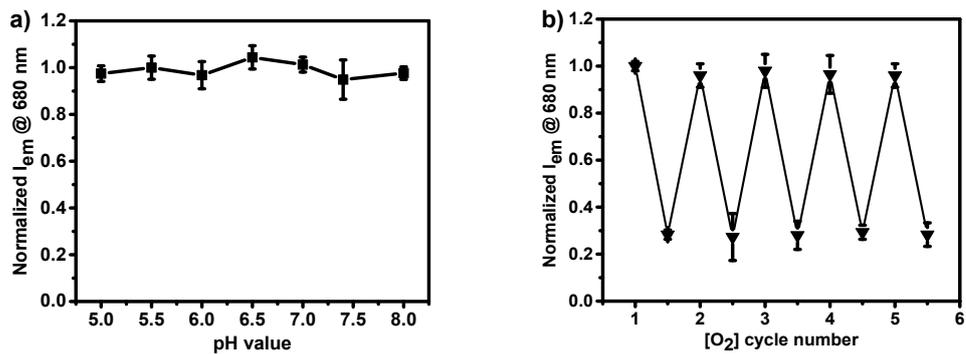
**Figure S6.** Stern-Volmer plots of **Ir-1** (10  $\mu\text{M}$ ) fixed from the oxygen quenched a) luminescence intensity and b) lifetime at 685 nm in toluene at 25  $^\circ\text{C}$ ; Stern-Volmer plots of **Ir-2** (10  $\mu\text{M}$ ) fixed from the oxygen quenched c) luminescence intensity and d) lifetime at 680 nm in toluene at 25  $^\circ\text{C}$ . Both the excitation wavelengths were 488 nm.



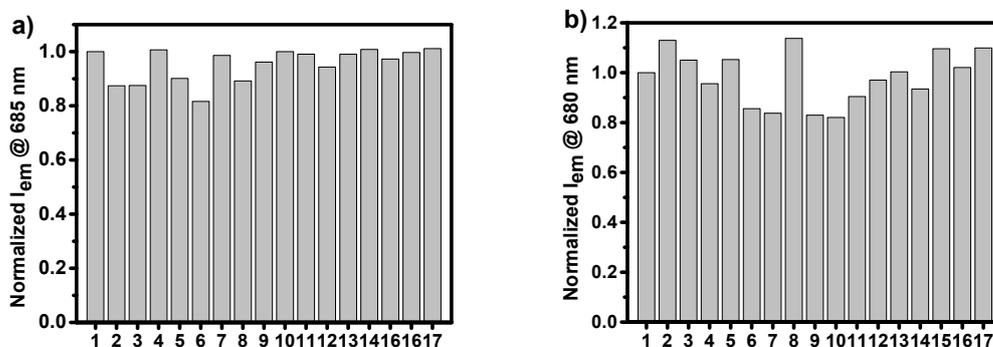
**Figure S8.** Photobleaching phosphorescence intensity of **Ir-1** (a, 10  $\mu$ M) and **Ir-2** (b, 10  $\mu$ M) in PBS buffer (pH 7.4, containing 10% DMSO, v/v) under continuous irradiation (488 nm, 150W Xe lamp).



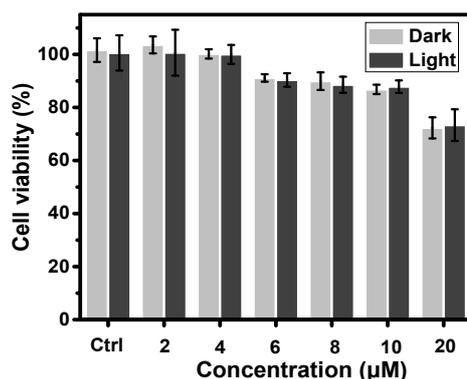
**Figure S9.** Serum-stability test of **Ir-1** (a, 10  $\mu$ M) and **Ir-2** (b, 10  $\mu$ M) at 37  $^{\circ}$ C, showing the emission intensity at different time points ( $\lambda_{ex}$ = 488 nm).



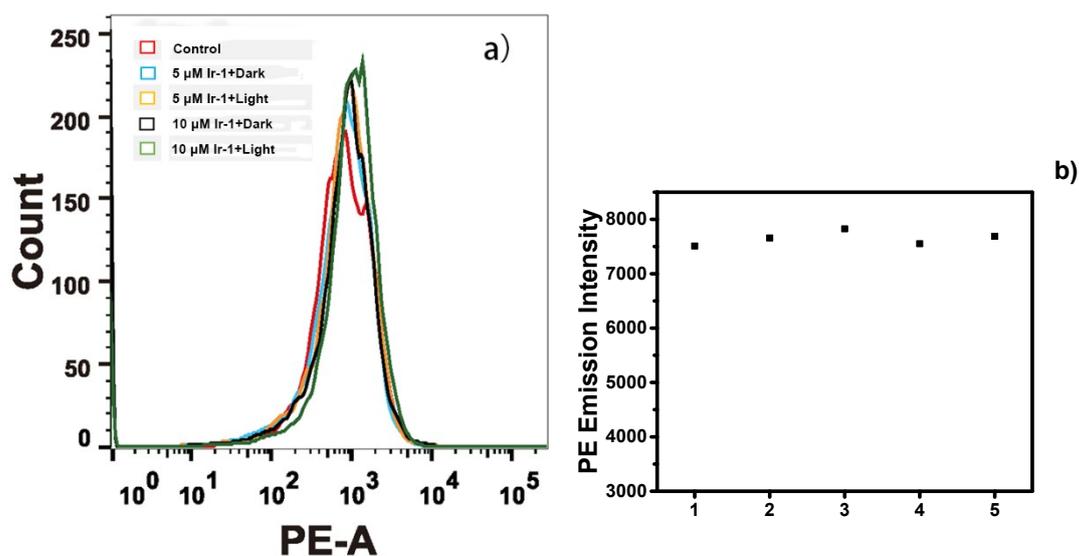
**Figure S10.** (a) Phosphorescence emission intensity of **Ir-2** in phosphate buffer solution of different pH values. (b) Phosphorescence emission intensity changes of **Ir-2** (10  $\mu\text{M}$ ) in toluene determined over consecutive  $[\text{O}_2]$  cycles.  $\lambda_{\text{ex}} = 488 \text{ nm}$ .



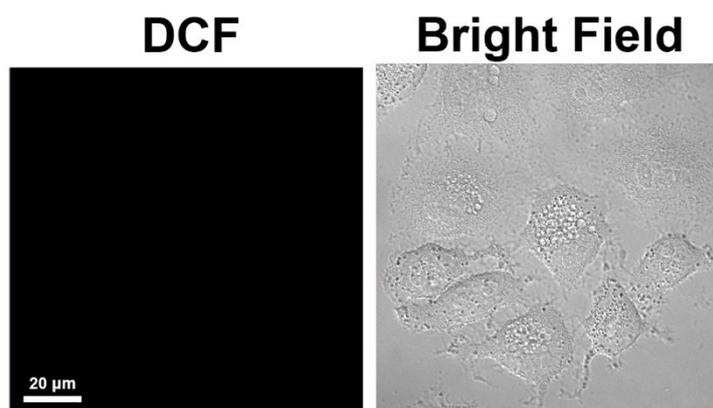
**Figure S11.** Normalized emission intensity of **Ir-1** (a, 10  $\mu\text{M}$ ) and **Ir-2** (b, 10  $\mu\text{M}$ ) in PBS buffer (pH 7.4, containing 10% DMSO, v/v) in the presence of different metal ions. 1: blank; 2:  $\text{Na}^+$ ; 3:  $\text{Mg}^{2+}$ ; 4:  $\text{K}^+$ ; 5:  $\text{Ca}^{2+}$  (2–5: 100  $\mu\text{M}$ ); 6:  $\text{Cr}^{3+}$ ; 7:  $\text{Mn}^{2+}$ ; 8:  $\text{Hg}^{2+}$ ; 9:  $\text{Fe}^{3+}$ ; 10:  $\text{Fe}^{2+}$ ; 11:  $\text{Cu}^{2+}$ ; 12:  $\text{Zn}^{2+}$ ; 13:  $\text{Co}^{2+}$ ; 14:  $\text{Cd}^{2+}$ ; 15:  $\text{Ba}^{2+}$ ; 16:  $\text{Al}^{3+}$ ; 17:  $\text{Ag}^+$ ; (6–17: 10  $\mu\text{M}$ ). The excitation wavelength was 488 nm.



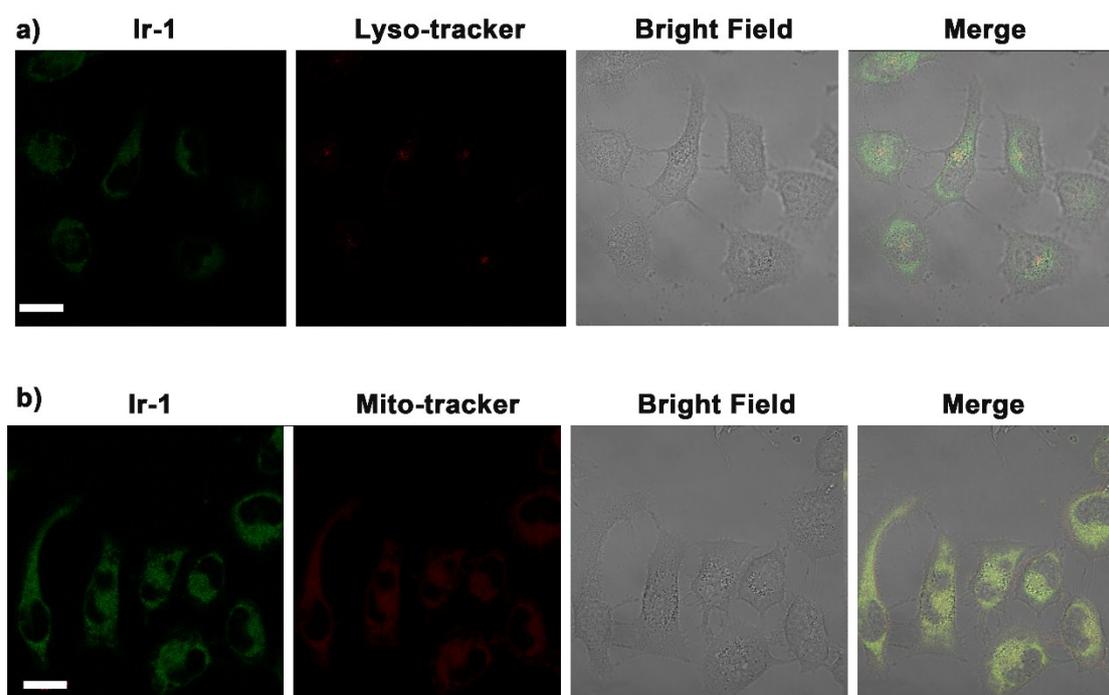
**Figure S12.** *In vitro* cytotoxicity and phototoxicity assay of **Ir-1** towards MCF-7 cells under different concentrations. Gray columns: the cells were incubated with **Ir-1** without irradiation under indicated concentrations. Black columns: the cells were irradiated with 450 nm laser (30  $J \cdot cm^{-2}$ , 300s) after staining with **Ir-1** for 4h.



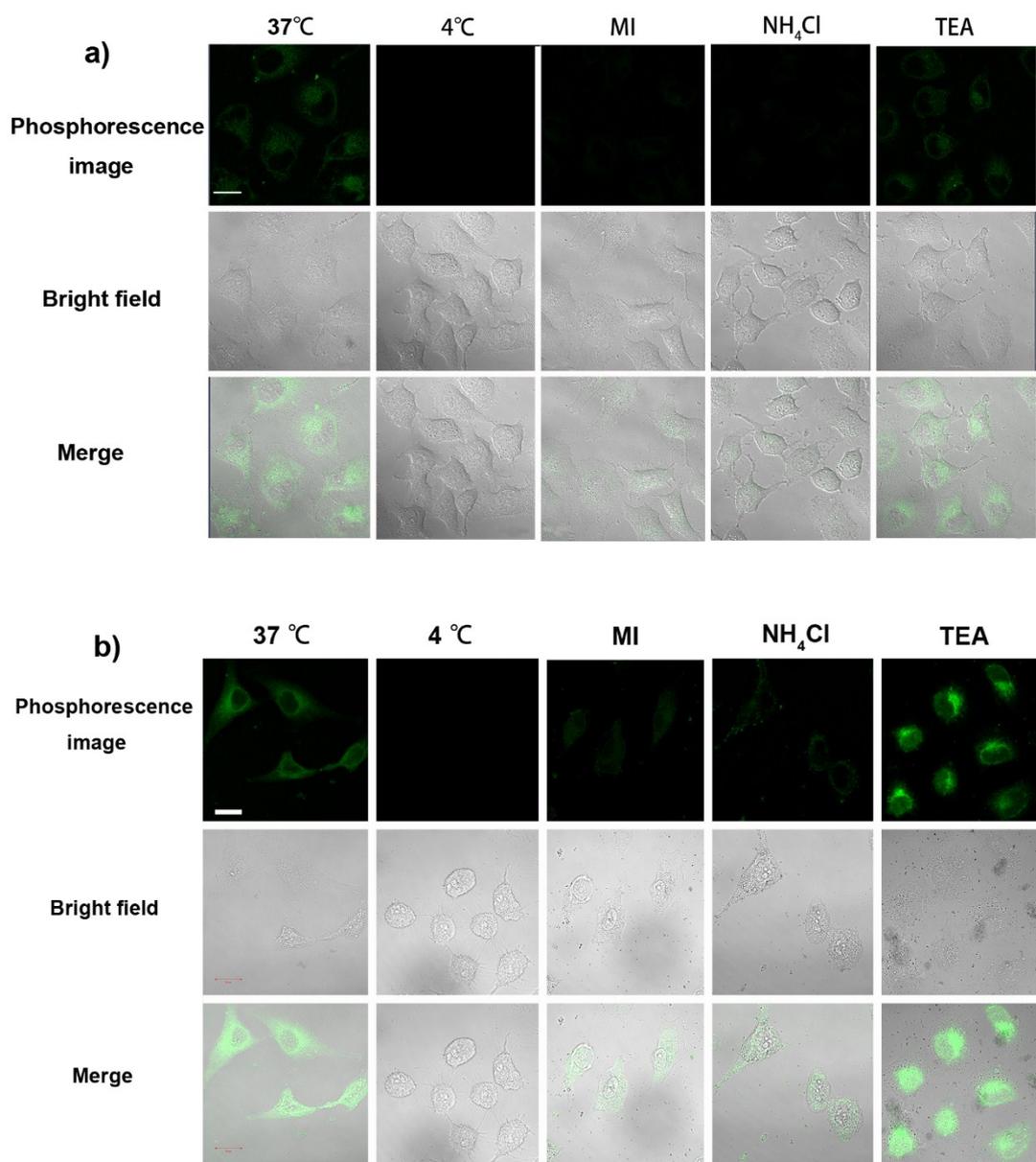
**Figure S13.** (a) Detection of apoptosis by flow cytometry after treating MCF-7 cells with **Ir-1** under different conditions. (b) The related PE fluorescence profile determined in (a). 1, control; 2, 5  $\mu M$  **Ir-1** + dark; 3, 5  $\mu M$  **Ir-1** + light; 4, 10  $\mu M$  **Ir-1** + dark; 5, 10  $\mu M$  **Ir-1** + light.



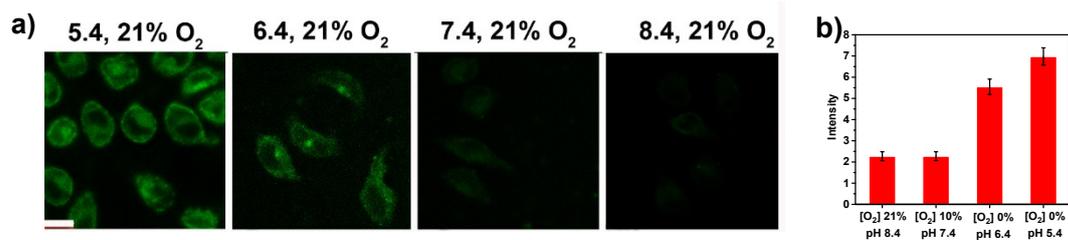
**Figure S14.** Confocal phosphorescence images of ROS generation stained with DCFH-DA after irradiation by 450 nm laser at the power density of  $30 \text{ J}\cdot\text{cm}^{-2}$  under 21%  $\text{O}_2$ . The MCF-7 cells were pretreated with **Ir-1** (10  $\mu\text{M}$  diluted in RPMI-1640 medium) and further incubated for 4h before irradiation.



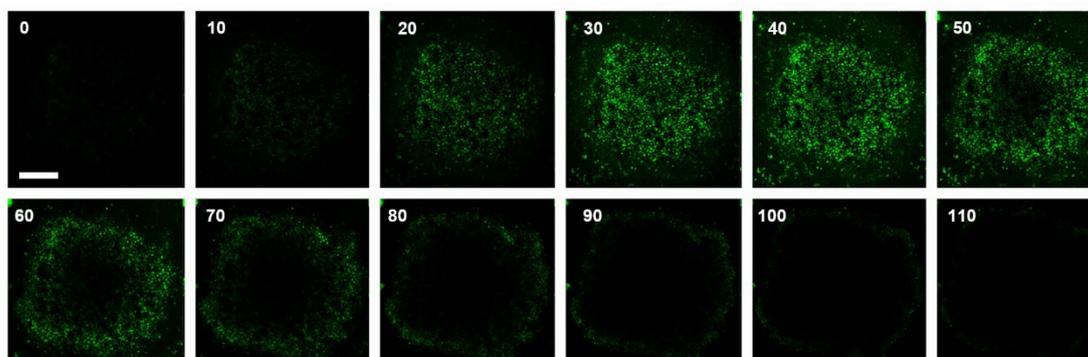
**Figure S15.** Confocal phosphorescence images of MCF-7 cells costained by **Ir-1** (10  $\mu\text{M}$  in RPMI-1640 medium with 0.1% DMSO,  $\lambda_{\text{ex}}=488 \text{ nm}$ , 2 h) and Lyso-tracker Deep Red (a, 10  $\mu\text{M}$ ,  $\lambda_{\text{ex}}=633 \text{ nm}$ , 30 min) or Mito-tracker Deep Red (b, 10  $\mu\text{M}$ ,  $\lambda_{\text{ex}}=633 \text{ nm}$ , 30 min). Scale bar: 20  $\mu\text{m}$ .



**Figure S16.** Cell uptake mechanism studies of **Ir-1** (a, 10  $\mu\text{M}$ ) and **Ir-2** (b, 10  $\mu\text{M}$ ) in the presence of different inhibitors/conditions. 37  $^{\circ}\text{C}$ : the cells were incubated with **Ir-1** or **Ir-2** at 37  $^{\circ}\text{C}$  for 2 h; 4  $^{\circ}\text{C}$ : the cells were incubated with **Ir-1** or **Ir-2** at 4  $^{\circ}\text{C}$  for 2 h; MI (Metabolic inhibitors): the cells were pretreated with 2-deoxy-D-glucose (50 mM) and oligomycin (5  $\mu\text{M}$ ) at 37  $^{\circ}\text{C}$  for 1 h and then incubated with **Ir-1** or **Ir-2** at 37  $^{\circ}\text{C}$  for 2 h.  $\text{NH}_4\text{Cl}$ : the cells were pretreated with  $\text{NH}_4\text{Cl}$  (50 mM) at 37  $^{\circ}\text{C}$  for 1 h and then incubated with **Ir-1** or **Ir-2** at 37  $^{\circ}\text{C}$  for 2 h; TEA (tetraethylammonium chloride): the cells were pretreated with TEA (1 mM) at 37  $^{\circ}\text{C}$  for 1 h and then incubated with **Ir-1** or **Ir-2** at 37  $^{\circ}\text{C}$  for 2 h. All the images share the same scale bar of 20  $\mu\text{m}$ .



**Figure S17.** a) Confocal imaging of MCF-7 cells stained by **Ir-1** (10  $\mu$ M in RPMI-1640 medium with 0.1% DMSO, 2 h, 37°C) upon further incubation with high K<sup>+</sup> buffers (137 mM NaCl, 120 mM KCl, 10.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>) of different pH (5.4–8.4) in the presence of 10.0 mM nigericin. Scale bar, 20  $\mu$ m. b) Average phosphorescence intensities of the cells under different conditions.



**Figure S18.** Z-stack images of large 3D multicellular spheroids incubated with **Ir-1** (10  $\mu$ M, pH=7.4) with intervals of 10  $\mu$ m.  $\lambda_{\text{ex}}$ =488 nm. Scale bar, 200  $\mu$ m.

**Table S1. Photo-physical data of Ir-1 and Ir-2<sup>a</sup>**

	$\lambda_{\text{abs}}$ (nm) <sup>b</sup>	$\text{Log}\epsilon_{\text{max}}$ <sup>b</sup>	$\lambda_{\text{em}}$ (nm) <sup>b</sup>	$\Phi_{\text{air}}$ (%) <sup>c</sup>	$\Phi_{\text{Ar}}$ (%) <sup>c</sup>	$\tau$ (ns) <sup>d</sup>	$\Phi_{\Delta}({}^1\text{O}_2)$ <sup>e</sup>
<b>Ir-1</b>	488	4.09	685	0.3	1.2	85	0.08
<b>Ir-2</b>	488	4.15	680	0.3	0.9	98	-

<sup>a</sup> All the photo-physical data was obtained under the O<sub>2</sub> concentration of 21% except specially noted.

<sup>b</sup> Absorption and emission spectra were recorded in PBS (pH 7.4, containing 10% DMSO, v/v) at room temperature.

<sup>c</sup>  $\Phi_{\text{air}}$  and  $\Phi_{\text{Ar}}$  refer to the phosphorescence quantum yield in air or Ar-saturated acetonitrile, the standard used was Ru(bpy)<sub>3</sub>Cl<sub>2</sub> ( $\Phi=0.018$ ,  $\lambda=450$  nm, air-saturated acetonitrile).

<sup>d</sup>  $\tau$  refers to the lifetime and was evaluated in PBS (pH 7.4, containing 10% DMSO, v/v).

<sup>e</sup>  $\Phi_{\Delta}({}^1\text{O}_2)$  refers to the singlet oxygen generation quantum yield in air-saturated acetonitrile.

**Table S2. Phosphorescence lifetime of Ir-1 (10  $\mu\text{M}$ ) and Ir-2 (10  $\mu\text{M}$ ) under different oxygen concentrations in toluene at 25 °C.  $\lambda_{\text{ex}} = 405$  nm.**

$\tau/\text{ns}$ \ / $[\text{O}_2]/\%$ <sup>a</sup>	0	2	4	6	10	15	21
<b>Ir-1<sup>b</sup></b>	892	730	640	576	424	307	206
<b>Ir-2<sup>c</sup></b>	646	532	471	422	289	245	198

<sup>a</sup> The concentration of oxygen in each sample solution was adjusted via bubbling corresponding gas mixed with Ar and O<sub>2</sub> for 20 min.

<sup>b</sup> The lifetime was measured at 685 nm.

<sup>c</sup> The lifetime was measured at 680 nm.

**Table S3. Phosphorescence lifetime of Ir-1 (10  $\mu$ M) and Ir-2 (10  $\mu$ M) at different pH values in PBS (containing 10% DMSO, v/v) at 25  $^{\circ}$ C<sup>a</sup>.  $\lambda_{\text{ex}} = 405$  nm.**

$\tau$ /ns \ pH	5.4	6.4	7.4	8.4	9.4
Ir-1 <sup>b</sup>	115	99	85	74	53
Ir-2 <sup>c</sup>	90	92	98	100	93

<sup>a</sup> All the lifetime values were obtained under 21% oxygen concentration.

<sup>b</sup> The lifetime was measured at 685 nm.

<sup>c</sup> The lifetime was measured at 680 nm.

**Table S4. Phosphorescence quantum yield of Ir-1 (10  $\mu$ M) at different pH values in PBS (containing 10% DMSO, v/v) at 25  $^{\circ}$ C under hypoxia and normoxia<sup>a</sup>.  $\lambda_{\text{ex}} = 405$  nm.**

$\Phi$ (%) \ pH	4.0	7.4	10.0
Hypoxia <sup>b</sup>	1.50	0.30	0.10
Normoxia <sup>c</sup>	0.90	0.20	0.08

<sup>a</sup> Ru(bpy)<sub>3</sub>Cl<sub>2</sub> ( $\Phi=0.018$ ,  $\lambda=450$  nm, air-saturated acetonitrile) was used as the standard to obtain the phosphorescence quantum yield values.

<sup>b</sup> Hypoxia was achieved via bubbling Ar for 20 min.

<sup>c</sup> Normoxia was achieved via bubbling O<sub>2</sub> for 20 min.

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