Supporting Information for

Red emitting two-photon fluorescent probe for dynamic imaging of redox balance meditated by superoxide anion and GSH in living cells and tissues

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Table of contents

Synthesis and the response mechanism of the probe

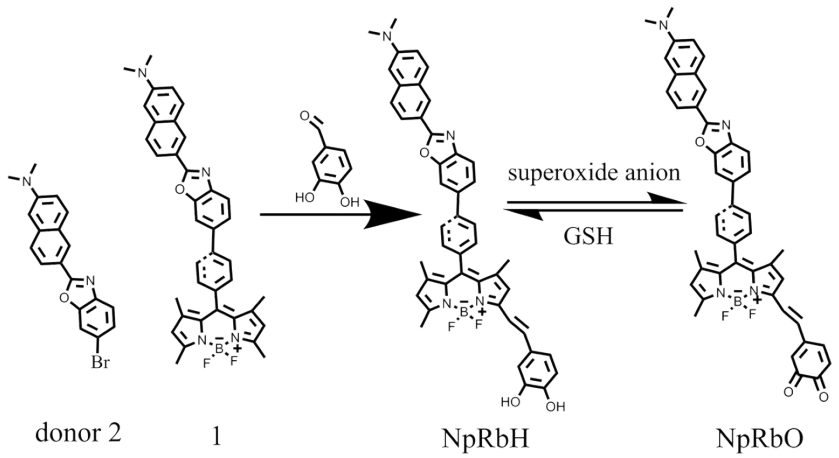


Fig. S1. Structures and synthetic route of probe **NpRbH** and the response mechanism of probe **NpRbH**.

Synthesis of probe NpRbH

Compound **1** and donor **2** was synthesized according to our previous work[1,2]. Compound **1** (305 mg, 0.5 mmol), 3,4-dihydroxy-benzaldehyde (70 mg, 0.5 mmol), glacial acetic acid (0.2mL) and piperidine (1 mL) was dissolved in dry toluene (100 mL) in a Dean-Stark apparatus and then refluxed for 12 h. The solvent was concentrated under reduced pressure. The residue was purified by the silica gel chromatography (dichloromethane /ethyl acetate, 20:1, v/v), which gave compound **NpRbH** as a dark-purple solid (36mg, 8%). ^{1}HNMR (DMSO-d6, 400 MHz) δ (ppm): δ 9.49 (s, 1H), 9.36 (s, 1H), 8.63 (s, 1H), 8.23 (s, 1H), 8.12 (d, J = 8.11, 1H), 8.03 (m, J = 8.01, 3H), 7.88(m, J = 7.86, 3H), 7.55(d, J = 7.54, 2H), 7.46(d, J = 7.44, 2H),7.35 (m, J = 7.31, 2H), 7.11 (s, 1H), 7.03 (s, 1H), 6.96 (s, 1H), 6.91 (d, J = 6.90, 1H), 6.81 (d, J = 6.80, 1H), 6.20 (s, 1H), 3.10 (s, 6H), 1.99 (s, 3H), 1.52 (s, 3H), 1.47 (s, 3H). MS (ESI): m/z 729.3 $[M-H]^-$, calcd for $C_{45}H_{37}BF_2N_4O_3$ 730.6.

Cell cytotoxic assays and two-photon fluorescence imaging

To evaluate the potential cytotoxicity of probe **NpRbH,** HeLa cells were seeded at 1×10^5 cells per well in 96-well plates and incubated for 24 h and 48 h. After that different concentration (2-20 μM) of probe **NpRbH** was added to the cells and these cells were cultured for an additional 24 h or 48 h. And then the cytotoxic effects of **NpRbH** was

determined using MTT assays. Following incubation, the RAW264.7 cells were washed three times with Dulbecco's phosphate buffered saline (DPBS) and imaged. The two-photon excitation wavelength of the femtosecond laser was fixed at 780 nm; the emission wavelengths were recorded at 470-530 and 550-650 nm respectively.

Two-photon fluorescence imaging of fresh mouse liver slices

Fresh tissue slices were prepared from the livers of nude mice. The slices were incubated with analytes at 37 °C. The slices were washed with DPBS three times, and then two-photon fluorescence microscopy images were collected. The excitation wavelength of the femtosecond laser was set at 780 nm, the emission wavelengths were recorded at 470-530 and 550-650 nm, respectively.

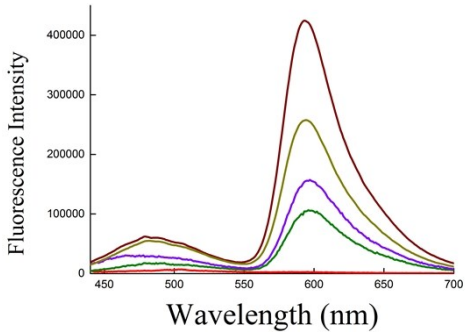


Fig. S2. Fluorescence emission spectra. The fluorescence performance of **NpRbH** in the different organic phase ratio. **NpRbH** (5 μM) in PBS/EtOH (containing EtOH 10, 15, 20, 30%, pH= 7.4, 10 mM). λ_{ex} = 400 nm.

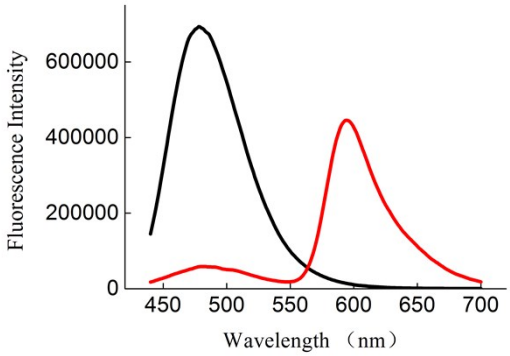


Fig. S3. Fluorescence emission spectra. The black and red lines represent donor **2** (5 μM), and **NpRbH** (5 μM) respectively, in PBS/EtOH (3/1, v/v, pH= 7.4, 10 mM), as the respective fluorescence responses; λ_{ex} = 400 nm.

Energy Transfer Efficiency (ETE) = [(fluorescence of donor-fluorescence of donor in cassette)/fluorescence of donor] × 100%.[1,2]

For **NpRbH**, **ETE**=(692056 -59734)/ 692056 × 100% = 91.37%.

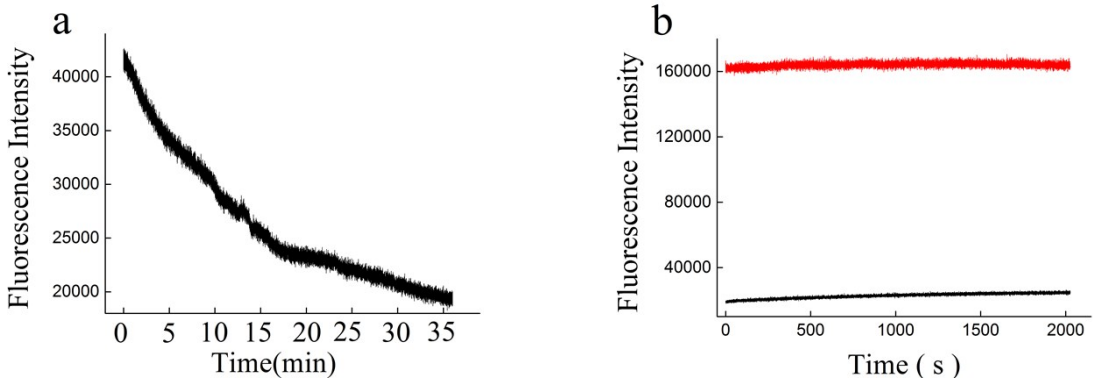


Fig. S4. (a) Time course of fluorescence change in detecting superoxide with **NpRbH**. (b) Photostability experiment of **NpRbH** before (red line) and after (black line) added $O_2^{\bullet-}$. In PBS/EtOH (3/1, v/v, pH= 7.4, 10 mM), as the respective fluorescence responses; λ_{ex} = 400 nm, λ_{em}= 596 nm.

Fluorescence titration and detection limit: The detection limit was determined from the fluorescence titration data. The fluorescence intensity increased linearly with the concentration of $O_2^{\bullet-}$ ranging from 0.33 μM to 4.0 μM. The detection limit was calculated to be 9.51×10^{-8} M based on 3σ/slope method.

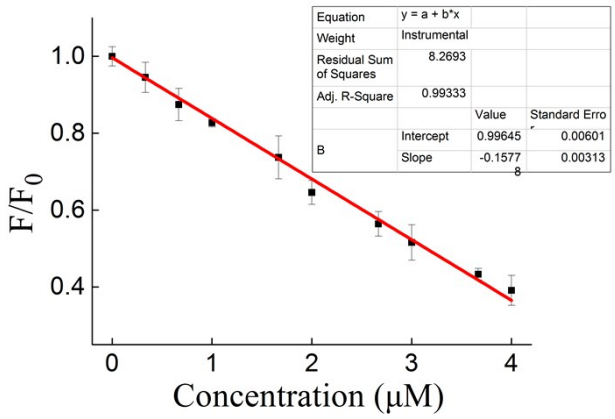


Fig. S5 The linear responses at low $O_2^{\bullet-}$ concentrations (0-4 μM). λex = 400 nm.

The reversibility of NpRbH

The proposed reaction mechanism of **NpRbH** with $O_2^{\bullet-}$ was shown in **Figure S1**. The structure of **NpRbO** was confirmed by MS (ESI): m/z 729.5 $[M+H]^+$, calcd 728.3. The fluorescence responses of **NpRbH** to different concentrations of GSH were showed in **Figure S5**. The fluorescence of **NpRbH** increases gradually with the increase of GSH concentrations, until the [GSH] reaches 2 mM. So 1.5 mM GSH was used to convert **NpRbH** into **NpRbO** in the reversible cycle experiment. The reversibility of the probe was tested (Figure S6). This reversible cycle can be repeated for three times more under the same conditions. The reversibility implied the advantage of **NpRbH** for dynamic determine $O_2^{\bullet-}$ in cells and in vivo.

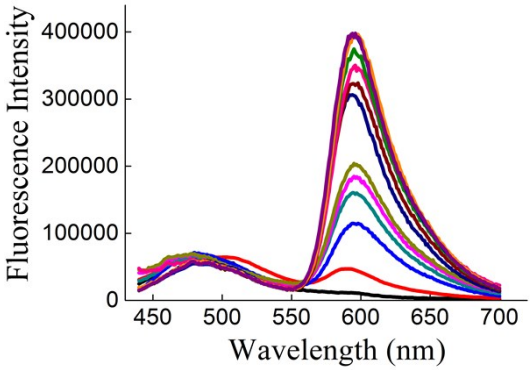


Fig. S6. The fluorescence emission spectra of **NpRbO** (5 μM) in the presence of different concentrations of GSH (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2 mM) in PBS buffered (10mM, pH=7.4) aqueous EtOH solution (3:1, v/v). λ_{ex} = 400 nm.

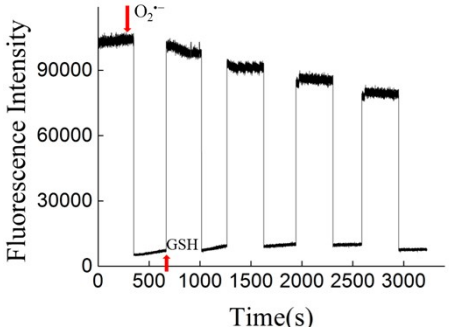


Fig. S7. The reversibility test of **NpRbH** for detection $O_2^{\bullet-}$ and GSH. **NpRbH** was added with 25 μM $O_2^{\bullet-}$, after 10 min, the solution was treated with 1 mM GSH. When the fluorescence returned to the baseline level, another 25 μM $O_2^{\bullet-}$ was added to the mixture after 10 min. λ_{ex} = 400 nm, λ_{em} = 596 nm.

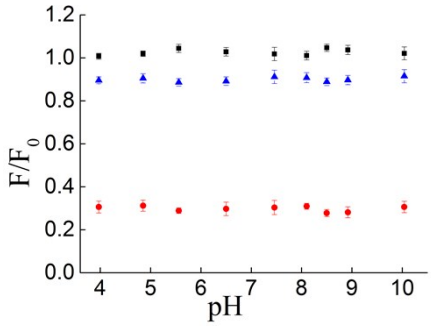


Fig. S8. Effect of pH on the fluorescence intensity of **NpRbH** (5 μM) in buffered/EtOH (3/1, v/v, pH= 4-9, 10 mM). Fluorescence responses are shown before (■), after (●) addition of $O_2^{\bullet-}$ (25 μM) and further addition of GSH (1mM) (▲), respectively. Where F_0 represents the fluorescence intensity of **NpRbH** (5 μM), the curve was plotted with the fluorescence intensity at 596 nm vs pH value. λ_{ex} = 400 nm.

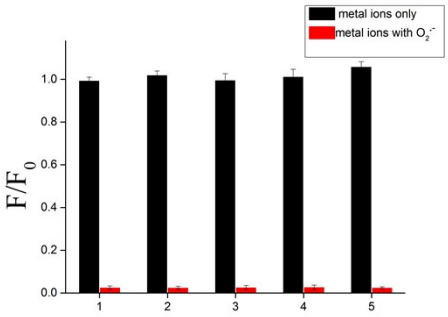


Fig. S9. Fluorescence responses of 5 μM **NpRbH** to various metal ions. The fluorescence intensity at λem=596 nm was plotted versus substances: 1-10. Mn^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+} (500 μM), $O_2^{\bullet-}$ (25μM). Data were acquired in buffered/EtOH (3/1, v/v, pH= 7.4, 10 mM), with λex = 400 nm.

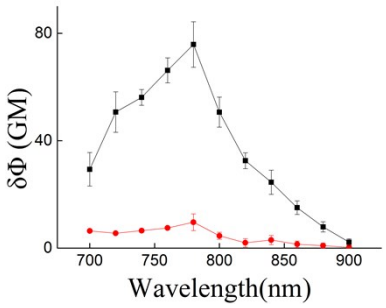


Fig. S10 TP absorption cross-section of **NpRbH** and **NpRbO** in PBS buffered (10 mM, pH = 7.4) aqueous EtOH solution (3:1, v/v). Black line represents the active absorption cross-section of **NpRbH** and red line represents the active absorption cross-section of **NpRbO**.

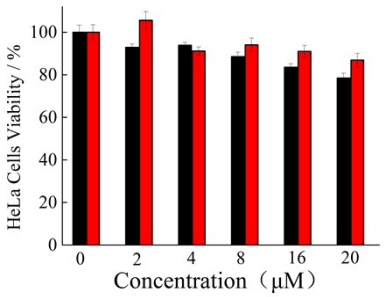


Fig. S11. Cytotoxicity of both **NpRbH** against HeLa cells as determined by MTT assay: HeLa cells were treated with **NpRbH** (0-20 μM). Black bar and red bar represents incubation for 48 h and 24 h respectively.

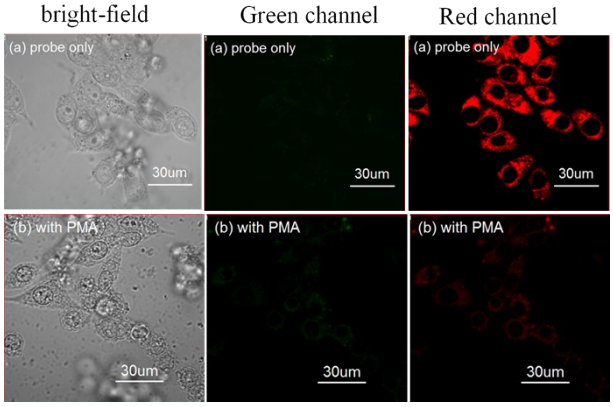
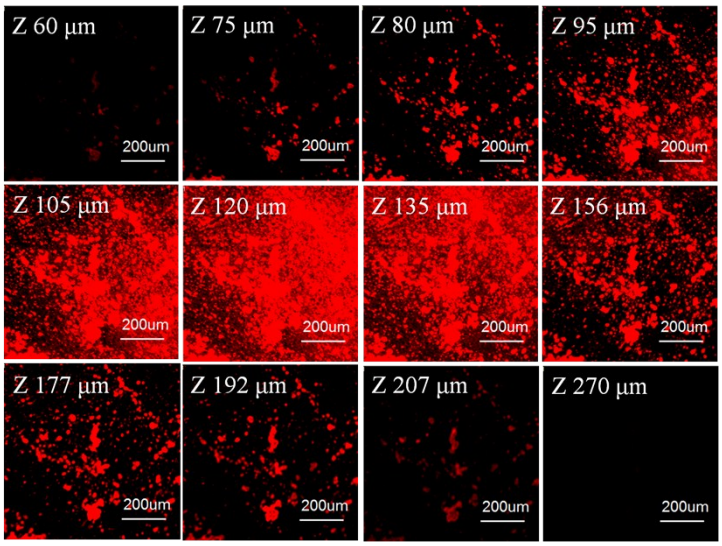
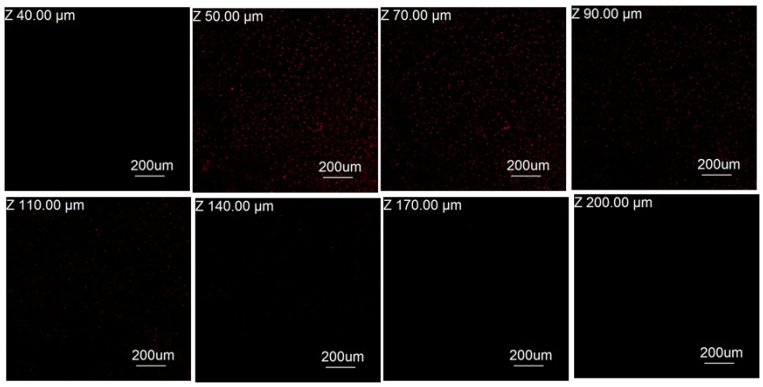


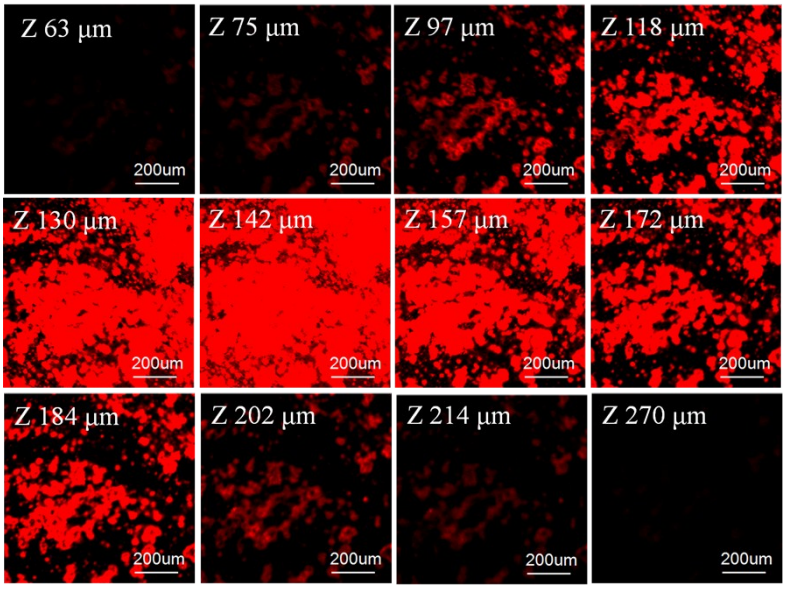
Fig. S12. TP fluorescence images of $O_2^{\bullet-}$/GSH reversible cycles in live RAW264.7 cells with **NpRbH** (2.5 μM). (a) Cells incubated with only probe **NpRbH** for 30 min.; (b) Cells were pretreated with PMA (5.0 μg/mL) for 30 min. Green channel λ_{em}=470-530 nm, red channel λ_{em}=550-650 nm. The results demonstrated that fluorescence signal coming from the donor remained very weak in the green channel due to the effective TBET process of the probe[2].



(a)



(b)



(c)

Fig. S13. Depth TP fluorescence images of: (a) **NpRbH** (5 μM) in tissues (0-300 μm).; (b) After added PMA (5 μg/mL) in tissues (0-300 μm) and (c) After treatment with PMA, flowed

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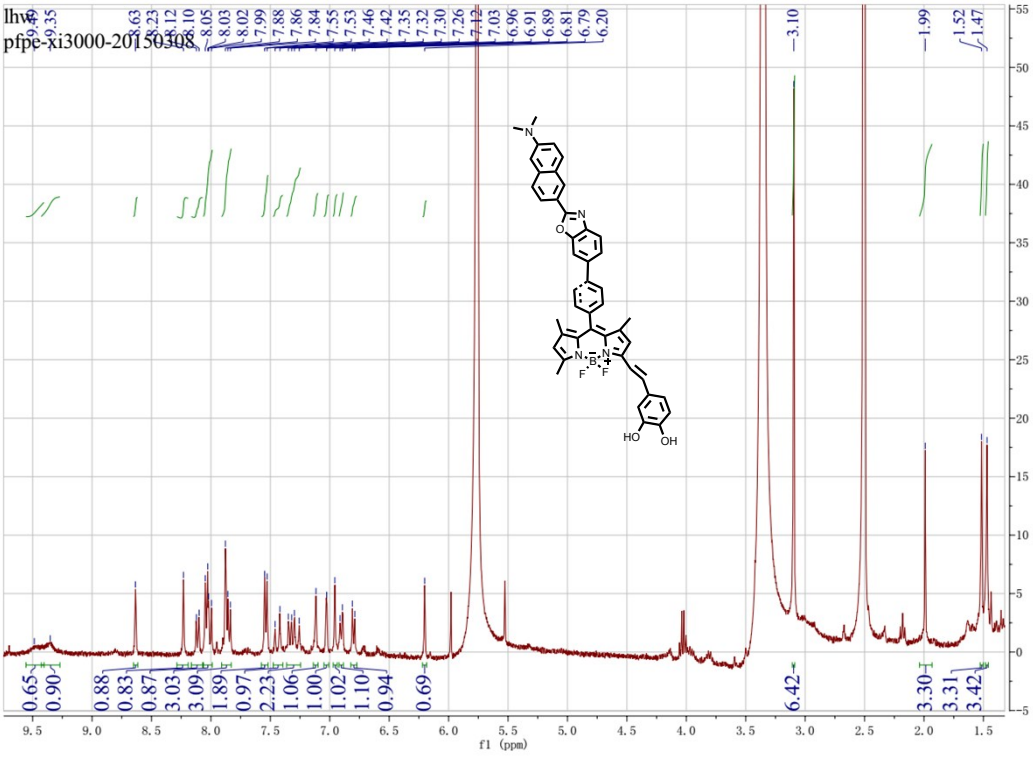
with GSH (1mM) for another 60 min in tissues (0-300 μm). Step size: 3 μm for a, c and 2 μm for b. Scale bars: 200 μm. λ_{ex}=780nm, red channel λ_{em}=550-650 nm.

References

1. H.-W. Liu, X.-B. Zhang, J. Zhang, Q.-Q. Wang, X.-X. Hu, P. Wang and W. Tan, *Anal. Chem.*, 2015, **87**, 8896-8903.

2. L. Zhou, X. Zhang, Q. Wang, Y. Lv, G. Mao, A. Luo, Y. Wu, Y. Wu, J. Zhang and W. Tan, *J. Am. Chem. Soc.*, 2014, **136**, 9838-9841.

Mass Spectra and ^{1}HNMR



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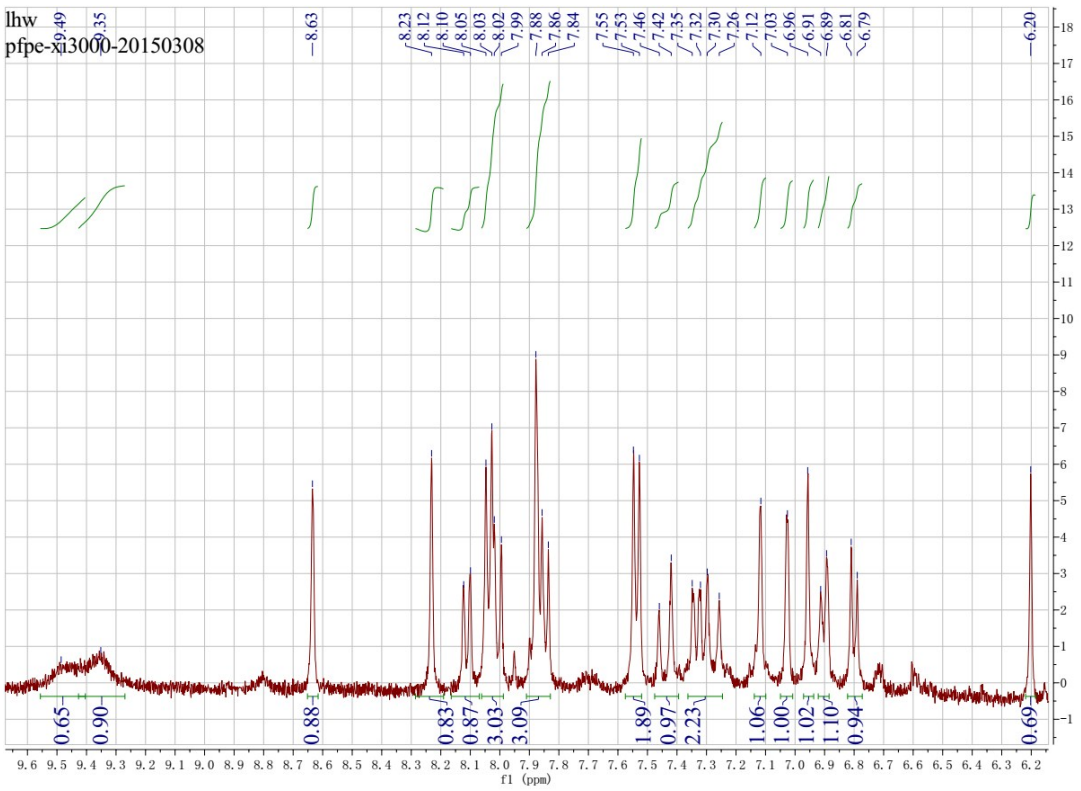

Fig. S14 [1]HNMR spectrum of the compound **NpRbH**

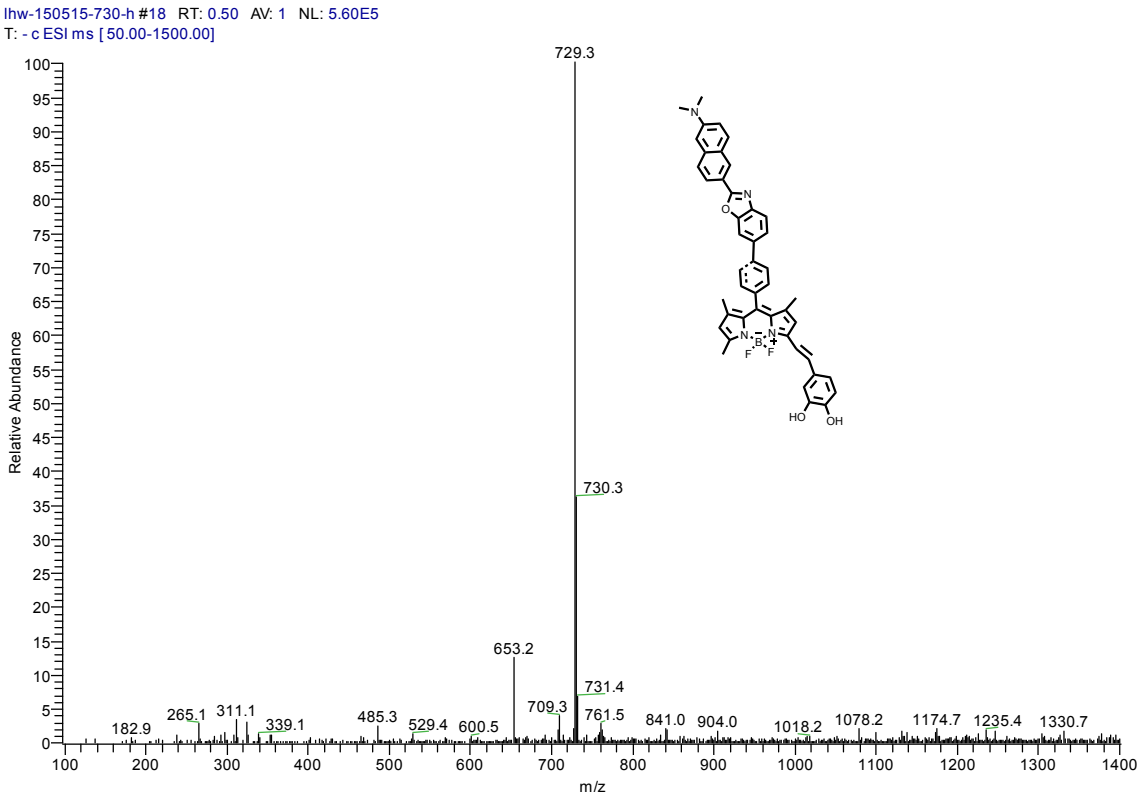

Fig. S15 ESI mass spectrum of the compound **NpRbH**.

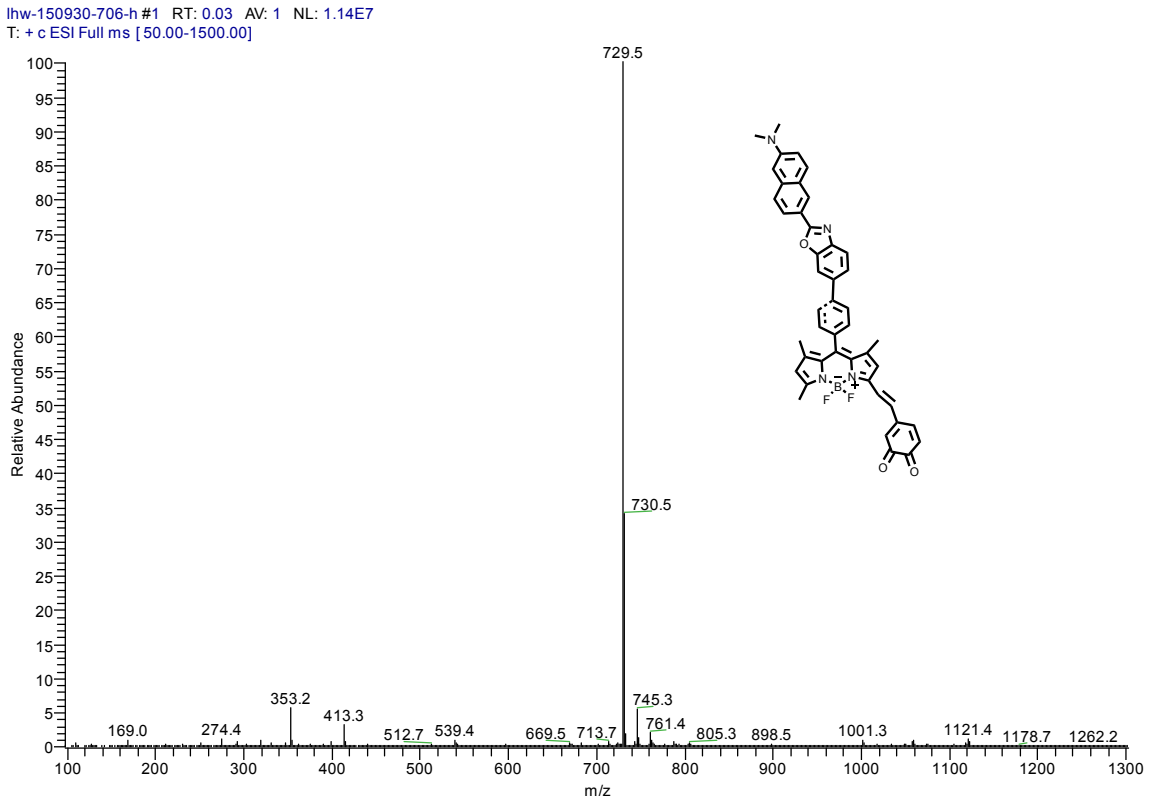

Fig. S16 ESI mass spectrum of the compound **NpRbO**.