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Supporting Information for

Development of cysteine-sensitive bimodal probes for in situ monitoring of early-stage pulmonary fibrosis progression and therapeutic effects

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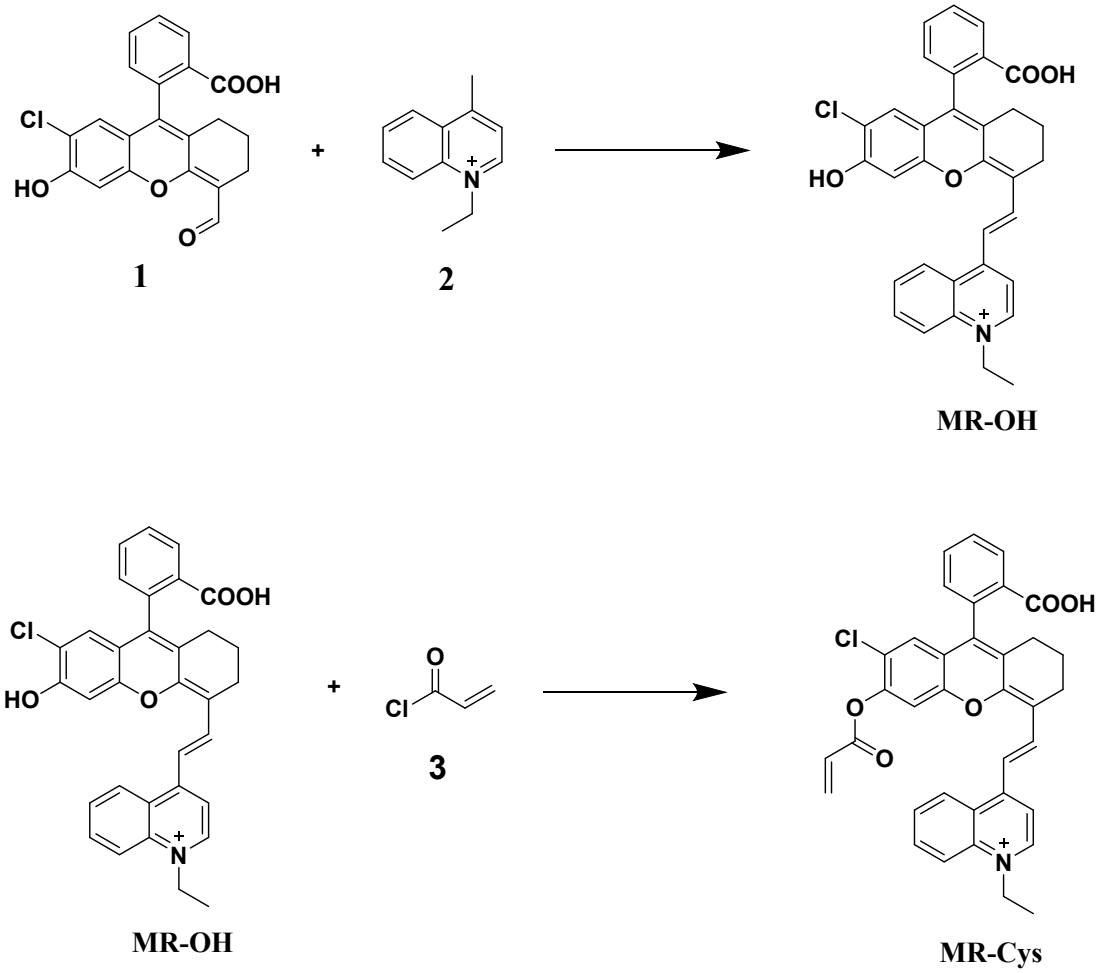
1. Materials and instruments

Unless specified otherwise, all chemicals and reagents were sourced from commercial providers. Ultra-pure water was utilized in all experimental procedures. NMR data, both 1H and 13C, were acquired using a Bruker Avance III HD 600 MHz spectrometer located in the United States. High-resolution mass spectrometry (HRMS) analyses were conducted on an Agilent 6550 Q-TOF instrument. UV-visible absorption spectrometry (UV-2700, Shimadzu) was used to record absorbance, while a Hitachi F-4700 fluorescence spectrophotometer was employed for fluorescence spectrum measurement. NIR fluorescence imaging of mice was performed using a Small Animal In Vivo Imaging System (IVIS Lumina Series III) with a laser power of 0.26 W, an excitation wavelength of 720 nm, and a reception range of 780-845 nm. All optoacoustic measurements were conducted using a LOIS 3D imaging system (TomoWave) for PA imaging, with an excitation wavelength of 780 nm and an energy setting of 250 mJ. TLC analysis was carried out on silica gel plates, and column chromatography was performed over silica gel (mesh 200-300), both sourced from Qingdao Ocean Chemicals. All aqueous solutions were prepared using ultrapure water obtained from a Milli-Q water purification system, which has a resistivity of 18.2 MΩ cm.



Scheme S1. Synthetic route for **MR-Cys**.

Reagents, conditions: a) C_2H_6O, 8 h; b) CH_2Cl_2, 0 °C, 3 h.

2. Synthesis of compounds

Synthesis of compound MR-OH. Synthesis of compound **1** and Compound **2** were synthesized according to our previous reports [1,2] Compound **1** (0.60 g, 1.6 mmol) and compound **2** (0.32 g, 1.86 mmol) were weighed, dissolved in anhydrous ethanol and heated to 80 °C, the solution changed from brownish yellow to dark blue, and the reaction was terminated after 8 h. The reaction solution was concentrated with a vacuum pump in a vacuum water bath and purified by column chromatography using the eluent CH_2Cl_2/MeOH (1/100, v/v) to afford the blue solid compound **MR-OH**. (Yield: 0.25g, 29 %). ^{1}HNMR (600 MHz, DMSO) δ 8.29 (d, J = 8.5 Hz, 1H), 8.16 (d,

J = 13.2 Hz, 1H), 8.02 (d, *J* = 3.5 Hz, 1H), 7.71 – 7.65 (m, 3H), 7.45 (dt, *J* = 7.3, 3.7 Hz, 2H), 7.43 – 7.35 (m, 1H), 7.17 (d, *J* = 7.7 Hz, 1H), 7.06 – 6.99 (m, 1H), 6.62 (d, *J* = 10.5 Hz, 2H), 6.51 (s, 1H), 4.27 (dd, *J* = 14.2, 7.1 Hz, 2H), 2.75 – 2.59 (m, 2H), 2.30 – 2.08 (m, 2H), 1.66 (s, 2H), 1.35 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, MeOD) δ 158.89 (d, *J* = 38.5 Hz), 116.30 (s), 114.39 (s), 48.01 (s), 47.86 (s), 47.72 (s), 47.58 (s), 47.44 (s), 47.30 (s), 47.15 (s). ESI-HRMS calcd for $C_{33}H_{27}ClNO_4^+$ $[M]^+$ 536.1623, found 536.1635.

3. General procedure for spectral measurement of MR-Cys in response to Cys

The stock solution of 10 mmol/L of the probe was prepared freshly in dry DMSO. For Cys response experiments, absorption and emission spectroscopy were monitored in PBS (pH = 8.0, 40% DMSO/PBS).

4. Cell culture and *in vitro* cytotoxicity test

A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (fetal bovine serum), 100 U/mL penicillin, and 100 μg/mL streptomycin with a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cytotoxicity of probe **MR-Cys** on A549 cells was detected by CCK8 assay. A549 cells with approximately 2 × 10^4 cells/mL were inoculated in 96-well plates and incubated with different concentrations of **MR-Cys** (0-50 μmol/L) for 24 h. Then 10 μL CCK-8 (5 mg/mL) was added to each well. After incubating for 4 h, the supernatant was sucked out and 100 μL dimethyl sulfoxide was added. The absorbance of the solution at 490 nm was recorded using a microplate reader.

$$\text{Cell viability (\%)} = (OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%.$$

OD_{sample} denotes the cells cultured with different concentrations of **MR-Cys**, $OD_{control}$ denotes the cells incubated with culture medium, OD_{blank} denotes only.

5. NIRF and PA Imaging of endogenous Cys

Cells were divided into four groups for different purposes: the first group was

treated with NEM (30 μL, 1 mmol/L) for 1 h and then incubated with **MR-Cys** (20 μmol/L) for 40 min; the second group was incubated with **MR-Cys** (20 μmol/L) only for 40 min; the third group was treated with NEM (30 μL, 1 mmol/L) for 1 h and then incubated with Cys (20 μmol/L) and **MR-Cys** (20 μmol/L) for 40 min; the fourth group was treated with NEM (30 μL, 1 mmol/L) for 1 h and then incubated with Cys (20 μmol/L) and **MR-Cys** (50 μmol/L) for 40 min. (λ_{ex} = 720 nm, λ_{em} = 780-845 nm). The excitation wavelength of all PA images was 780 nm.

6. Animal model

The 6-week-old female BALB/c mice were obtained from Guangxi Medical University (Nanning, China). All animal experiments were reviewed and approved by the Animal Care and Experiment Committee of Guangxi University (protocol number: Gxu-2022-173).

We established a mouse model of pulmonary fibrosis, i.e., confirmed diabetes mellitus, by administering 5 mg/Kg bleomycin (BLM) dissolved in saline (37.5 mg/ml) via tracheal tube and then reared for 14 days. The pulmonary fibrosis mice were then treated with OFEV 10 days later, **MR-Cys** was injected into normal mice, pulmonary fibrosis mice and mice treated with OFEV.

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7. NIRF and PA Imaging of exogenous Cys in live mice

The female BALB/c mice (4-5 weeks) were selected for *in vivo* imaging of exogenous Cys. First, the hair of the mouse's back was shaved with a razor, and then depilated with a depilatory cream, then different treated solutions were subcutaneously injected into the thigh muscle of the mice. The first group was treated with NEM (30 μL, 1 mmol/L) for 1 hours and then incubated with injections of **MR-Cys** (20 μmol/L, 100 μL) for 30 minutes; the second group was injected with probe **MR-Cys** (20 μmol/L, 100 μL) for 30 minutes; the third group was treated with NEM (30 μL, 1 mmol/L) for

1 hours and then incubated with injections of Cys (The third group was treated with NEM (30 μL, 1 mmol/L) for 1 hours and then injected with Cys (20 μmol/L, 100 μL) and incubated with probe **MR-Cys** (20 μmol/L, 100 μL) for 40 minutes; the fourth group was treated with NEM (30 μL, 1 mmol/L) for 1 hours and then injected with Cys (50 μmol/L, 100 μL) and incubated with probe **MR-Cys** (20 μmol/L, 100 μL) for 40 minutes. Then, the mice subjected to different treatments were anesthetized with isoflurane (2%), and fluorescence and photoacoustic imaging of the mouse legs were performed. Fluorescence imaging was done at Guangxi University (Small Animal Imaging System, IVIS Lumina III, and USA). The excitation wavelength for all fluorescence images was 720 nm and the reception range was 780-845 nm. Photoacoustic imaging was conducted at Guangxi University (LOIS-3D, TomoWave Laboratories, and USA). The excitation wavelength of all PA images was 780 nm.

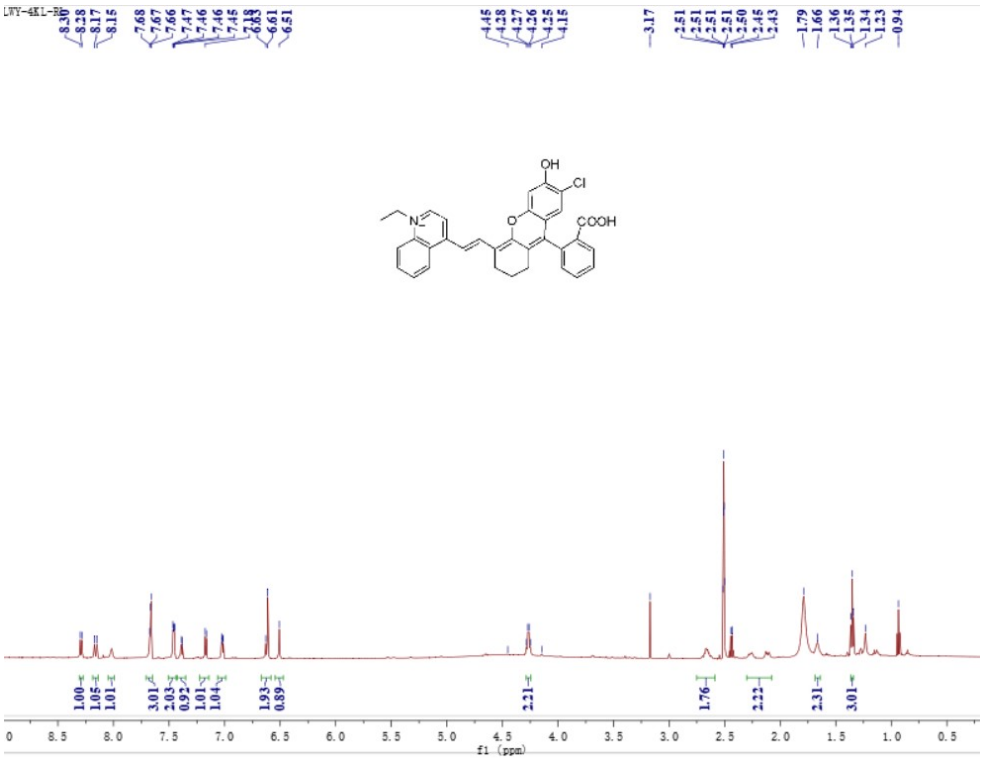

Fig. S1. [1]H NMR spectrum of compound **MR-OH** in DMSO-d6.

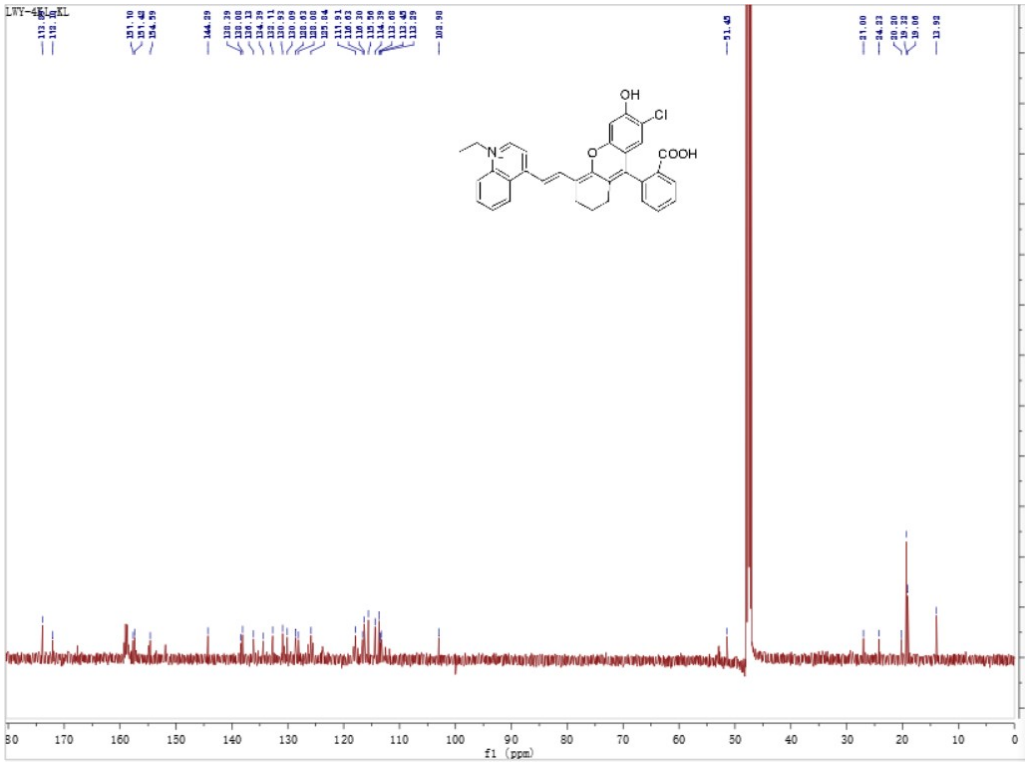

Fig. S2. [13]C NMR spectrum of **MR-OH** in DMSO-d6

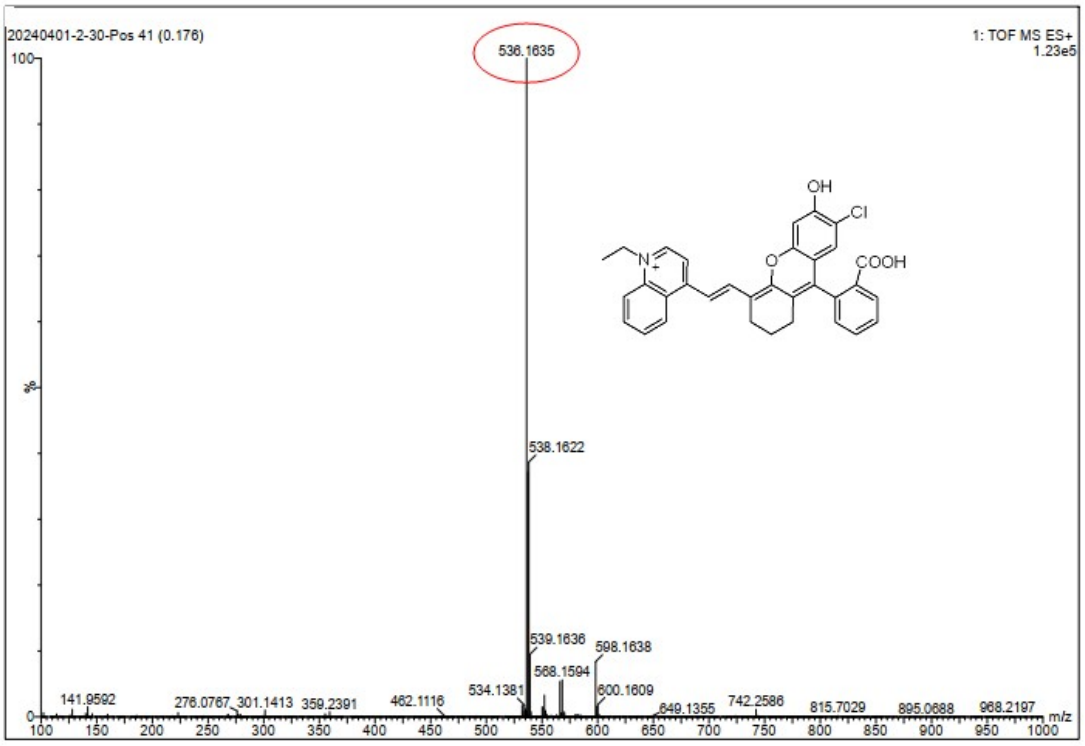

Fig. S3. High-resolution mass spectrum (HRMS) of **MR-OH**

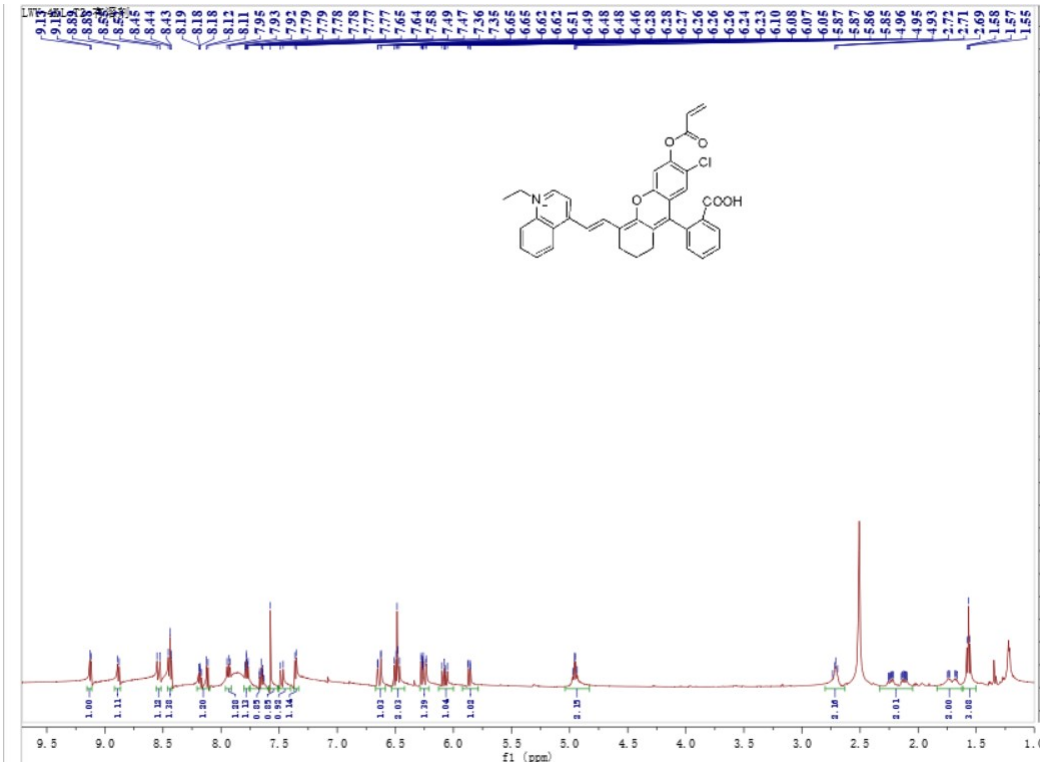

Fig. S4. ^{1}H NMR spectrum of **MR-Cys** in DMSO-d6.

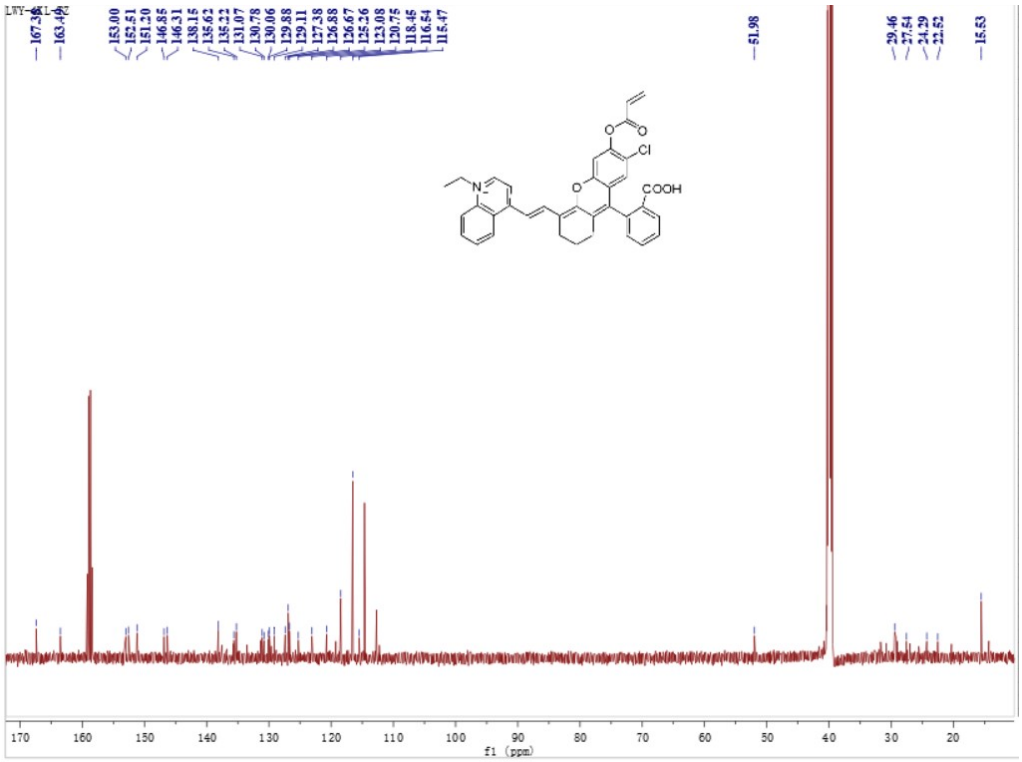

Fig. S5. ^{13}C NMR spectrum of **MR-Cys** in DMSO-d6.

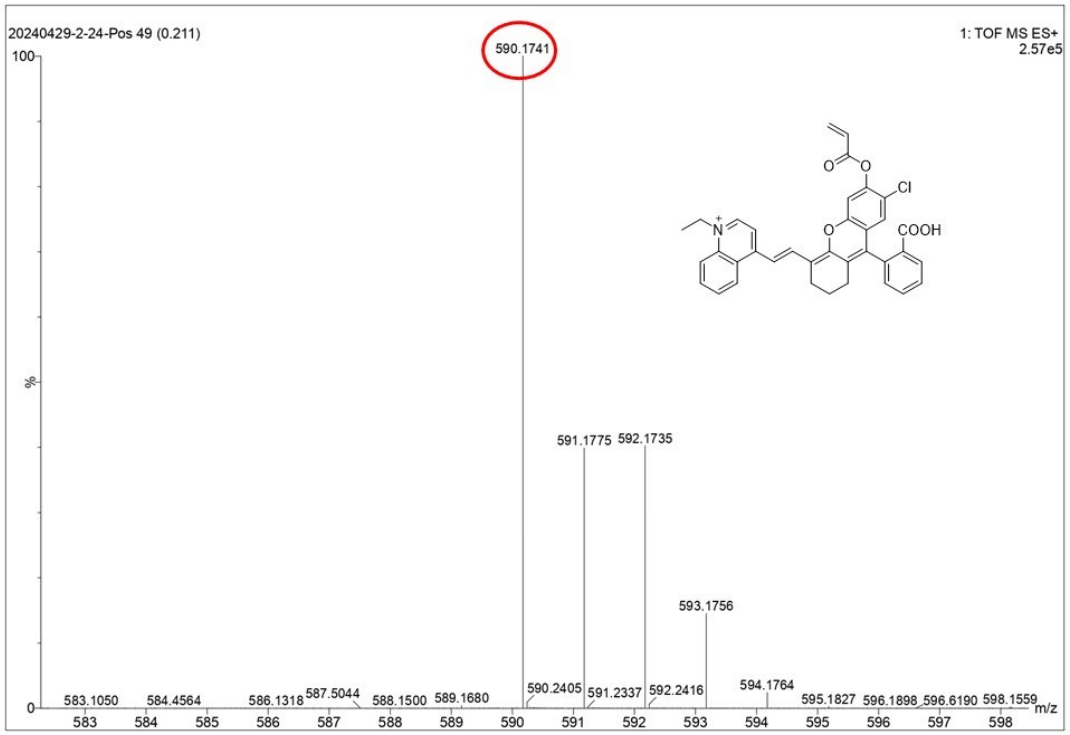

Fig. S6. High-resolution mass spectrum (HRMS) of **MR-Cys**

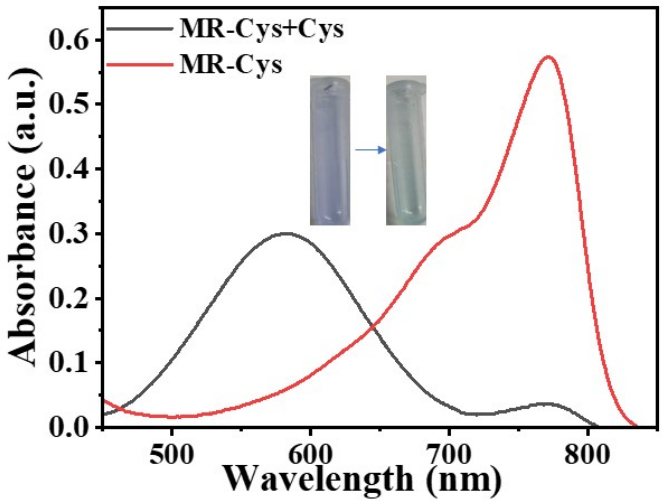

Fig. S7. The absorption spectra before and after the probe **MR-Cys** (10 μmol/L) reaction with Cys (50 μmol/L). At 25 ℃, the spectra were detected after mixing in 40% DMSO/PBS pH=8.0 buffer for 20 min. (B) Fluorescent spectra of **MR-Cys** (10 μmol/L) with and without Cys (50 μmol/L).

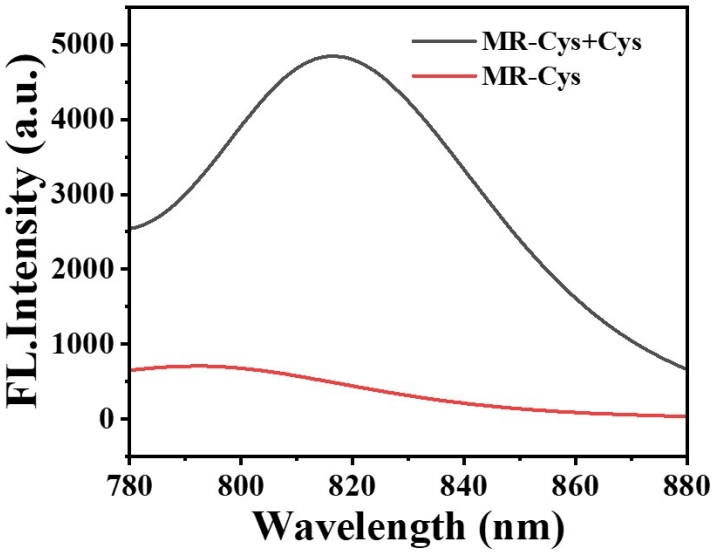

Fig. S8. Fluorescent spectra of **MR-Cys** (10 μmol/L) with and without Cys (50 μmol/L).

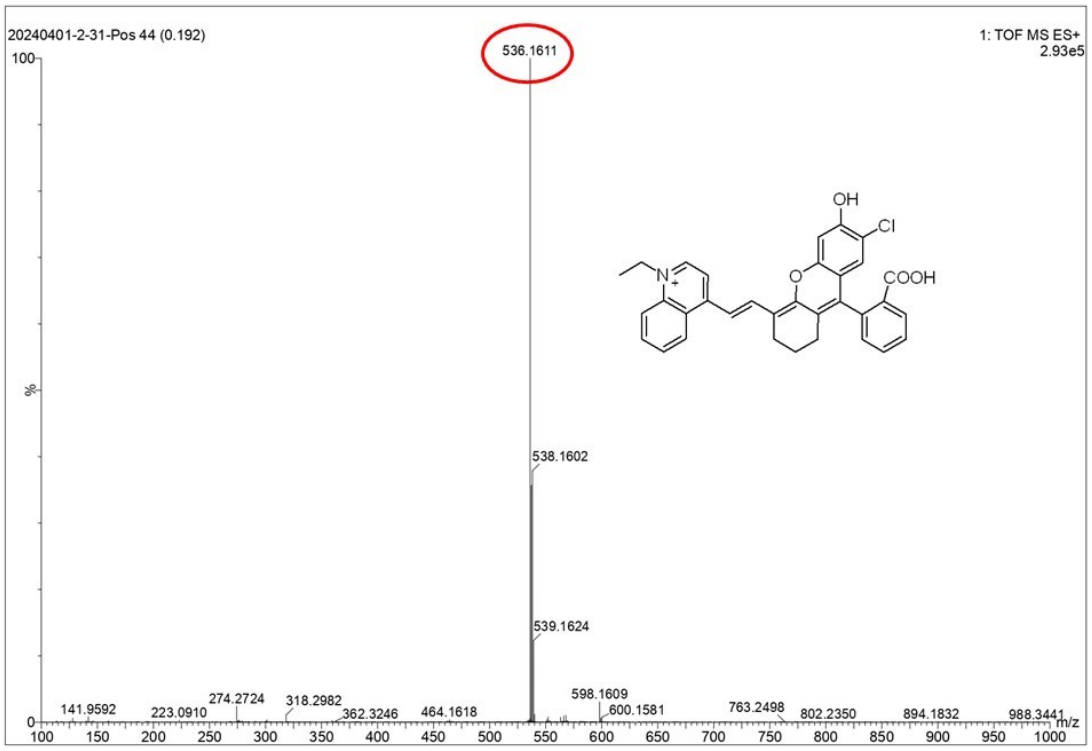

Fig. S9. High-resolution mass spectrum (HRMS) of **MR-Cys** + Cys.

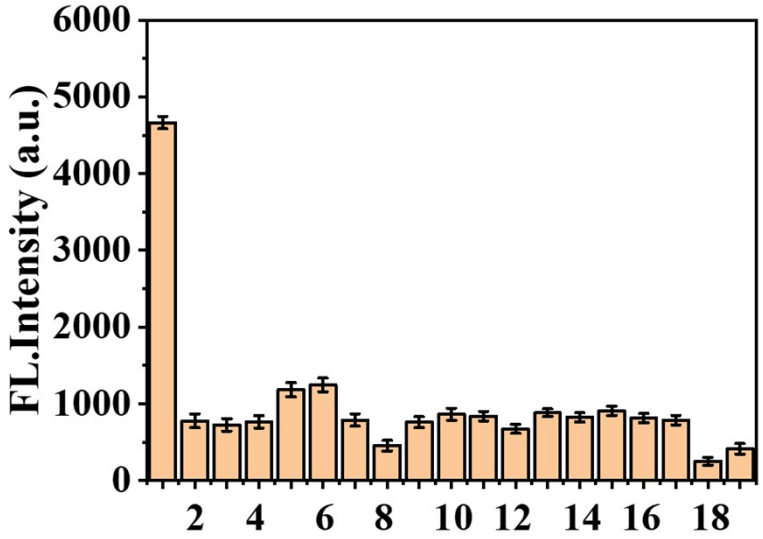

Fig. S10. The absorption intensity of **MR-Cys** (10 μmol/L) at 815 nm for a range of analytes (500 μmol/L each) including (1) Cys, (2) H_2O_2, (3) Co^{2+}, (4) Cu^{2+}, (5) Hcy, (6) GSH, (7) Mn^{2+}, (8) SO_3^{2-}, (9) OAc^-, (10) $S_2O_3^{2-}$, (11) Ser, (12) Zn^{2+}, (13) O^{2-}, (14) Aln, (15) ASP, (16) Br^-, (17) Ca^{2+}, (18) blank, (19) $ONOO^-$. Data are presented as the means ± s.d. (n = 3 independent samples)

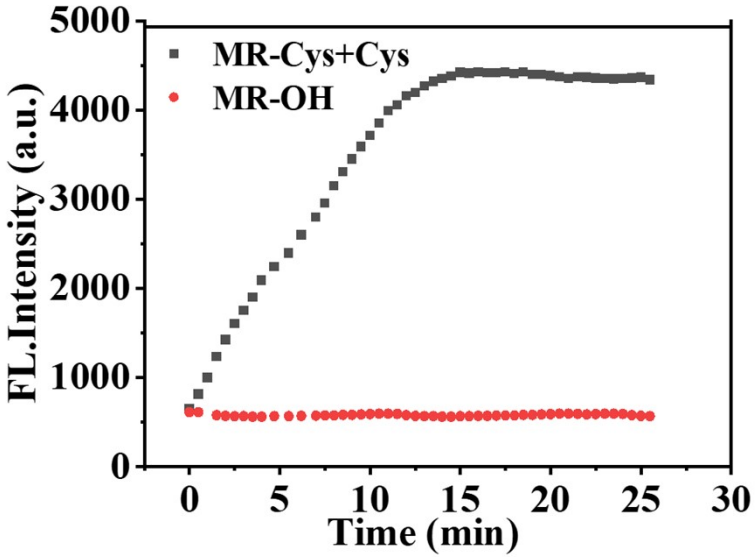

Fig. S11. Changes in fluorescence intensity of the probe **MR-Cys** (10 μmol/L) after reaction with Cys (50 μmol/L) and changes in fluorescence intensity of the probe over a 30 minutes period.

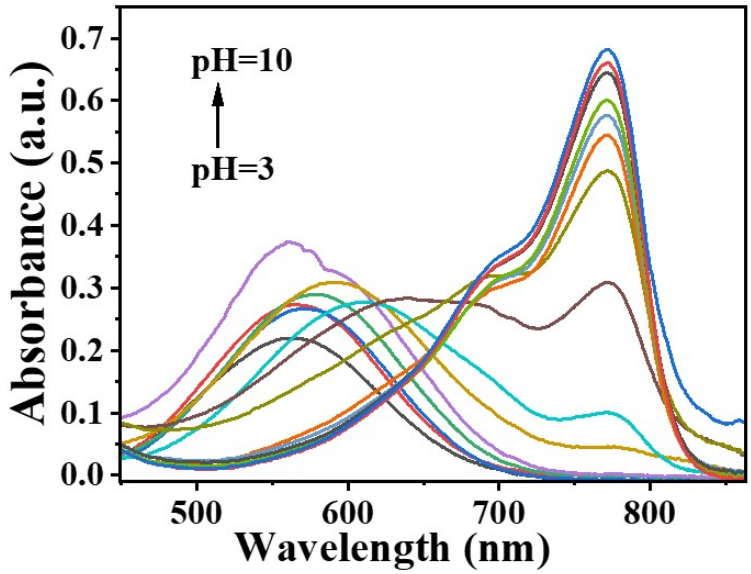

Fig. S12. The effect of absorption intensity of **MR-Cys** (10 μmol/L) with Cys (50 μmol/L) at different pH values.

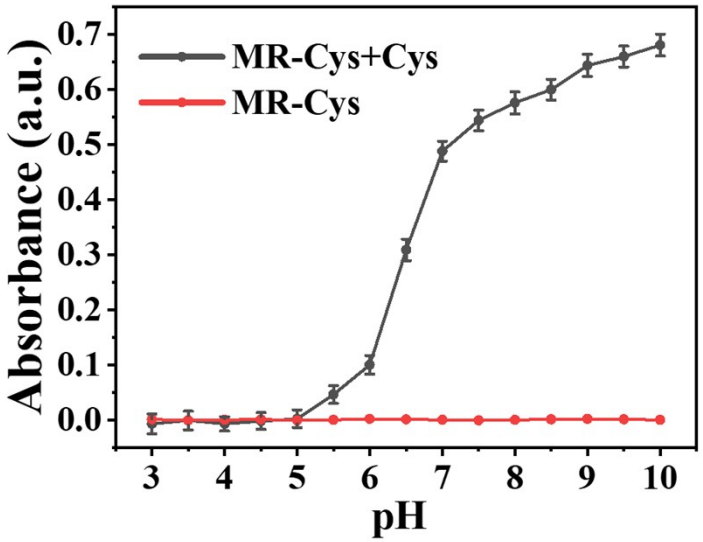

Fig. S13. The linear relationship between Abs_{770} and different pH values. Data are presented as the means ± s.d. (n = 3 independent samples)

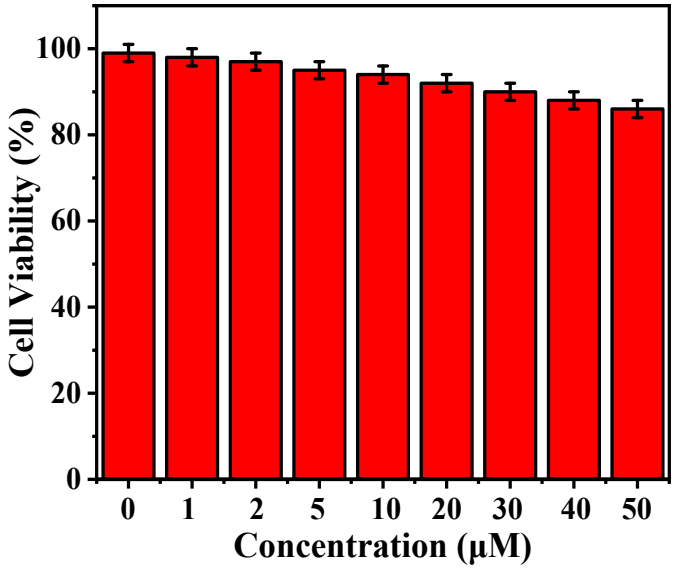

Fig. S14. Survival rate of HapG-2 cells after incubation with different concentrations of **MR-Cys**. Error bars represent mean values ± SD (n = 6 independent samples).

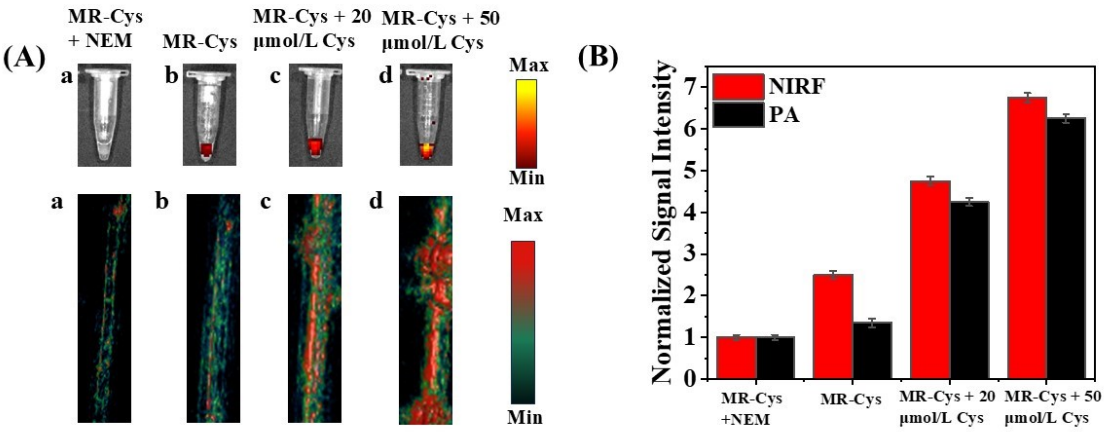

Fig. S15. (A) Respresentative fluorescence imaging and photoacoustic imaging of HapeG2 cells. (a) The first group was treated with NEM (30 μL) for 1 hours, and then incubated with the probe **MR-Cys** (20 μmol/L) for 30 minutes; (b) In the second group, the probe **MR-Cys** (20 μmol/L) was added for 30 minutes; (c) The third group was treated with NEM (30 μL) for 1 h, followed by the addition of Cys (20 μmol/L) and incubation with the probe MR-Cys (20 μmol/L) for 40 min; (d) The fourth group was treated with NEM (30 μL) for 1 h, followed by the addition of Cys (50 μmol/L) and incubation with the probe **MR-Cys** (20 μmol/L) for 40 min. (B) NIRF/PA signal intensity values from (A). Data are presented as the means ± s.d. n = 3 independent experiments, similar results were obtained.

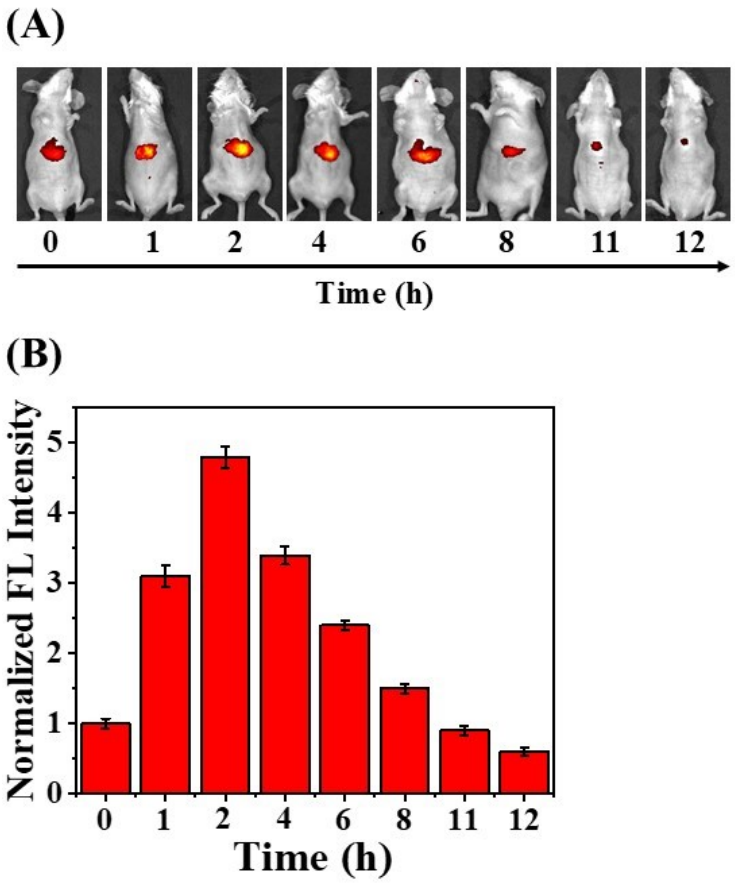

Fig. S16. (A) Representative time-dependent NIRF imaging after the **MR-Cys** system (containing 50 μmol/L Cys, 100 μL) was injected into the tail vein. **(B)** Normalized NIRF signal intensity values from (A). Data are presented as the means ± s.d. (n = 8 mice, similar results were obtained.)

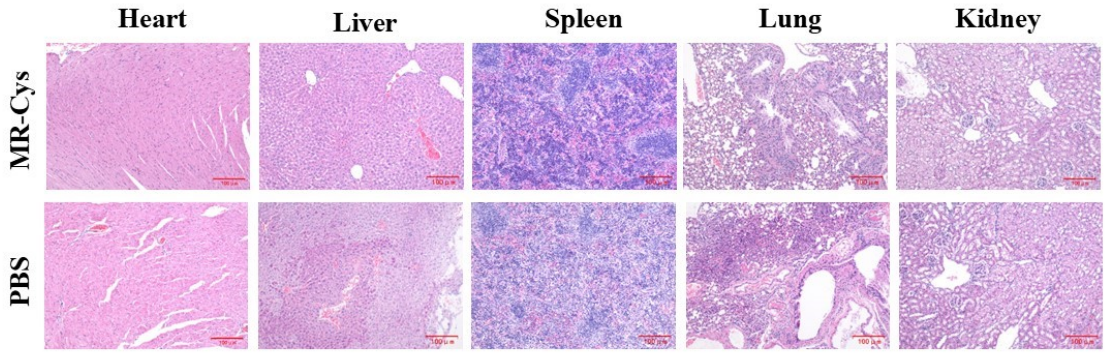

Fig. S17. Representative H&E staining images of the heart, liver, spleen, lungs, and kidneys after tail vein injection of 20 μmol/L **MR-Cys** +50 μmol/L Cys (100 μL) and PBS (0.01 mol/L, 100 μL) for 12 hours. Scale bar: 100 μm.

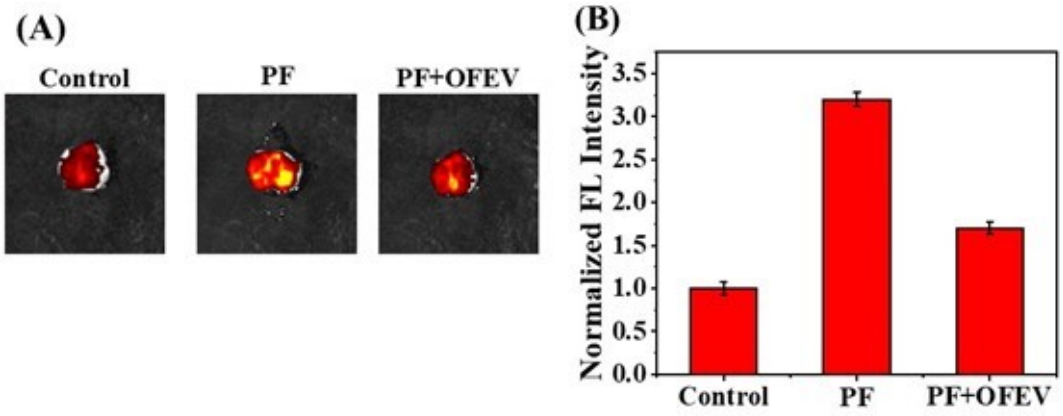

Fig. S18. (A) The fluorescence imaging of the *ex vivo* lungs from the control group, PF group, and PF + OFEV group. **(B)** NIRF signal intensity values from (A). Data are presented as the means ± s.d. (n = 8 mice, similar results were obtained.)

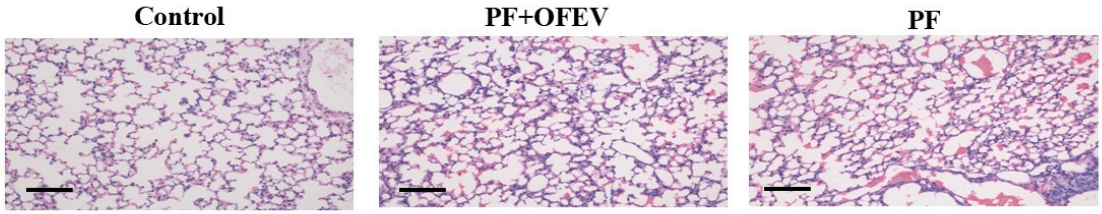

Fig. S19. H&E staining images of the lung for the control group, PF + OFEV group, and PF group. Scale bar: 20x, 100 μm.

References

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2. X. Xiong, F. Song, G. Chen, W. Sun, J. Wang, P. Gao, Y. Zhang, B. Qiao, W. Li, S. Sun, J. Fan and X. Peng, *Chem. - Eur. J.*, 2013, **19**, 6538-6545.