Supporting Information

Novel small-molecule fluorophores for *in vivo* NIR-IIa and NIR-IIb imaging

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Author contributions

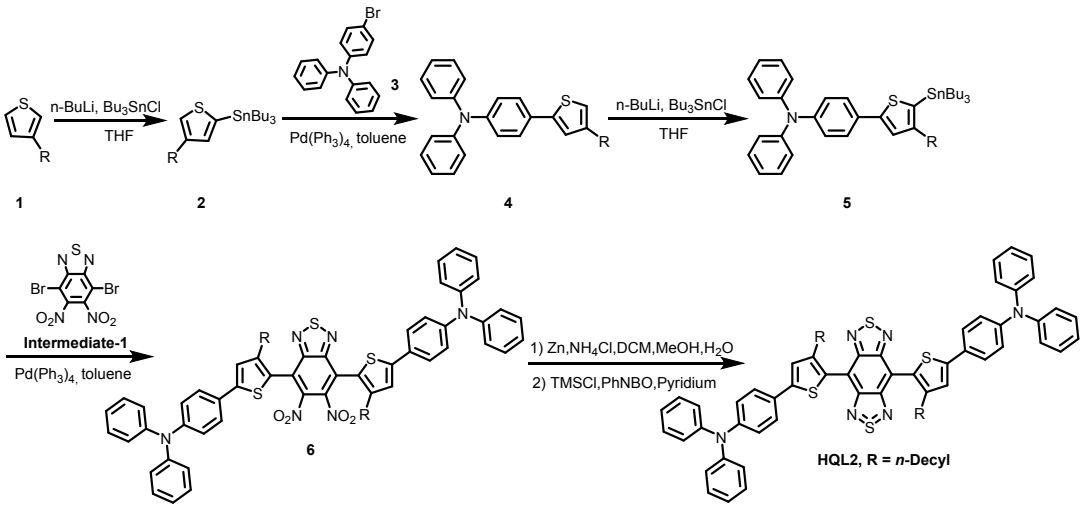
Y. Xiao conceived and designed the experiments. Q. Li, Q. Ding, Y. Li, X. Zeng, Y. Liu, S. Lu performed the experiments. Q. Li, X. Zeng, Q. Ding, Y. Li, Y. Liu, H. Zhou, X. Meng, X. Wang, J. Wu, Z. Deng and Y. Xiao analyzed the data, Y. Xiao and Q. Li wrote the manuscript. All authors discussed the results and commented on the manuscript.

Materials and General Procedure

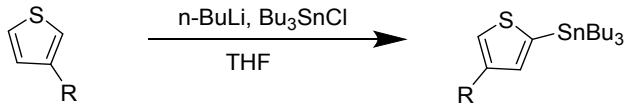
The synthesis reagents were purchased from commercial suppliers (such as Aldrich, Adamas, Energy Chemical, Sinopharm Group Co., Ltd.) and used without further purification unless otherwise noted. Tetrahydrofuran (THF) was freshly distilled from sodium/benzophenone. Dimethylformamide (DMF) and Dichloromethane (CH_2Cl_2) were distilled from calcium hydride. Anhydrous pyridine was freshly distilled using calcium hydride. 4,7-bis(7-bromo-2,3-dihydrothieno[3,4-*b*][1,4]dioxin-5-yl)-5,6-dinitrobenzo[*c*][1,2,5]thiadiazole (intermediate_**1**) was synthesized according to our previous report.[1] ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ at room temperature using a Bruker AV400 magnetic resonance spectrometer. ESI-MS were performed on Finnigan LCQ advantage mass spectrometer. MALDI-TOF-MS characteristics were performed on an AB SCIEX 5800 MALDI TOF mass spectrometer. Analytical and preparative TLC were performed on silica gel plates, and column chromatography was conducted over silica gel (mesh 200-300), both of which were obtained from the Qingdao Ocean Chemicals. UV-vis-NIR spectra were tested with a SHIMADZU UV-2600 or PerkinElmer Lambda 25 spectrophotometer. NIR fluorescence spectrum was performed on an Applied Nano Fluorescence spectrometer at room temperature with an excitation laser source of 785 nm and 808 nm. The NIR-II *in vivo* imaging system was purchased from Suzhou NIR-Optics Technologies CO., Ltd.

Synthesis and characterization

The synthetic route of HQL2



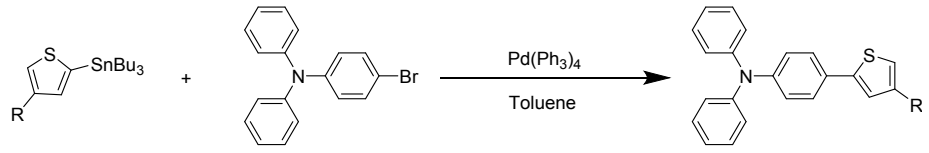
Synthesis of tributyl(3-dodecylcyclopenta-1,3-dien-1-yl)stannane



1 **2**

Compound **1** (6 g, 0.02 mol) was dissolved in 35 mL of dry THF in a rounded-bottom flask under a N_2 atmosphere. 11.4 mL of *n*-butyllithium (2.5 M in hexane) was added dropwise at −78 °C. After stirring for 1 h, tributyltin chloride (9.28 g, 0.028 mol) was added in one portion. Then the reaction was warm to room temperature and stirred overnight. Finally, the solution was purged into cold water. The organic phase was separated, and the aqueous layer was twice extracted with 50 mL of ether. The organic layers were collected and dried over anhydrous $MgSO_4$. After removing the solvent, the crude product tributyl(3-dodecylcyclopenta-1,3-dien-1-yl)stannane **2** as light yellow oil (5.4 g) was obtained and used in the next step without further purification.

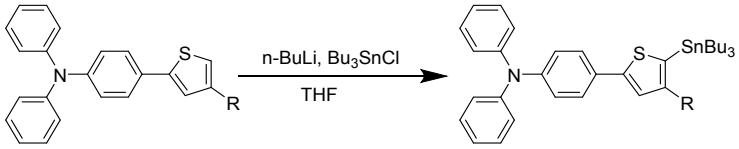
Synthesis of compound 4



2 **3** **4**

A solution of compound **2** (3 g, 5.5 mmol) in Toluene (15 mL) was treated with 4-bromo-N,N-diphenylaniline (1.79 g, 5.5 mmol) and tetrakis(triphenylphosphine) palladium (0) (638 mg, 0.5 mmol), heated at 110° C under a N_2 atmosphere for 8 hours, cooled to ambient temperature, poured into ethyl acetate (10 mL), washed with saturated KF, and brine, dried with anhydrous Na_2SO_4, filtered, and concentrated. The concentrate was purified by column chromatography on silica gel to give the desired product **4** (1.5 g, yield 55.6%) as colorless oil.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56-7.54 (m, 2H), 7.35-7.32 (m, 4H), 7.22-7.11 (m, 8H), 6.88(s, 1H), 2.69-2.68 (m, 2H), 1.74 (s, 2H), 1.39(m, 18H), 1.01-1.00(m,3H).^{13}C NMR (101 MHz, $CDCl_3$) δ 147.7, 147.2, 144.3, 144.0, 129.4, 129.1, 126.6, 124.5, 124.0, 123.8, 123.1, 118.8, 32.1, 30.8, 30.6, 29.9, 29.7, 29.6, 22.9, 14.3. ESI-MS Calcd for: $C_{34}H_{42}NS^+$ ($[M+H]^+$): 496.30. Found: 496.37.

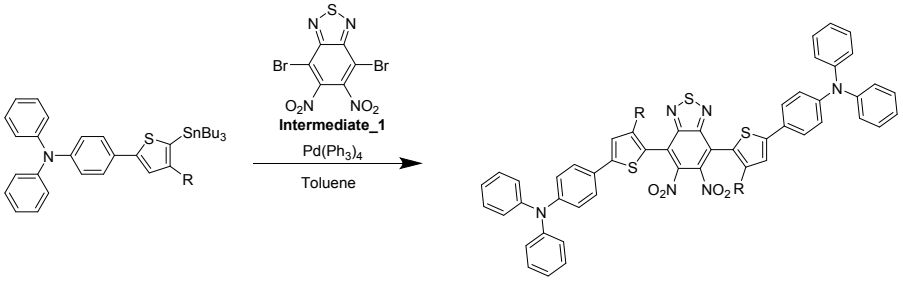
Synthesis of compound 5



4 5

Compound **4** (1.5 g, 3 mmol) was dissolved in 20 mL of dry THF in a rounded-bottom flask under a N_2 atmosphere. 1.4 mL of *n*-butyllithium (2.5 M in hexane) was added dropwise at −78 °C. After stirring for 1 h, tributyltin chloride (1.18 g, 3.6 mmol) was added in one portion. Then the reaction was warm to room temperature and stirred overnight. Finally, the solution was purged into cold water. The organic phase was separated, and the aqueous layer was twice extracted with 50 mL of ether. The organic layers were collected and dried over anhydrous $MgSO_4$. After removing the solvent, the crude product 4-(4-dodecyl-5-(tributylstannyl)thiophen-2-yl)-N,N-diphenylaniline **5** as light yellow oil (1.1 g) was obtained and used in the next step without further purification.

Synthesis of compound 6



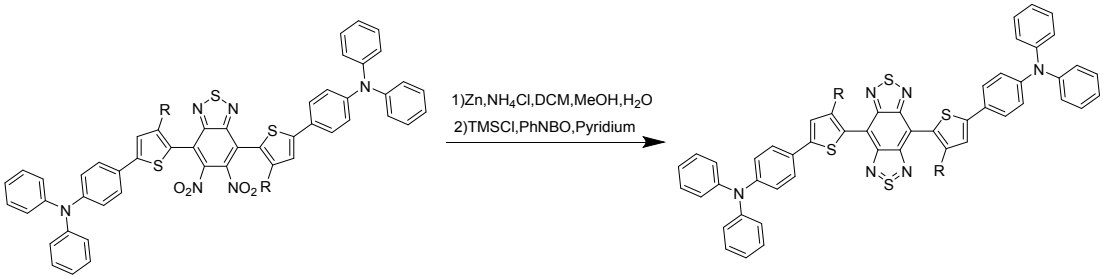
5 6

To a solution of compound **5**（1.1 g, 1.2 mmol）and intermediate_**1** (4,7-dibromo-5,6-dinitrobenzo[c][1,2,5]thiadiazole) (370 mg,0.9 mmol) in Toluene (20 mL) was added tetrakis(triphenylphosphine) palladium (0) (223 mg, 0.19 mmol) at a N_2 atmosphere and degassed for 3 times. Then the mixture was stirred at 110 °C for 10 hours. After reaction, the mixture was cooled to ambient temperature, and poured in to 100 mL water, and extracted with EA (50 mL x3), washed with brine (100 mL), dried with anhydrous Na_2SO_4, concentrated and purified with column chromatography on silica gel to give the desired product **6** (0.85 g, yield 51%) as purple solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 (d, *J* = 8.6 Hz, 2H), 7.37-7.29 (m, *8*H), 7.26(s,2H), 7.17 (d, *J* = 7.9 Hz, 2H), 7.12-7.06 (m, 16H), 2.48-2.45 (m, 4H), 1.64-1.60 (m, 36H), 0.96 (t, *J* = 7.3 Hz, 3H), ^{13}C NMR (101 MHz, $CDCl_3$) δ 153.4, 148.1, 147.3, 146.5, 144.0, 129.4, 127.1, 126.8, 124.8, 124.0,123.8, 123.4, 123.2, 121.0, 31.9, 29.7, 27.9, 26.9, 22.7, 17.6, 14.2, 13.7. ESI-MS Calcd for:

$C_{74}H_{81}N_6O_4S_3^+$ ([M+H]$^+$): 1213.54. Found: 1213.68.

Synthesis of compound HQL2

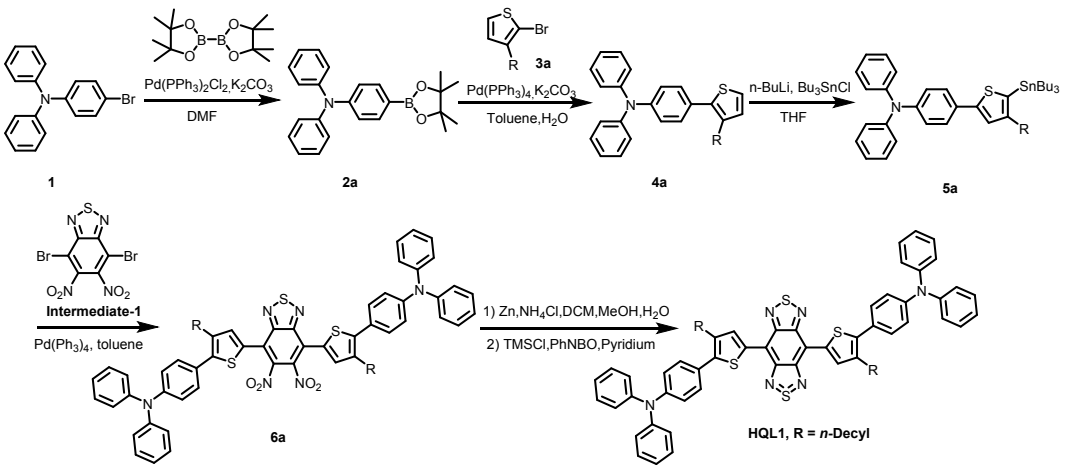


Zinc dust (500 mg, 9.4 mmol) and NH_4Cl (200 mg, 3.07 mmol) were added to a stirred solution of compound **6** (100 mg, 0.08 mmol) in dichloromethane (5 mL) and 90% methanol (5 mL) under an N_2 atmosphere. After being stirred at room temperature for 4 h, the solution was filtered through Celite pad, diluted with dichloromethane, and washed with water, saturated aqueous $NaHCO_3$, and saturated aqueous brine. The organic phase was dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum to afford a yellow solid which was used into the next step without further purification.

To a dark yellow solution in anhydrous pyridine (1 mL) was added N-thionylaniline (0.45mL, 4.02 mmol, 560 mg) and chlorotrimethylsilane (0.45 mL, 5.1 mmol, 560 mg). The mixture was heated in an oil bath at 80 °C for 12 h. The reaction mixture was allowed to cool down to room temperature, poured into iced water and 1M HCl (aq.), extracted with ethyl acetate. The combined organic layer was washed saturated aqueous brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (15:1 petroleum ether: ethyl acetate) to yield the product **HQL2** as light blue solid (15 mg, two step 45% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, *J* = 8.3 Hz, 4H), 7.41 (s, 2H)7.32 (t, *J* = 8.3 Hz, 8H), 7.23-7.04 (m, *16*H), 2.64 (t, *J* = 7.8 Hz, 4H), 1.67-1.60 (m, 4H), 1.42-1.08 (m, 36H), 0.90 (t, *J* = 6.6 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 153.3, 147.6, 147.5, 147.0, 145.7, 129.4, 128.2, 126.8, 124.8, 124.7, 123.4, 123.2, 116.1, 32.0, 30.5, 30.4, 29.7, 29.4, 25.7, 22.7, 14.2. MALDI-TOF-MS Calcd for: $C_{74}H_{81}N_6S_4^+$ ([M+1]$^+$): 1181.53, found: 1181.2.(Fig. S7)

The synthesis route of HQL1



The compound **HQL1** was obtained from 4-bromo-N,N-diphenylaniline by the method similar to that of **HQL2.**

Cell Culture and Animal Model

Human hepatocyte cells L02 were purchased from the China Center for Type Culture Collection (CCTCC). All cells were grown in a humidified atmosphere at 37 °C with 5% CO_2 atmosphere. L02 cells were maintained in Mimumum Essentiul Medium (MEM, Gibco), supplemented with 10% fetal bovine serum, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin. For U87 subcutaneous tumor model establishment, U87 cells (roughly 2×10^6 in 75 μL of FBS-free DMEM medium) were subcutaneous injected into the right back leg of the 6-week-old female athymic nude mice (nu/nu) which were purchased from Charles River Laboratories (Beijing, China). The tumors were allowed to reach ~200 mm^3 for small animal fluorescence imaging studies (tumor volume = Length*Width*Width/2). All animal experiments were performed according to the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of Wuhan University.

Preparation and Characterization of HQL2 dots

HQL2 (5 mg) and DSPE-PEG5K (40 mg) were completely dissolved in THF (1 mL). Then the mixture was added dropwise into one grade water (10 mL) under strong sonication. After 5 min sonication, the organic solvent was removed under N_2 flow, then the resulting mixture was washed several times using a 30 kDa centrifugal filter units and concentrated to yield **HQL2** dots (~ 5 mg/mL). The resultant was stored in dark at 4 °C for further usage. The size and morphology were

characterized by Transmission electron microscopy (TEM) images on a JEM-2100 TEM system at an accelerating voltage of 200 kV. The hydrodynamic diameter and zeta potentials were measured using a Malvern Zetasizer Nano ZS. The UV-vis-NIR spectra of **HQL2** dots was tested with a PerkinElmer Lambda 25 UV-Vis spectrophotometer. The corresponding NIR fluorescence spectrum was recorded on an Applied Nano Fluorescence spectrometer at room temperature with an excitation laser source of 808 nm.

The cell viability assay of HQL2 dots

The cytotoxicity of **HQL2** dots was investigated by a standard MTT assay. The cell viability was measured using human hepatocyte cells L02 for 24 h incubation. The L02 cells were seeded in a 96-well plate (around 5000 cells per well). After 12 h, the medium was substituted with the fresh medium contained **HQL2** dots with different concentrations. Followed by incubation for another 24 h, then a standard MTT method was performed for measuring the cell viability.

***In vivo* vascular Imaging.**

All NIR-II fluorescent images were collected using a NIR-II imaging system with the indium-gallium-arsenide (InGaAs) camera (Princeton Instruments). The excitation light source was an 808 nm diode laser. The laser power density was 90 mW cm^{-2} with different filter (1000 nm LP, 1250 nm LP, 1320 nm LP and 1550 nm LP) during *in vivo* imaging. The mice were anesthesized by intraperitoneal injection of pentobarbital sodium solution (50 mg kg^{-1}) during the NIR-II imaging. For *in vivo* vascular imaging, the C57 Balb/c female mice and the athymic nude mice (nu/nu) bearing subcutaneous U87 tumor were given **HQL2** dots (0.2 mL, 5 mg/mL) via tail vein injection. After injection, the mice were mounted in the prone position and supine position beneath the laser for vascular imaging.

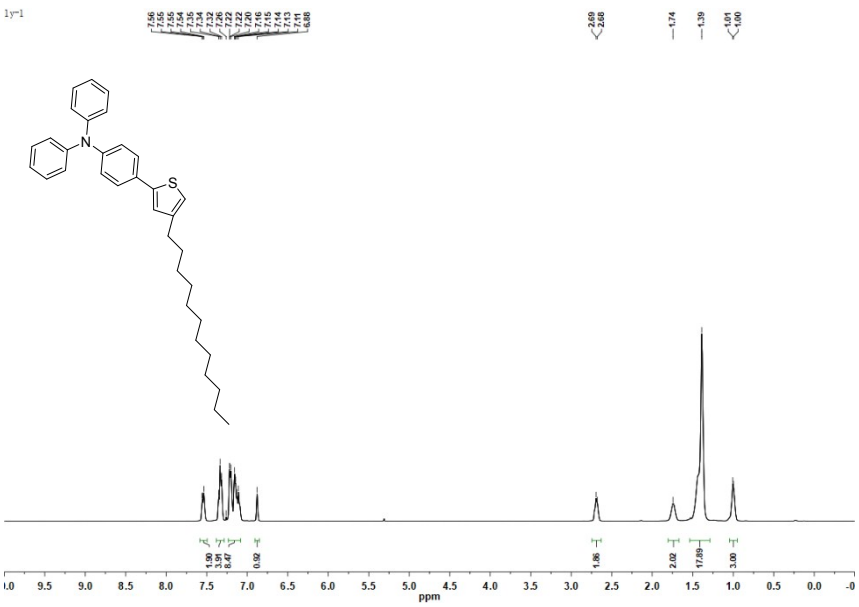


Figure S1. ^{1}H NMR of compound **4**

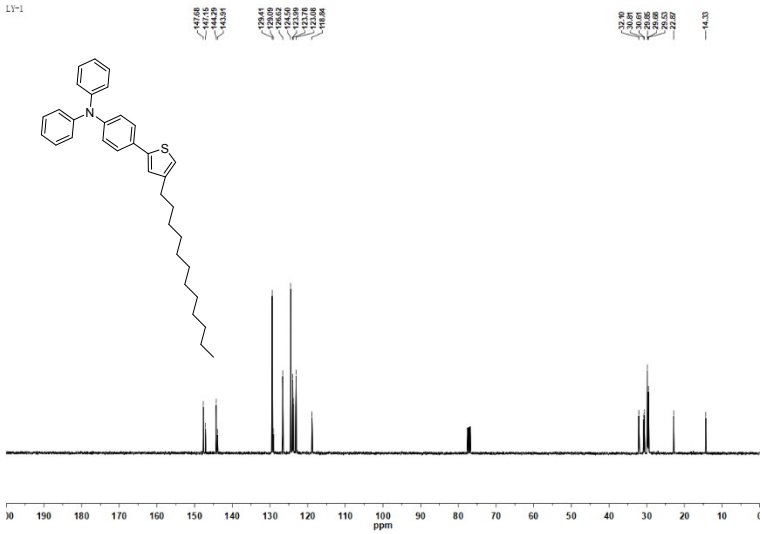


Figure S2. ^{13}C NMR of compound **4**

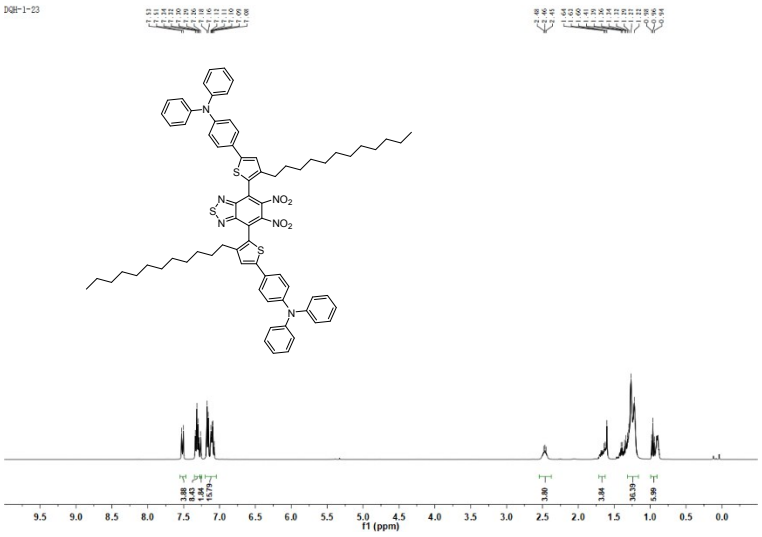


Figure S3. ^{1}H NMR of compound **6**

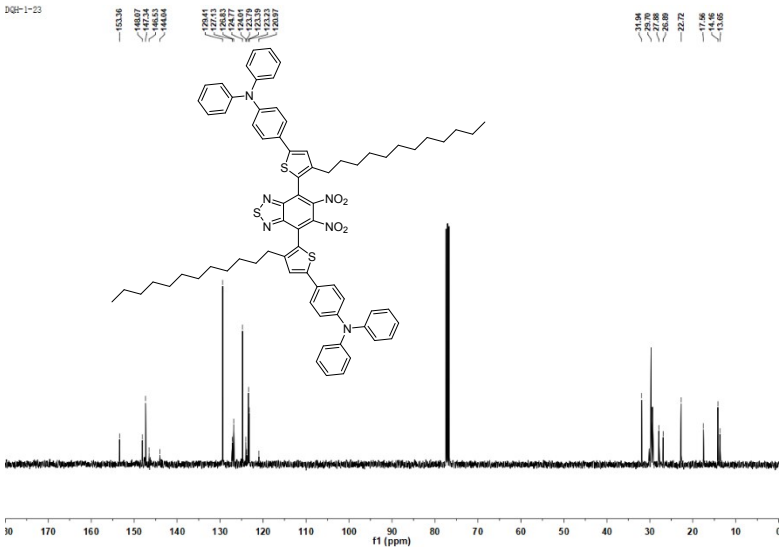


Figure S4. ^{13}C NMR of compound **6**

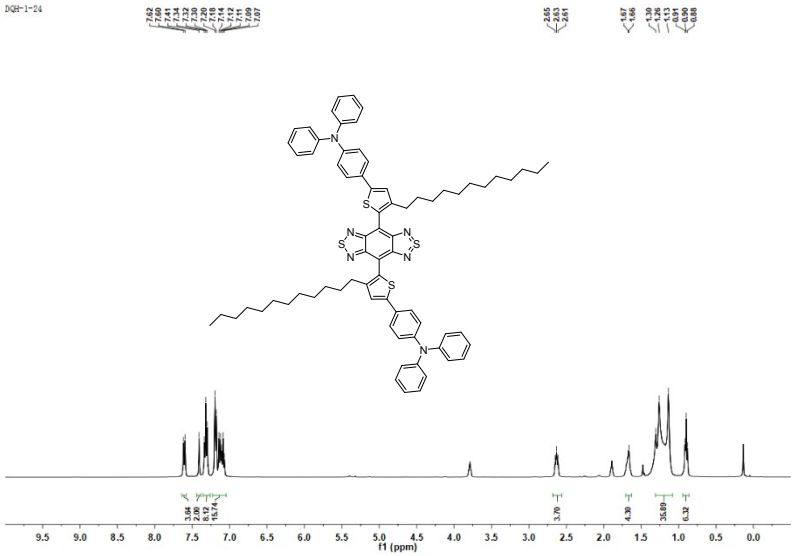


Figure S5. ^{1}H NMR of compound **HQL2**

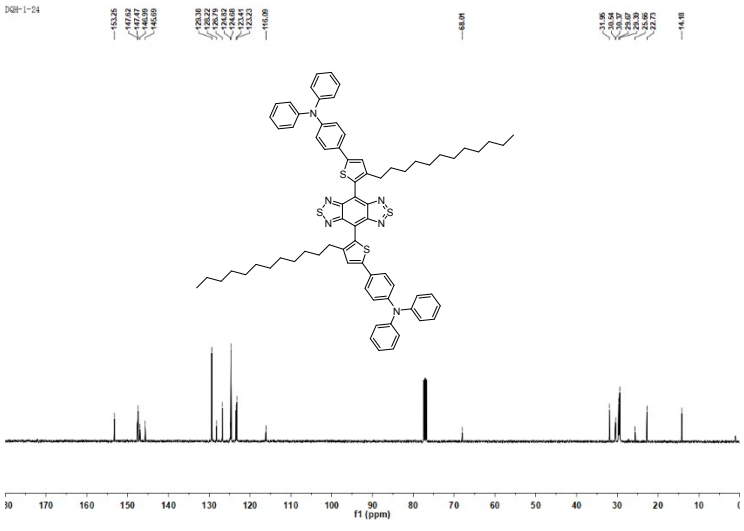


Figure S6. ^{13}C NMR of compound **HQL2**

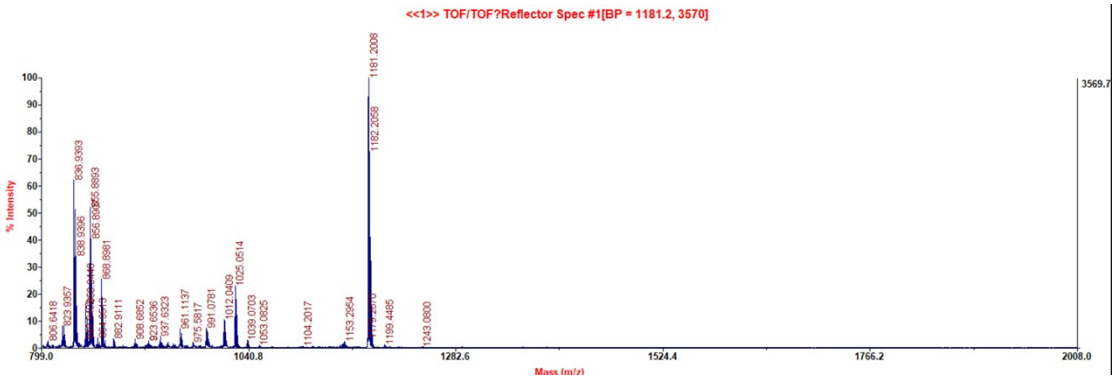


Figure S7. The MALDI-TOF-MS spectrum of **HQL2**

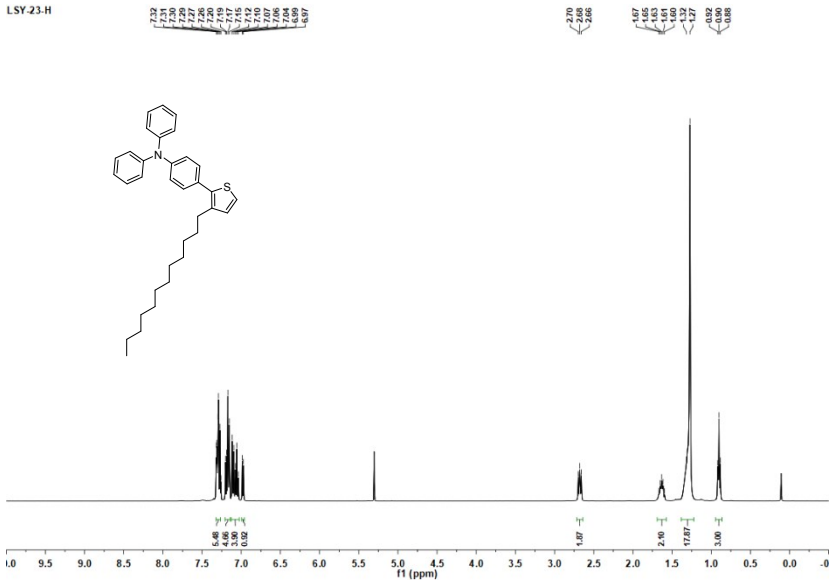


Figure S8. ^{1}H NMR of **4a**

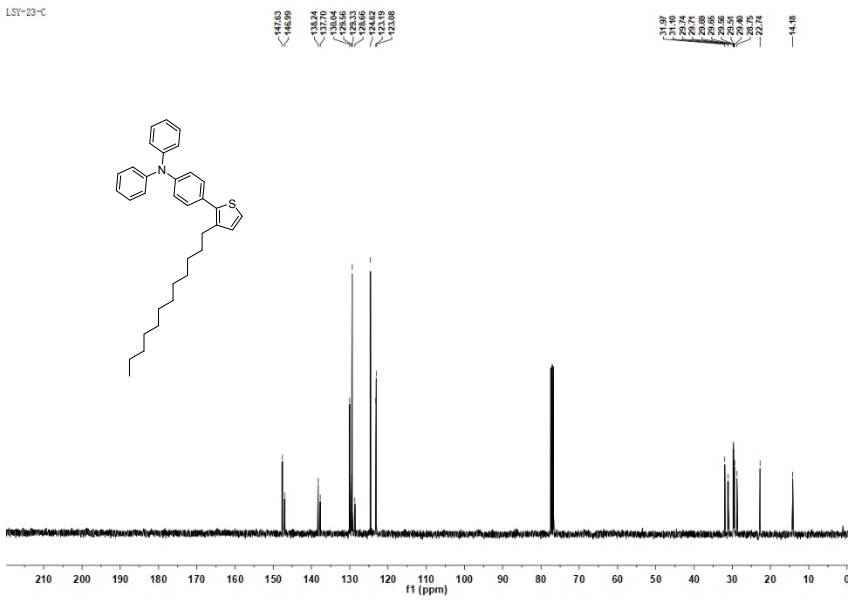


Figure S9. ^{13}C NMR of **4a**

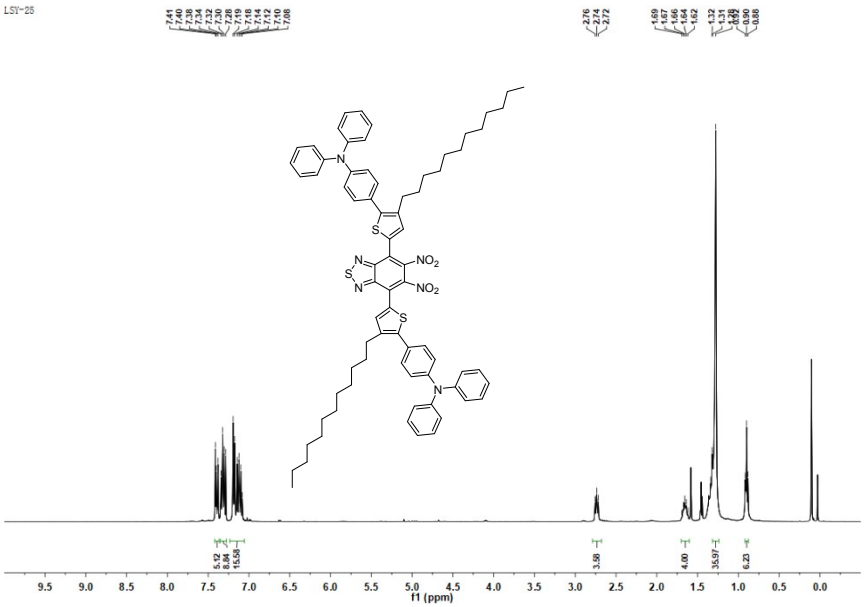


Figure S10. ^{1}H NMR of compound **6a**

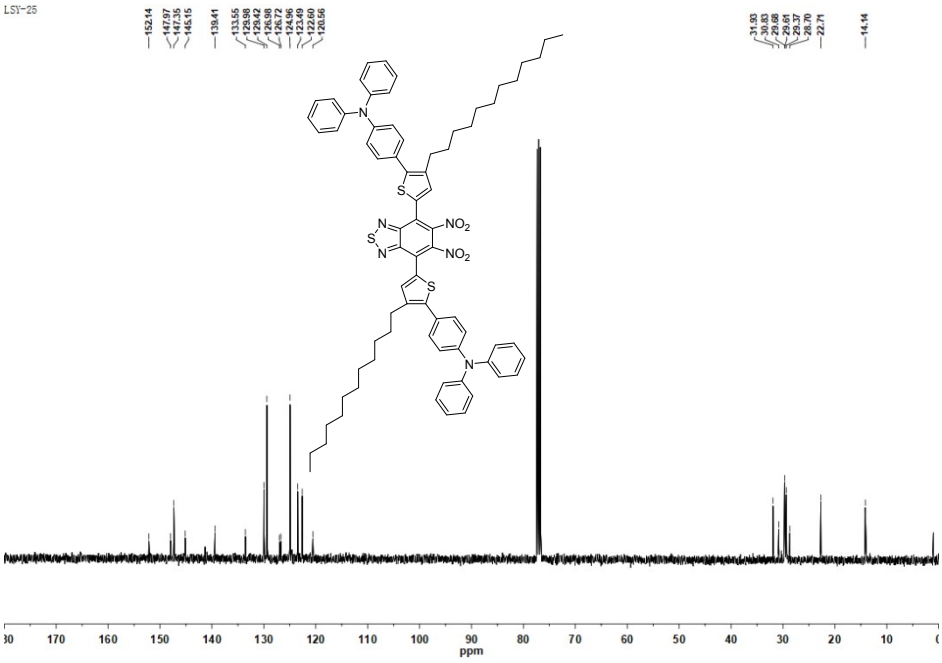


Figure S11. ^{13}C NMR of compound **6a**

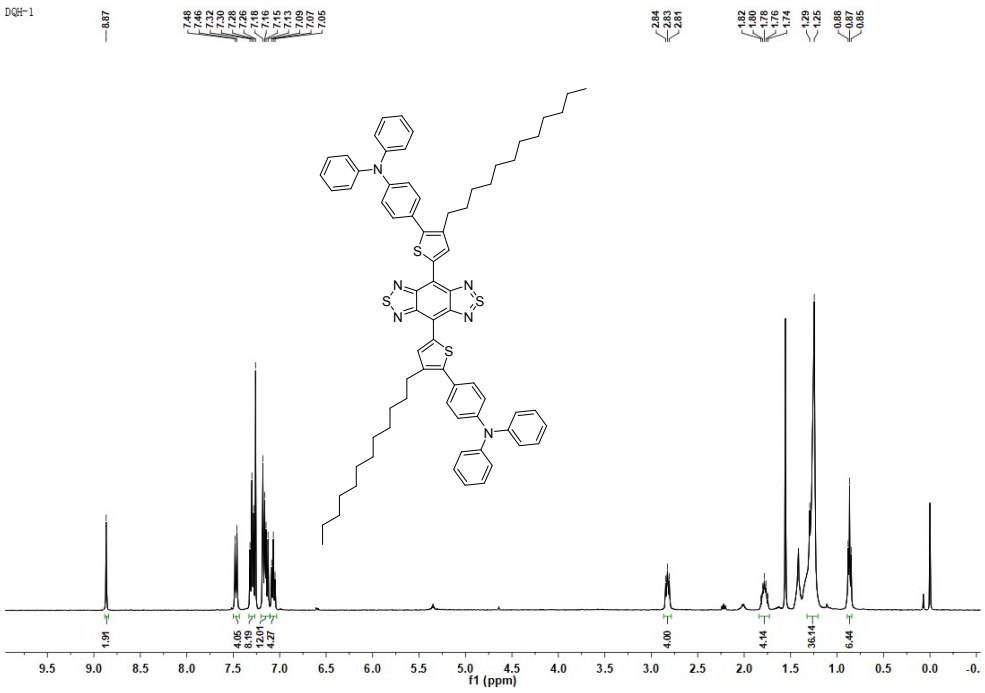


Figure S12. ^{1}H NMR of **HQL1**

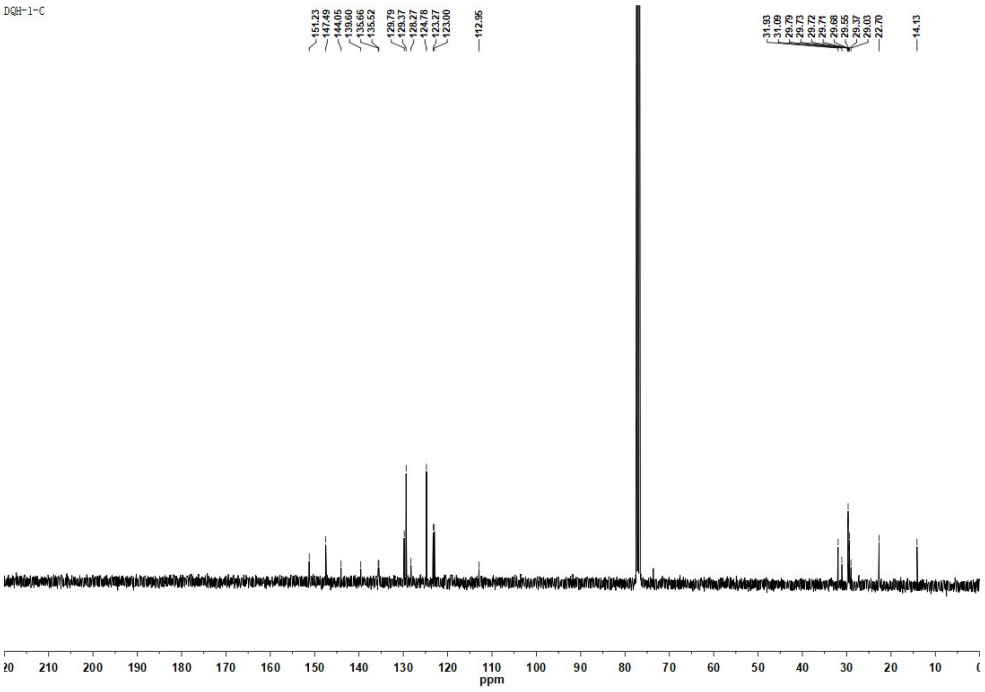


Figure S13. ^{13}C NMR of **HQL1**

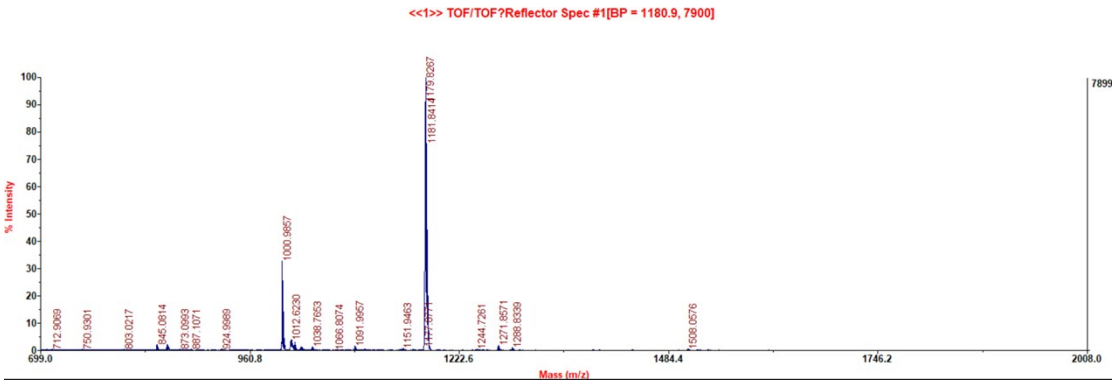


Figure S14. The MALDI-TOF-MS spectrum of **HQL1**

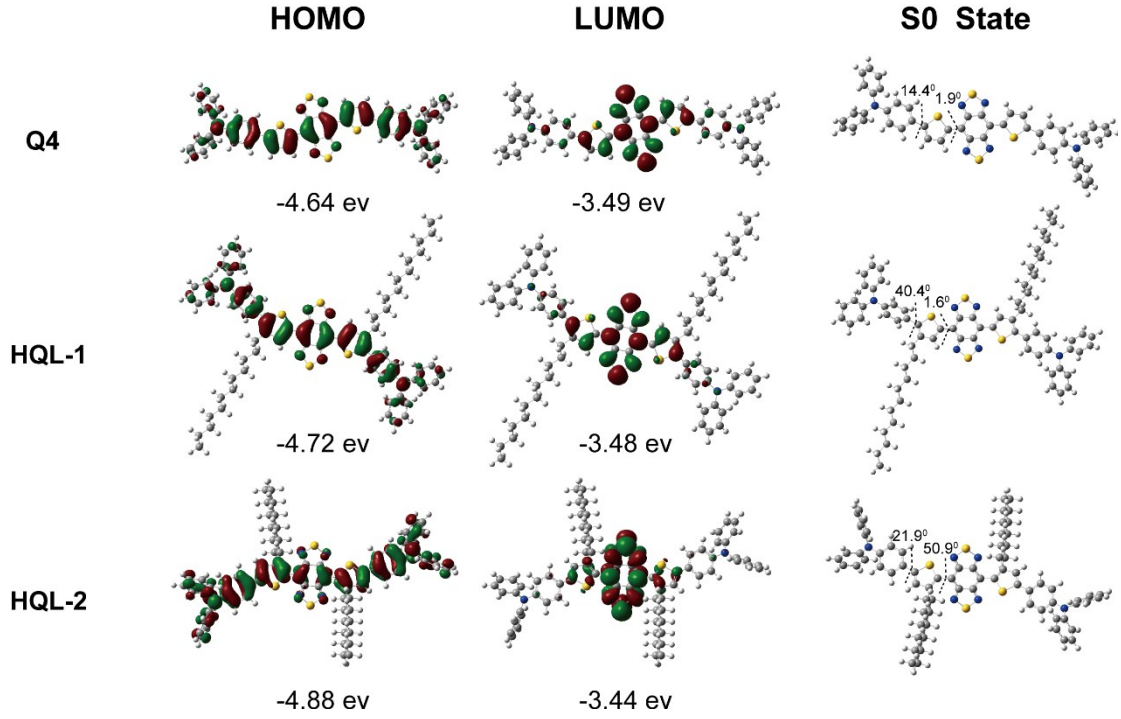


Figure S15. HOMO and LUMO orbital surfaces of **HQL1**、**HQL2** and **Q4** using DFT B3LYP/6-31G(d) scrf = (cpcm, solvent=dichloromethane) method. And the Optimized groud-state (S_0) of these molecules and the relative dihedral angles.

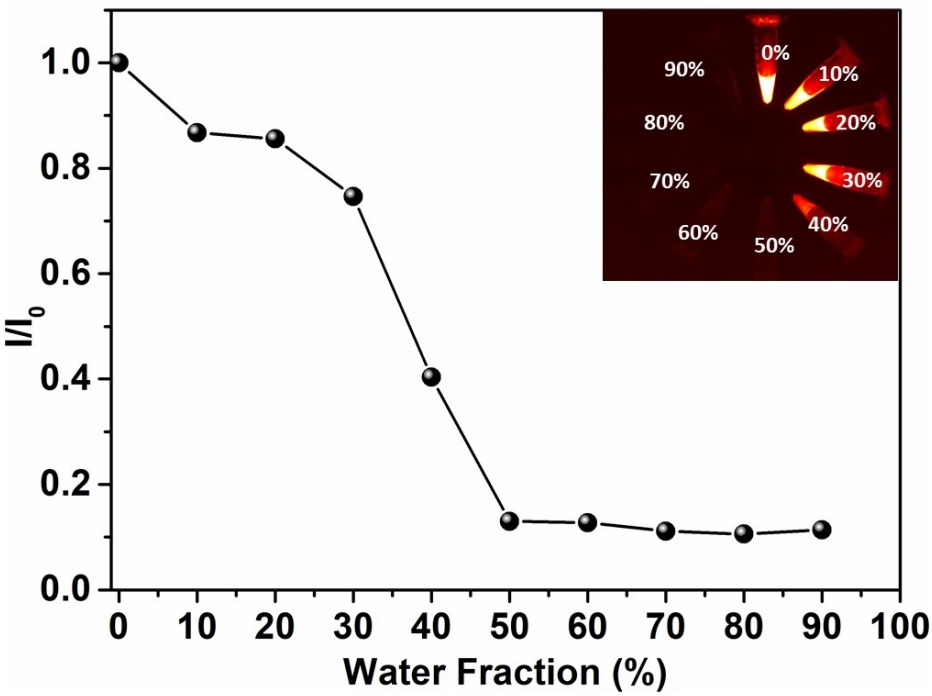


Figure S16. The fluorescence intensity ratio(I/I_0) of **HQL1** in different f_w, I_0 is the fluorescence intensity of **HQL1** in pure THF and the fluorescence image of **HQL1** in different f_w with 808 nm excitation (5 ms exposure time, 90 mW cm^{-2})

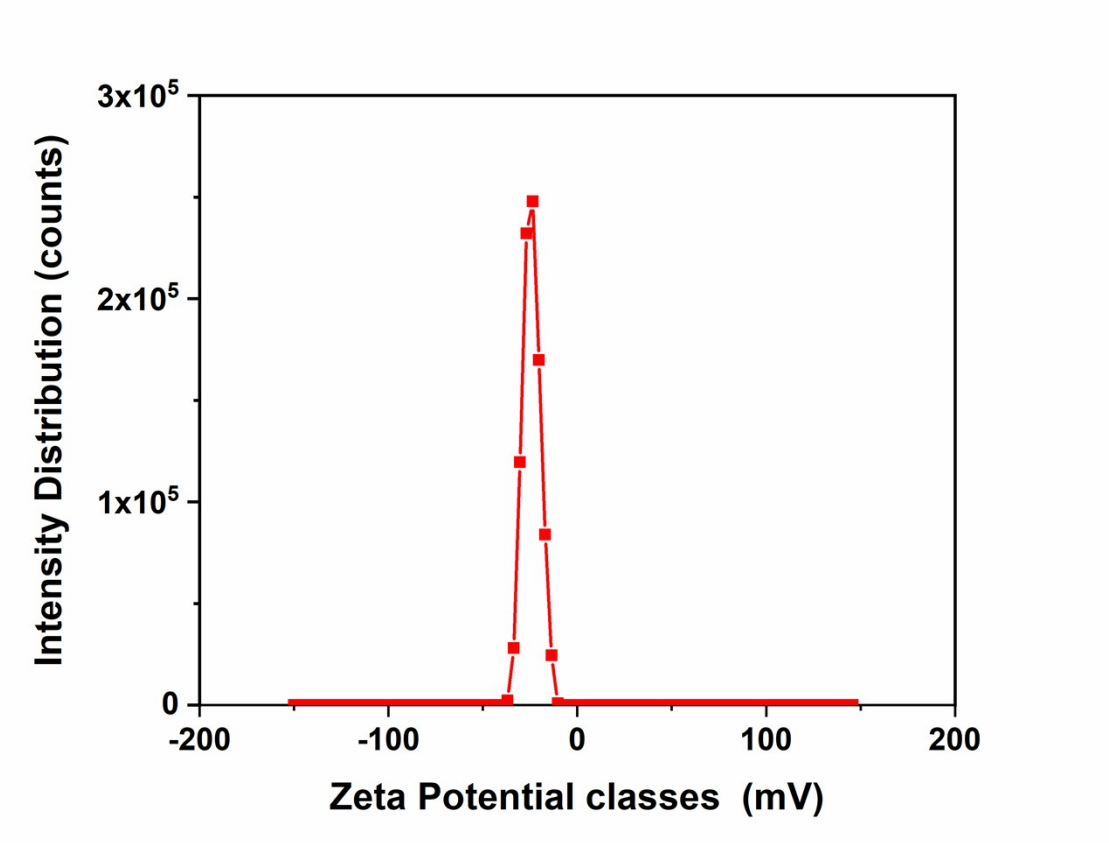


Figure S17.The Zeta potential of **HQL2** dots

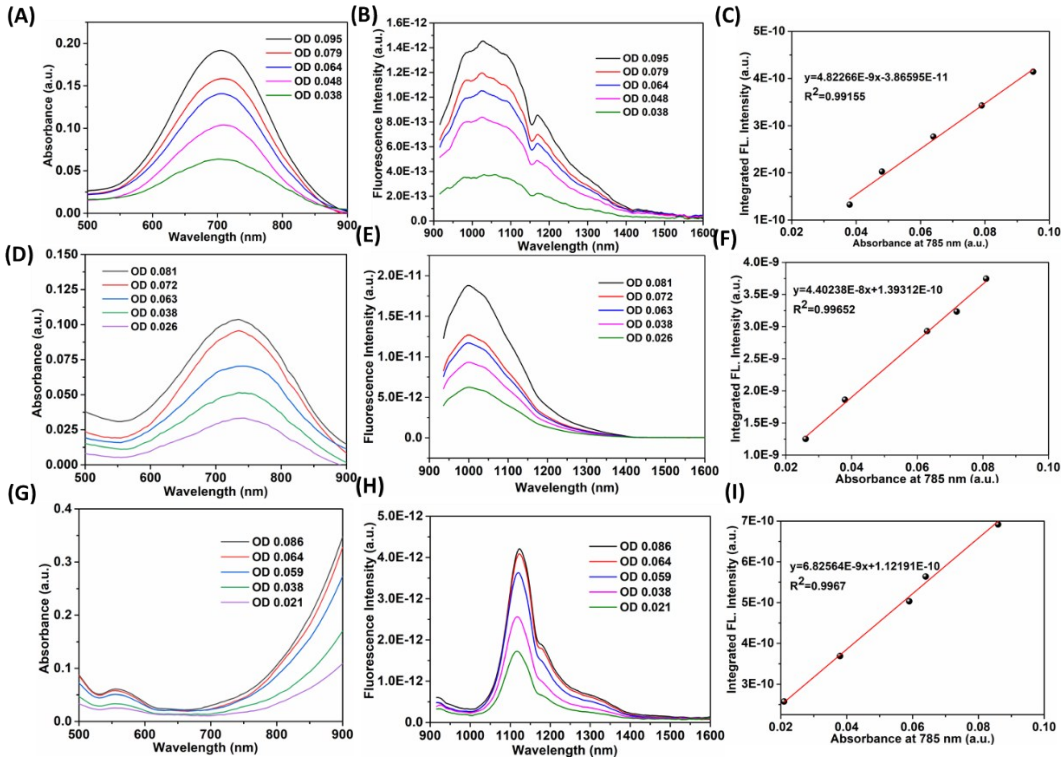


Figure S18. Absorbance (A, D, G) and fluorescence (B, E, H) spectra of **HQL2** in DCM (A, B), **HQL2** dots in water (D, E), and **IR-26** in DCE (G, H). The slope of **HQL2** in DCM (C), **HQL2** dots in water (F), and **IR-26** in DCE (I). Fluorescence quantum yield measurements of **HQL2** in DCM and **HQL2** dots in water were calculated according to a standard equation in the previous literature [2]. The equation was below:

$$QY_{sam} = QY_{ref} \times \frac{S_{sam}}{S_{ref}} \times \left(\frac{n_{sam}}{n_{ref}}\right)^2$$

Where QY_{sam} is the QY of **HQL2** dots, QY_{ref} is the quantum yield of IR-26 (~0.05%), S_{sam} and S_{ref} are the slopes obtained by linear fitting of the integrated fluorescence intensity of **HQL2** dots (1300-1600 nm) or (1500-1600 nm) and IR-26 (1000-1600 nm) against the absorbance at 785 nm. n_{sam} and n_{ref} are the refractive indices of their respective solvents (water:1.333 and DCM: 1.42)

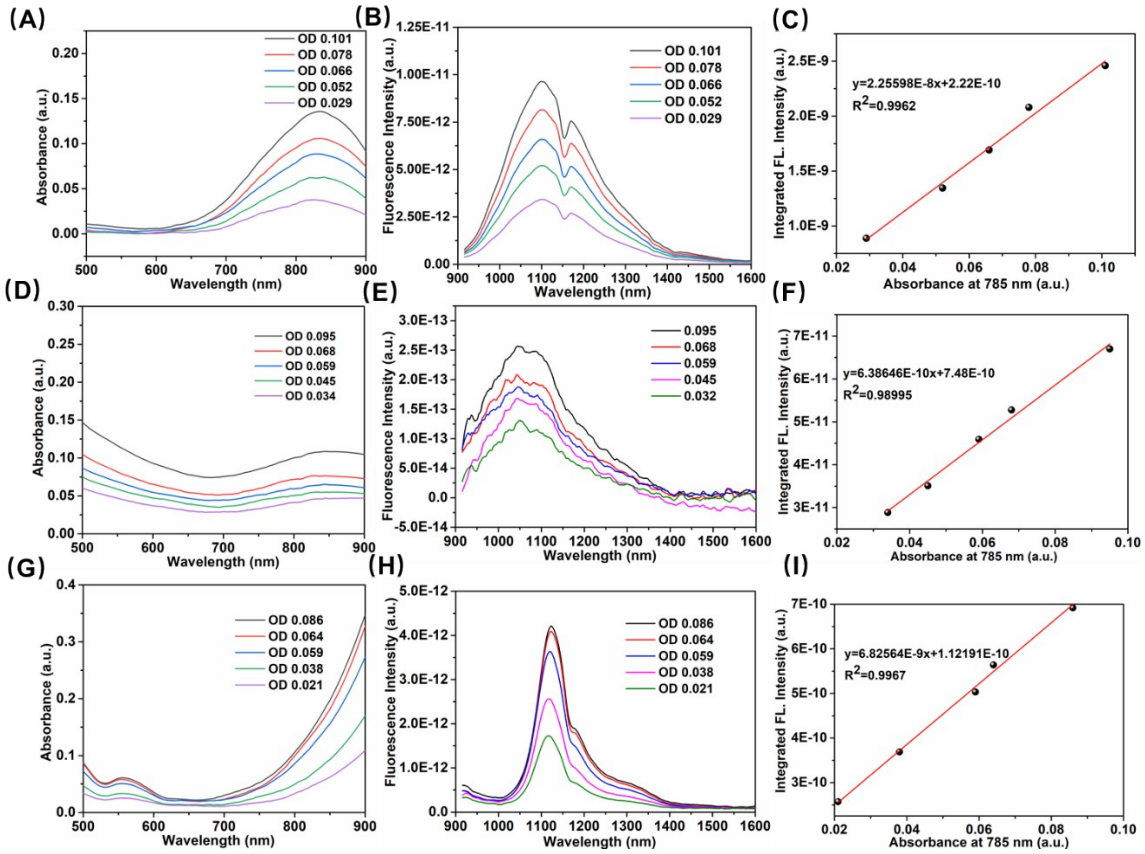


Figure S19. Absorbance (A, D, G) and fluorescence (B, E, H) spectra of **HQL1** in DCM (A, B), **HQL1** dots in water (D, E), and **IR-26** in DCE (G, H). The slope of **HQL1** in DCM (C), **HQL1** dots in water (F), and **IR-26** in DCE (I). The method of calculation fluorescence quantum yield of **HQL1** in DCM and **HQL1** dots in water were similar to the QY of **HQL2.**

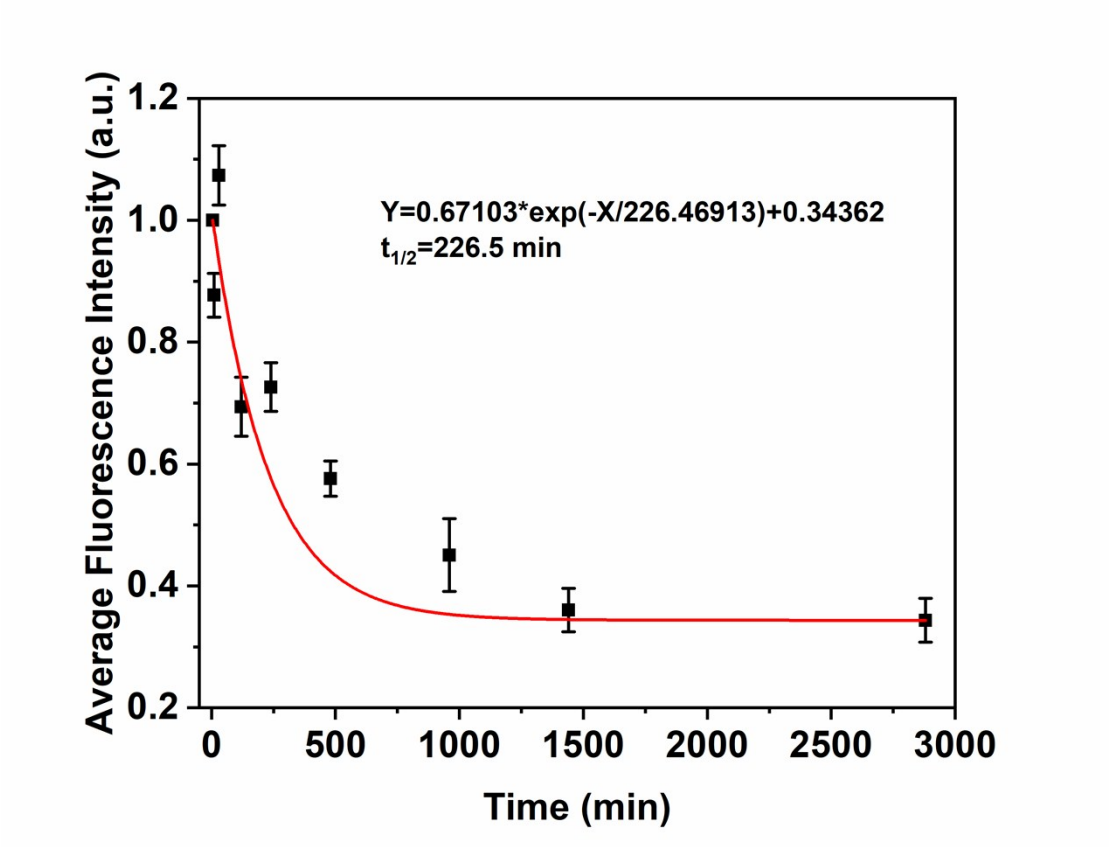


Figure S20. The blood half-life circulation curve of **HQL2** dots in female KM mice was determined to be ~226.5 min by fitting the data from the mean fluorescence intensity of blood samples at different time points after tail vein injection of **HQL2** dots using a first-order exponential decay.

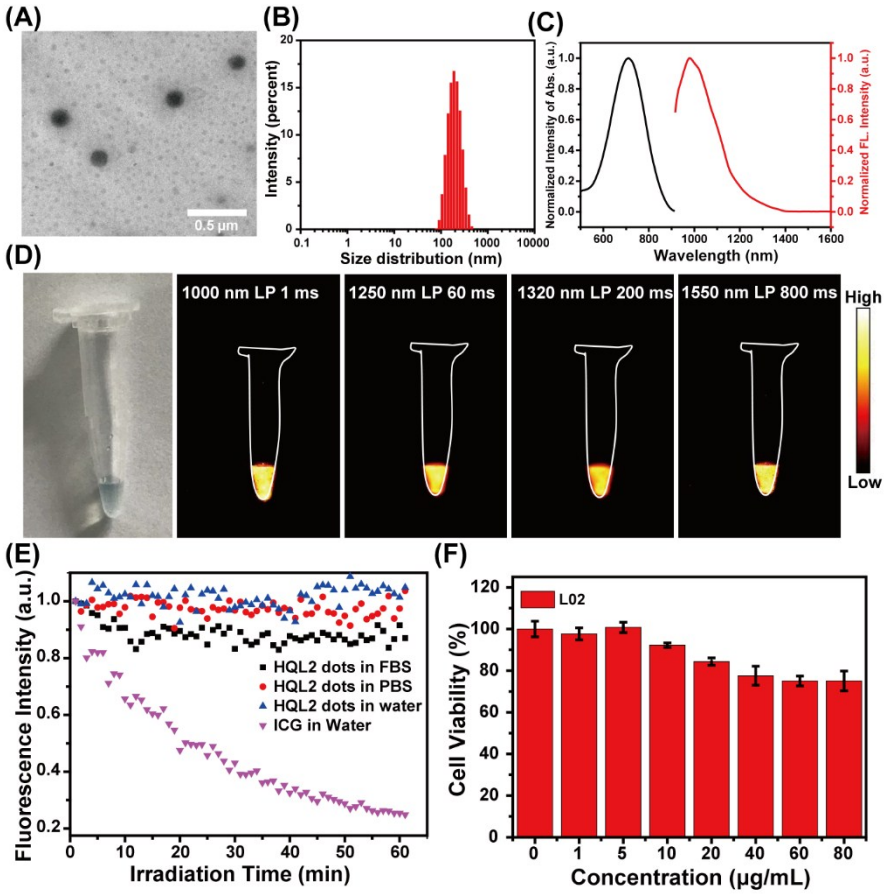


Fig. S21 (A) The acquired TEM image and (B) DLS results of a sample of the **HQL2** dots. (C) The acquired UV-Vis-NIR absorption spectrum (black line) and fluorescence emission spectrum (red line) of the **HQL2** dots in water.(D) Digital photograph of the **HQL2** dots (left) and fluorescence signals of the dots (right) under di□erent long-pass (LP) filters with an 808 nm-wavelength laser excitation (90 mW cm^{-2}). (E) The photostability levels of the **HQL2** dots in water, PBS and FBS media. ICG in water was used as a reference. (These experiments were conducted with an 808 nm excitation, 180 mW cm^{-2}.) (F) The viabilities of various samples of L02 human hepatocyte cells incubated with di□erent concentrations of **HQL2** dots for 24 h.

Reference

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