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**Supporting Information**

# Mitochondrial Membrane Potential-Independent Near-Infrared Fluorescent Probes for Viscosity-Exclusive Imaging

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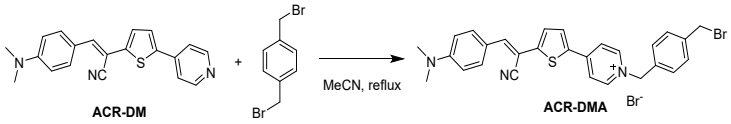
**Content**

## Materials and Instruments

All reagents were purchased from commercial suppliers and used without further purification. CCK-8 Detection kit and penicillin-streptomycin solution was purchased from biosharp. Dulbecco's modified Eagle's medium was purchased from Gibco. Fetal bovine serum was purchased from Wuhan Punosai Life Technology Co., LTD. Monensin was purchased from Suzhou Jialiang Technology Co., LTD. Lipopolysaccharide and nystatin were purchased from Adamas. Fluorescence spectra were obtained using a Horiba Duetta spectrofluorimeter with a 10 mm quartz cuvette. UV-vis absorption spectra were recorded on a Hitachi PharmaSpec UV-1900 UV-Visible spectrophotometer. The pH measurements were performed with a Mettler-Toledo Delta 320 pH meter. Silica gel GF 254 was used for TLC analysis. Silica gel (200-300 mesh) column chromatography was used for purification. NMR spectra were measured on an AVANCE NEO 400 NMR instrument. Chemical shifts are given in ppm, relative to an internal reference TMS as the internal standard. The following abbreviations were used in $^1$H NMR: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet. High resolution mass spectra were recorded on a Finnigan LCQDECAAglientESI-MS mass spectrometer. Confocal fluorescence images were recorded using a Nikon A1R$^+$ confocal laser scanning microscope. PerkinElmer IVIS spectrum imaging system was used for *in vivo* imaging.

## Synthesis routine of ACR-DMA




**Scheme S1** Synthesis routes of ACR-DMA

ACR-DM was synthesized according to the routine in the literature.[1] ACR-DM (70 mg, 1 mmol) was dissolved in 10 ml acetonitrile and then dibromo-p-xylene (1115 mg, 20 mmol) was added. The mixture was stirred at 50 °C for 24 h. The solvent was removed, and the mixture was purified by flash chromatography with silica gel (methylene dichloride/methanol = 20:1, v/v) to afford 59.8 mg of ACR-DMA (48% yield). $^1$H NMR (400 MHz, DMSO-$d_6$): $^1$H NMR (400 MHz，DMSO-$d_6$)，$\delta$. 9.04 (d，2H，$J$ = 7.2 Hz)，8.32 (d，2H，$J$ = 7.2 Hz)，8.27 (d，1H，$J$ = 4.0 Hz)，7.92-7.85 (m，3H)，7.59 (d，1H，$J$ = 4.0 Hz)，7.53 (d，4H，$J$ = 1.6 Hz)，6.86 (d，2H，$J$ = 9.2 Hz)，5.75 (s，2H)，4.72 (s，2H)，3.08 (s，6H)；$^{13}$C NMR (100 MHz，DMSO-$d_6$)，$\delta$. 152.5，148.3，147.6，144.6，143.8，134.7，134.0，132.0，130.1，129.1，

128.7，127.2，126.1，122.2，120.0，118.0，111.9，95.2，62.4，33.7，29.1.
HRMS (ESI): m/z $[M-Br]^+$ calculated for $C_{28}H_{25}BrN_3S$: 514.0947; found: 514.0918.

## Optical Study

The stock solution of ACR-DMA (5 mM) was prepared by dissolving powder in 1 mL DMSO solution. The photophysical property of ACR-DMA (5 μM) were carried out by dispersing 3 μL stock solution into 3 mL different solvents. ACR-DMA was added to PBS or different viscosity systems with different pH or different analyte, shaken well and ultrasounded for 20 min to eliminate air bubbles, after which the spectra were recorded at 25 °C. For all the measurements, the detailed test conditions were shown below: the excitation slits and emission slits were 10 nm, and the excitation wavelength was 498 nm.

The fluorescence quantum yield is determined with cresol violet (Φs=0.58 in ethanol) as the reference, and the fluorescence quantum yield is calculated as follows:

$$\Phi x=\Phi s(As\times Fx/Ax\times Fs)\ (nx/ns)^2 \quad (1)$$

Φx: fluorescence quantum yield of the probe, Ax/As: absorbance of the probe/reference, Fx/Fs: integral area of the emission spectrum of the probe/reference, n: solvent refraction coefficient

The relationship between fluorescence response performance and viscosity of ACR-DMA was calculated by FÖrster-Hoffmann equation:

$$\text{Log } I=C+x \log \eta \quad (2)$$

I: fluorescence intensity, C: constant, x: sensitivity of fluorescence probe to viscosity, η: viscosity

## Cell culture

HeLa cells and 4T1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin-streptomycin solution. Cells were maintained in a humidified incubator at 37 °C in the presence of 5% $CO_2$.

## cytotoxicity evaluation

The HeLa cells were digested. About 5000 cells per well were inoculated into 96-well plates and incubated with 100 μL of medium overnight to cell density to about 80%. The medium was replaced with 100 μL of fresh medium containing different concentrations (0, 5, 10, 20, 30 μM) of ACR-DMA. Then, after the cells were incubated

for 12 h, the medium was discarded. After adding fresh medium and incubating for another 24 h, 10 μL of CCK-8 solution was added to each well and mixed with 90 μL of medium, and then incubated for another 1 h. The absorbance at 450 nm was measured.

Cell viability (%) = ($OD_{450\ sample}$-$OD_{450\ blank}$/$OD_{control}$-$OD_{450\ blank}$) × 100%. （3）

**Cell imaging**

Before imaging, 1 mL of HeLa cells suspension was inoculated into a glass bottom confocal dish with the density of $1\times10^5$ /mL. The cells were incubated for 24 h. Imaging experiments were performed when the cells density reached about 70%.

**Co-localization experiment**

HeLa cells were co-incubated with LIQ-3, Lyso-Tracker or BODIPY and ACR-DMA (5 μM) for 30 min, then washed 3 times with PBS, and fluorescence images were recorded by laser scanning confocal microscopy (CLSM). ACR-DMA: $\lambda_{ex}$ = 488 nm, $\lambda_{em}$ =620-750 nm. LIQ-3: $\lambda_{ex}$ = 405 nm, $\lambda_{em}$ =500-580 nm. Lyso-Tracker: $\lambda_{ex}$ = 488 nm, $\lambda_{em}$ =500-550 nm. BODIPY: $\lambda_{ex}$ = 488 nm, $\lambda_{em}$ =480-550 nm.

**Mitochondrial immobilization assay**

HeLa cells were co-incubated with LIQ-3 (5 μM) and ACR-DMA (5 μM) for 30 min, and then washed 3 times with PBS. 20 μM CCCP was added and incubated for 0-30 min to disrupt the mitochondrial membrane potential, and fluorescence images were recorded with CLSM at different time points.

**Imaging of viscosity in living cells**

HeLa cells were incubated with Nys (10 μM), Mon (10 μM) for 30 min or LPS (100 μg/mL) for 60 min to induce intracellular viscosity changes, and washed 3 times with PBS. Fluorescence imaging was performed by adding 5 μM ACR-DMA for 30 min of incubation.

**Animal experimental approval**

The 6-week-old BALB/c female mice (SPF) were provided by Chengdu Dashuo Laboratory Animal Co., LTD. All animal experiments were in accordance with institutional animal use and care regulations approved by the Model Animal Research Center of Affiliated Hospital of Southwest Jiaotong University.

**Construction and imaging of 4T1 tumor-bearing mice model**

4T1 cells were digested with trypsin and diluted to $1\times10^7$ per unit with PBS to obtain cell suspension. The underarm of the right forelimb was depilated and disinfected. After subcutaneous injection of 4T1 cell suspension, the model was successfully constructed when the tumor volume reached 80-100 $mm^3$. The heart, liver, spleen, lung, kidney and tumor from mice were incubated with ACR-DMA (20 μM). Tumor sizes were measured as the length (A) and the width (B), and the tumor volume (V) was calculated according to the following formula: $V = (A \times B^2)/2$. Fluorescence images were recorded with an IVIS Spectrum *in vivo* optical imaging system.

**Construction and imaging of mice model with abnormal viscosity**

Firstly, the abdomen of the mice was depilated and disinfected. The subcutaneous viscosity changes were induced by subcutaneous injection of Nys (1 mM, 100 μL), Mon (1 mM, 100 μL) and LPS (2.5 μg/mL, 100 μL), respectively. ACR-DMA (1mM, 100 μL) was injected subcutaneously 4 h after model construction, and *in vivo* imaging was performed 30 min later. Fluorescence images were recorded with an IVIS Spectrum *in vivo* optical imaging system.

**Construction and imaging of mice model with kidney injury**

Mice were injected with dibenzoic acid (30 mg/kg, 100 μL) through a tail vein and stimulated for 24-36 h to form a kidney injury model. The acute kidney injury model was established by intraperitoneal injection of vancomycin (Van) for 7 consecutive days. ACR-DMA (1 mM, 100 μL) was injected into the tail vein and *in vivo* imaging was performed 2 h later. The heart, liver, spleen, lung and kidney obtained from mice were washed with saline for 3 times, and the organs were imaged. Fluorescence images were recorded with an IVIS Spectrum *in vivo* optical imaging system.

## Supplementary tables

**Table S1.** Photophysical properties of ACR-DMA in different solvents.

| Solvent | $\lambda_{abs}$（nm） | $\lambda_{em}$（nm） | $\Phi_F$[a]（%） | △SS[b]/nm |
|---|---|---|---|---|
| Water | 498 | 747 | 0.20 | 249 |
| DMSO | 516 | 747 | 2.04 | 231 |
| Glycerol | 528 | 709 | 25.24 | 181 |
| Acetonitrile | 512 | 757 | 0.89 | 245 |
| DMF | 514 | 762 | 1.50 | 248 |
| Methanol | 514 | 734 | 1.53 | 220 |
| EtOH | 520 | 730 | 1.20 | 210 |
| Acetone | 512 | 759 | 1.46 | 247 |
| THF | 506 | 699 | 4.06 | 193 |
| Ethyl acetate | 502 | 698 | 2.06 | 196 |
| Hexyl hydride | 520 | 710 | 0.09 | 190 |

**Table S2.** The fluorescence intensity of ACR-DMA in water-glycerol mixture with different glycerol fraction.

| Log $I_{710}$ | Log η | $I_{710}$ | Viscosity (cP) | Glycerol fraction (vol %) |
|---|---|---|---|---|
| 1.33 | 0.08672 | 21.63 | 1.22 | 10 |
| 1.53 | 0.39881 | 34.22 | 2.51 | 30 |
| 1.71 | 0.61962 | 51.13 | 4.17 | 40 |
| 1.91 | 0.8428 | 80.38 | 6.96 | 50 |
| 2.08 | 1.09202 | 120.13 | 12.36 | 60 |
| 2.31 | 1.44295 | 203.92 | 27.73 | 70 |
| 2.53 | 1.80754 | 338.69 | 64.20 | 80 |
| 2.82 | 2.38238 | 666.55 | 241.2 | 90 |
| 3.15 | 2.97543 | 1403.82 | 945 | 100 |

## Supplementary figures

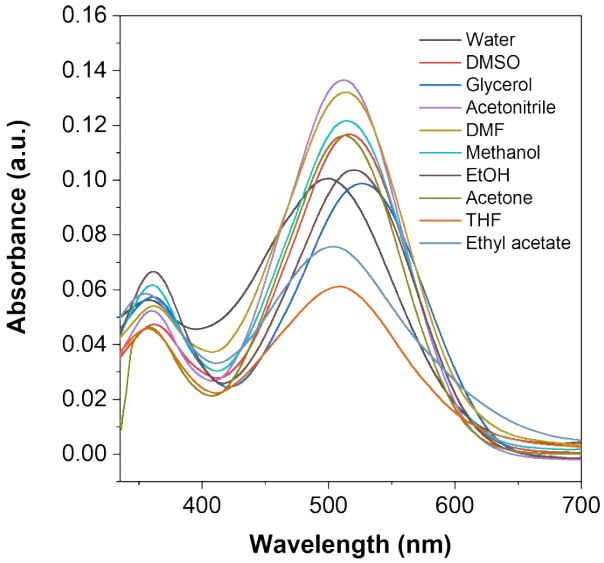



**Figure S1**. Absorbance spectra of ACR-DMA (5 μM) in various solvents with different polarity.

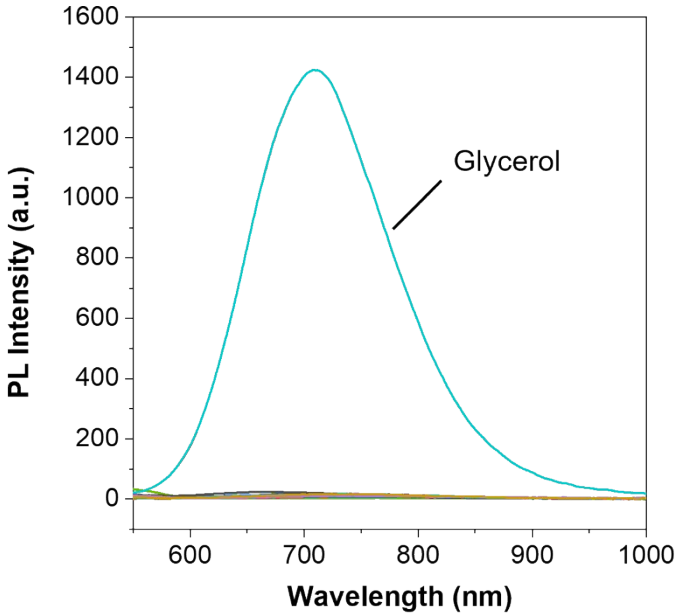



**Figure S2**. (A) PL spectra of ACR-DMA (5 μM) in PBS with different analytes or in glycerol. (Metal ions and anions: 200 μM. amino acid, RSS and ROS: 500 μM). (1) blank, (2) $Ca^{2+}$, (3) $Cu^{2+}$, (4) $Fe^{2+}$, (5) $Fe^{3+}$, (6) $K^{+}$, (7) $Mg^{2+}$, (8) $Na^{+}$, (9) $Ni^{2+}$, (10) $Zn^{2+}$, (11) $Cl^{-}$, (12) $CO_3^{2-}$, (13) $HCO_3^{-}$, (14) $CH_3COO^{-}$, (15) $C_2O_4^{2-}$, (16) $SO_4^{2-}$, (17) Gly, (18) Glu, (19) Trp, (20) GSH, (21) $SO_3^{2-}$, (22) $S_2O_3^{2-}$, (23) $HSO_3^{-}$, (24) $HS^{-}$, (25) $NO_2^{-}$, (26) $ONOO^{-}$, (27) $^1O_2$ , (28) · OH, (29) $ClO^{-}$, (30) $H_2O_2$, (31) Glycerol.

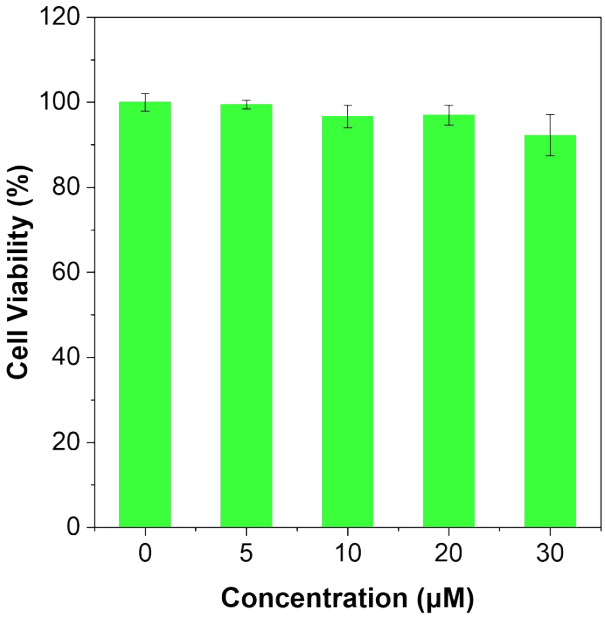



**Figure S3**. Survival rate of HeLa cells after incubation with different concentrations of ACR-DMA.

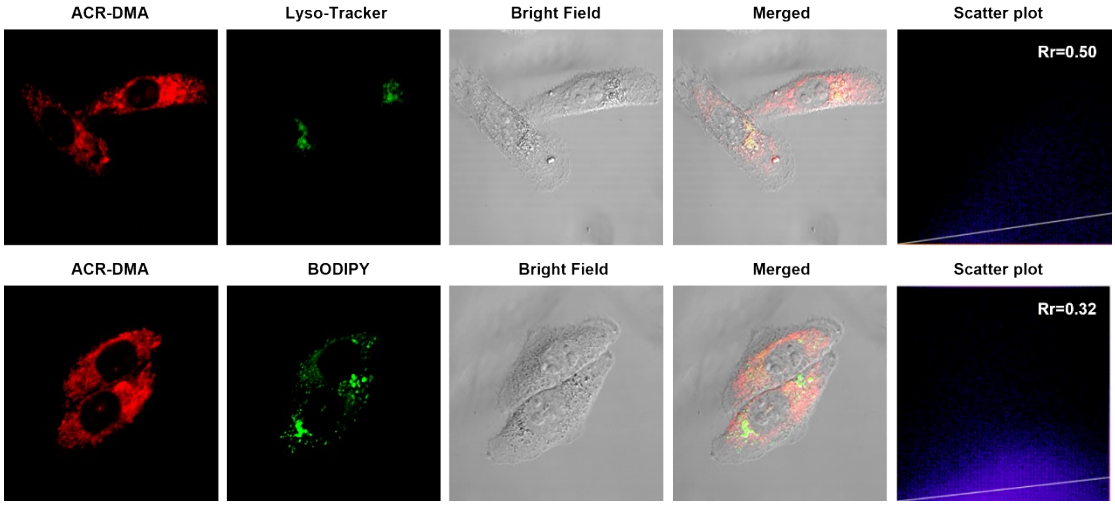



**Figure S4**. Fluorescence images of HeLa cells co-stained with ACR-DMA (5 μM) and Lyso-Tracker or BODIPY (5 μM) for 30 min. ACR-DMA: λex = 488 nm, λem =620-750 nm. Lyso-Tracker: λex = 488 nm, λem =500-550 nm. BODIPY: λex = 488 nm, λem =480-550 nm.

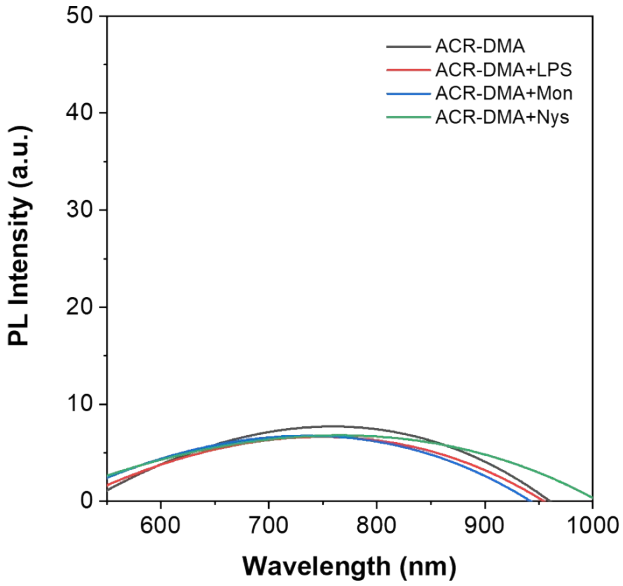



**Figure S5**. PL spectrum of ACR-DMA in the absence or presence of LPS, Mon and Nys.

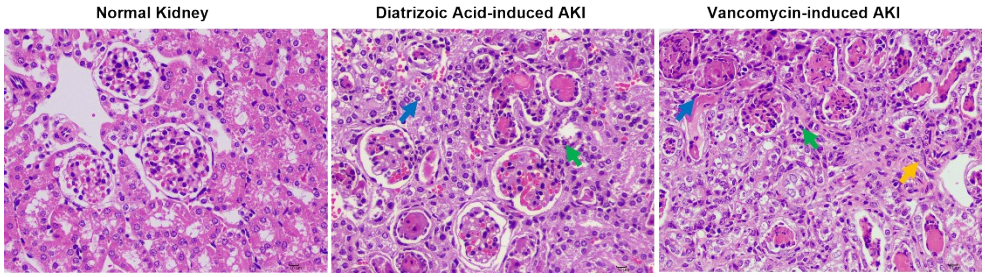



**Figure S6.** H&E staining of kidney tissues from normal mice, diatrizoic acid-induced acute kidney injury mice, and vancomycin-induced acute kidney injury mice, respectively.

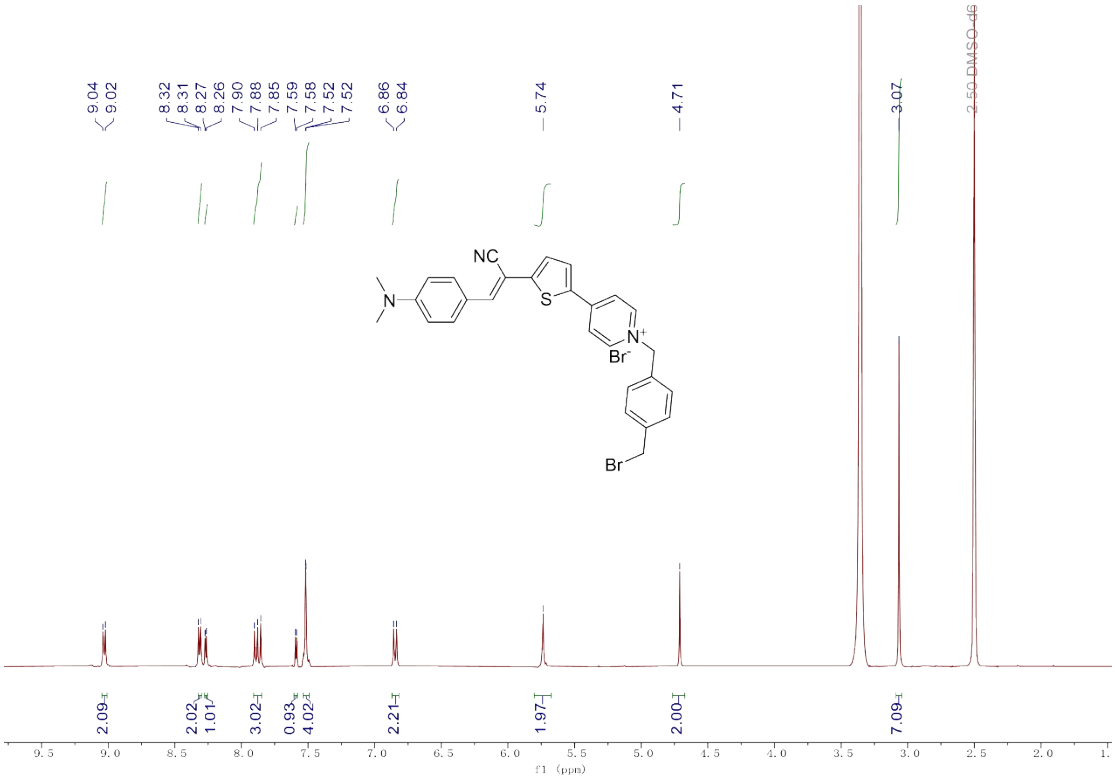



**Figure S7**. $^{1}$H NMR of ACR-DMA in DMSO-$d_6$.

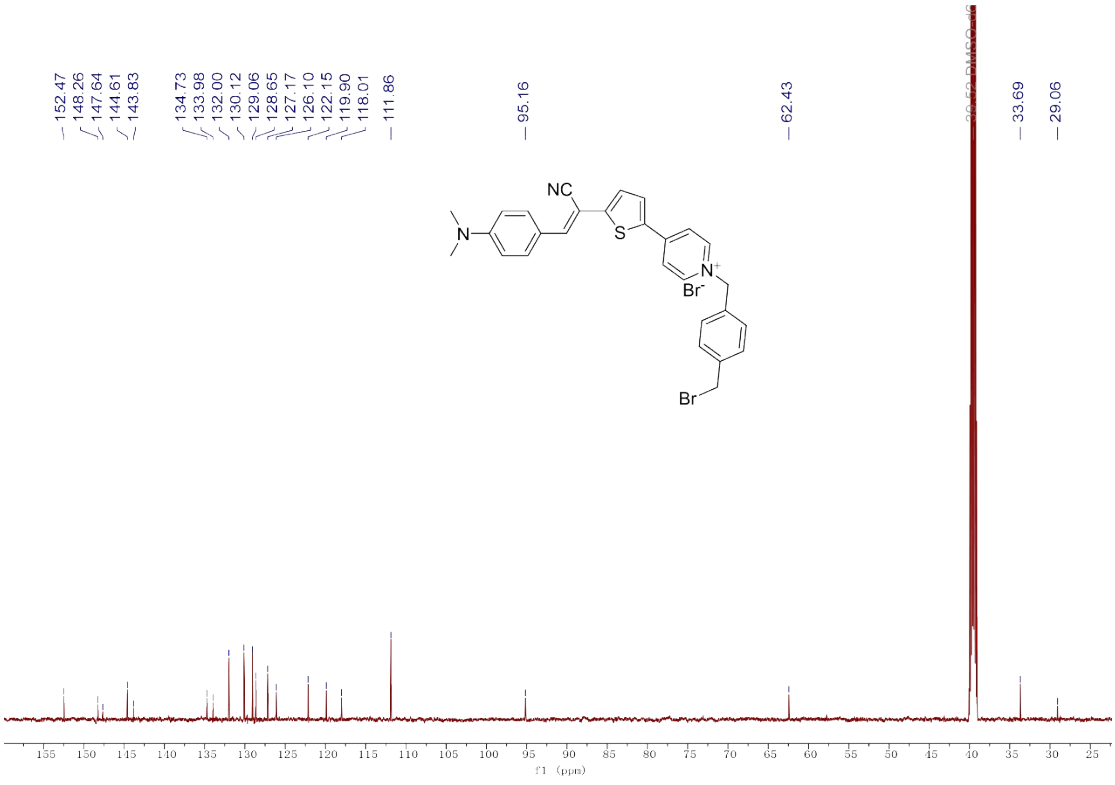



**Figure S8**. $^{13}$C NMR of ACR-DMA in DMSO-$d_6$.

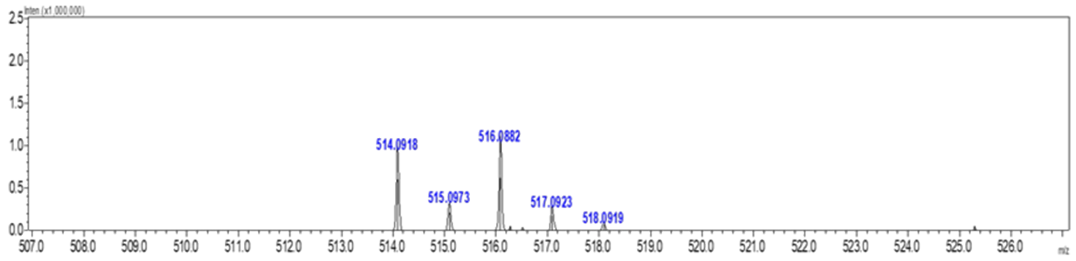



**Figure S9**. HRMS of ACR-DMA.

## References

1 F.-W. Xia, B.-W. Guo, Y. Zhao, J.-L. Wang, Y. Chen, X. Pan, X. Li, J.-X. Song, Y. Wan, S. Feng, M.-Y. Wu, *Adv. Mater*, 2023, **35**, 2309797.