Supporting Information for

A red-emitting mitochondria targetable fluorescent probe for detecting viscosity in Hela, zebrafish, and mice

Xi Gong, Rui Guo, Xiaoya Li, Yingjie Yang, Weiying Lin*

Institute of Optical Materials and Chemical Biology, Guangxi Key Laboratory of Electrochemical Energy Materials, School of Chemistry and Chemical Engineering, Guangxi University, Nanning, Guangxi 530004, P. R. China.

E-mail: weiyinglin2013@163.com

Table of Contents

Materials and general information

All reagents, unless stated otherwise, were obtained from commercial suppliers, and utilized without any additional purification. Lipopolysaccharides (LPS) and nystatin (Nys) were purchased from Sigma-Aldrich and Thermo Fisher Scientific respectively. MTT was purchased from Hangzhou Fude Biological Technology CO., LTD. PEG (average Mn 5000) was purchased from Bide Pharmatech Co., Ltd. , China and prepared as a stock solution on demand. Ultrapure water with a resistivity of 18.2 MΩ cm, obtained from a Milli-Q water purification system, was used to prepare all aqueous solutions. MTT assay was performed on CMax Plus (Molecular Devices, USA). High-resolution mass spectrometry (HRMS) analysis was conducted using the Thermo Scientific Q Exactive Focus Orbitrap LC-MS/MS System. 1H NMR and 13C NMR spectra were recorded on a Bruker AVANCE III HD 600 MHz NMR spectrometer using TMS as an internal standard. Absorption spectra of the probe were obtained using a Shimadzu UV-2700 UV-Visible Spectrophotometer, and fluorescent spectra were recorded with a HITACHI Fluorescence Spectrophotometer F-4700. A PHS-3E pH meter was used to conduct the pH measurements. The viscosity measurement of the methanol-glycerol system is achieved through the NDJ-8S instrument (Shanghai Youyi Instrument Co., Ltd., China). A microplate reader used in MTT assay was SpectraMax Absorbance Reader CM. A Leica TCS SP8 X White Light Laser Confocal Microscope (Wetzlar, Germany) was used to conduct fluorescence imaging of cells and zebra fishes. The figures generated by the instrument were processed using Leica Application Suite X (Version: 3.5.6), which could automatically calculate Pearson's correlation coefficient between two figures. The Manders' colocalization coefficients was automatically calculated by the ImageJ (version: 1.53e) software with the JoCoP plugin. The IVIS Lumina Series III (USA) was utilized to conduct fluorescence imaging of live mice.

Detection fluorescence intensity of probe at different pH values

The solvents were obtained by mixing glycerol and PBS buffer with different values of pH (3.5 - 11.5). The volume ratio of glycerol to PBS buffer is 2:8. Subsequently, the probe GX-VS (10 μM) is mixed with the aforementioned solution and allowed to stand for 30 min before fluorescence spectrum detection.

Photostability test

Mix the probe (10 μM) with glycerol or methanol. Then, perform spectral testing experiments using the Hitachi F-4700 fluorescence spectrophotometer and its accompanying FL Solutions software. During the testing, if the Shutter control checkbox under the Instrument tab in the software is checked, it indicates no light interference (light-off), while setting it unchecked means there will be interference from external light (light-on). The excitation wavelength for the test is set to 545 nm, with measurements taken every minute for a total of 60 times.

Viscosity determination and spectral measurement

The solvents used in the experiment were obtained by combining methanol-glycerol systems in various ratios. The viscosity of each solvent was determined using an NDJ-8S rotary viscometer and each viscosity value was recorded (**Fig.S1**). To prepare solutions of **GX-VS** with different viscosities, the stock solution (1.0 mM) was added in 30 μL increments to 3 mL of the solvent mixture (methanol-glycerol system), resulting in a final concentration of the **GX-VS** probe at 10.0 μM. To remove any air bubbles, the solutions were sonicated for 30 minutes. Subsequently, the solutions were left to stand at a constant room temperature for 0.5 hour before being analysed using a UV spectrophotometer and a fluorescence spectrophotometer. For all measurements, the excitation wavelength, excitation slit widths, and emission slit widths are 545 nm, 10 nm, and 10 nm, respectively.

The Förster-Hoffmann equation

The relationship between the fluorescence emission intensity of the probe **GX-VS** and the solvent viscosity could be formulated by the Förster-Hoffmann equation:

$$\log I = C + x \log \eta$$

Where η is the viscosity, I is the emission intensity, C is a constant, and x is the gradient which reflects the sensitivity of the probe to viscosity.

MTT assay

The standard MTT assay was used to study the vitro cytotoxicity of probe **GX-VS** on Hela cells (**Fig.S3**). The cells were seeded in 96-well plates (from Solabio Life Sciences in Beijing, China) at a density of 5×10^3 cells/well and treated with varying concentrations of the probe (ranging from 0 to 50μM) for 24 hours. After this, 10 μL of MTT (5 mg/mL in PBS) was added to each well and incubated for an additional 4 hours. The media was then removed, and 100 μL of DMSO was added to dissolve the formazan crystals. The plate was shaken for approximately 10 minutes, and the absorbance at 490 nm was measured using a microplate reader for each well.

$$The\ cell\ viability\ (\%) = \frac{ODsample - Mean\ ODblank}{Mean\ ODcontrol - Mean\ ODblank} * 100\ \%$$

OD_{sample} represents the optical density of each sample well (cells + probes with different concentrations).
Mean OD_{blank} represents the mean optical density of the 6 blank well (PBS only).
Mean $OD_{control}$ represents the mean optical density of the 6 control wells (cells + zero concentration of the probe).

Cell culture and fluorescence imaging

The HeLa cells were grown in a culture medium consisting of 90% DMEM (Dulbecco's Modified Eagle Medium), supplemented with 10% FBS (fetal bovine serum) and 1% antibiotics (100 U/ml penicillin and 100 μg/mL streptomycin, Hyclone), and maintained at 37 °C with 5% CO_2. The cells were seeded into 35 mm glass-bottom culture dishes (Nest) at an appropriate density. To treat the HeLa cells, **GX-VS** (10 μM)

was added and incubated for 30 minutes at 37 °C. The cells were then washed three times with PBS buffer (pH = 7.4) and incubated with lipopolysaccharide and nystatin (final concentration of 10 μM) for an additional 30 minutes at 37 °C. Afterward, the cells were washed three times with PBS buffer. Fluorescence imaging was performed using a Leica TCS SP8 CARS confocal microscope with a 63* objective lens. The fluorescence images of the slices were acquired using 545 nm excitation and fluorescence emission windows of 620-680 nm.

Colocalization Experiment

After being exposed to a culture medium containing 10 μM of the probe and 1 μM of Mito-Tracker Green (λ_{ex} = 488 nm, fluorescence emission window: 500-550 nm) for 30 minutes, Hela cells underwent a co-localization experiment.

Fluorescence imaging in living zebrafishes

Wild type zebra fishes were purchased from the Nanjing EzeRinka Company Limited. All procedures for this study were approved by the Animal Ethical Experimentation Committee of Guangxi University according to the requirements of the National Act on the use of experimental animals (China). For the fluorescence imaging experiments, 3-day-old zebrafishes were transferred into a 30 mm glass culture dishes using a disposable sterilized dropper. The probe **GX-VS** (10 μM) was added for incubated for 30 min, followed by washing away gently. Then 10 μM Lipopolysaccharides were put into dishes respectively for another 30 min. After that, the zebrafishes were transferred into new glass bottom dishes for imaging. Prior to the imaging, we adopted 1% agarose gel for immobilization of zebrafishes, and put zebrafishes onto agarose with a little media to ready imaging. The imaging experiments were recorded through a Leica TCS SP8 CARS confocal microscope with a 4*objective lens. The fluorescence emission was collected at TRICT channel (580-660 nm) upon excitation at 580 nm.

In vivo inflammation experiments and fluorescence imaging in living mice

Guangxi University procured Kunming mice that were approximately 4 weeks old. The animals were raised in a standard laboratory environment and all procedures were conducted in compliance with the Guidelines for Care and Use of Laboratory Animals of Guangxi University. The Animal Ethics Committee of Guangxi University approved all animal procedures. The mice were randomly assigned to facilitate further experimental investigations and were fasted for 12 hours prior to the experiment.

The mice were administered an IP injection of LPS (1 mg in 400 μL saline) and after 1 hours, they were anesthetized with gaseous ether. The abdominal fur was removed using an electric shaver and the mice were subsequently given an IP injection of **GX-VS** (400 μM in DMSO). Control groups were also prepared, consisting of untreated mice and unstimulated mice that were only given an IP injection of **GX-VS** (400 μM in DMSO). Imaging of the mice was performed using a IVIS Lumina Series III with an excitation filter of 600 nm and an emission filter of 670 nm, 30 minutes after the injection of **GX-VS**.

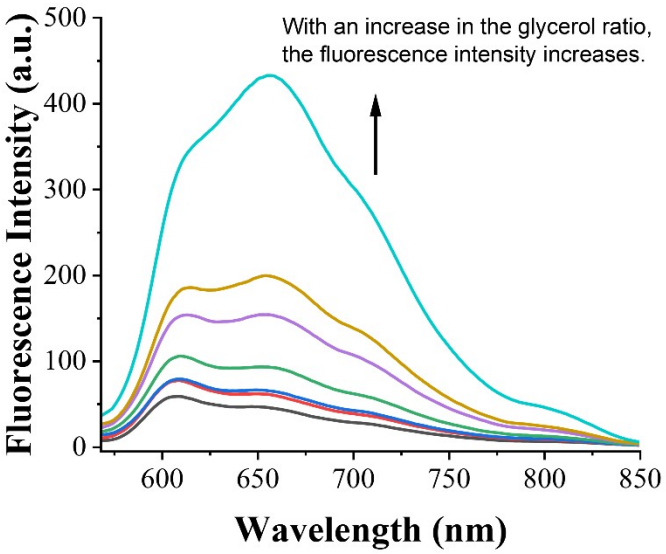

Fig.S1. Fluorescence spectra of **GX-VS** (10 μM) with the variation of solution viscosity (methanol-glycerol system). λ_{ex}= 545 nm, slit width: dex = dem = 10 nm. The curves in the graph, from bottom to top, correspond to the volume ratios of glycerol to methanol as follows: 0:100, 10:90, 20:80, 30:70. 40:60, 50:50 and 60:40.

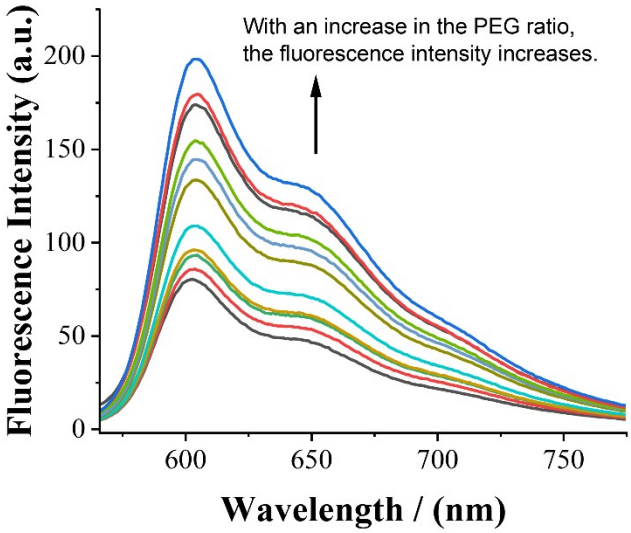

Fig.S2. Fluorescence intensity of **GX-VS** (10 μM) in PEG (Polyethylene Glycol) - methanol medium. Different viscosity PEG (stock aqueous solution concentration: 50 mM) - methanol systems were achieved by varying the mixing ratios of the two components (volume ratio) from 0:100 to 100:0. λ_{ex} = 545 nm, slit width: dex = dem = 10 nm.

H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
G	PBS	PBS	0	1	2	5	10	20	30	40	50	PBS
F	PBS	PBS	0	1	2	5	10	20	30	40	50	PBS
E	PBS	PBS	0	1	2	5	10	20	30	40	50	PBS
D	PBS	PBS	0	1	2	5	10	20	30	40	50	PBS
C	PBS	PBS	0	1	2	5	10	20	30	40	50	PBS
B	PBS	PBS	0	1	2	5	10	20	30	40	50	PBS
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
	1	2 (blank)	3 (control)	4	5	6	7	8	9	10	11	12

Fig.S3. Schematic diagram of a 96-well plate used in the MTT assay. The second column represents the blank group, the third column represents the control group with a probe concentration of 0, and the fourth to eleventh columns represent groups with probe concentrations of 1, 2, 5, 10, 20, 30, 40, and 50 μM, respectively.

Gly/MeOH (V/V ratio)	Viscosity η (cP)	log η	Fluorescense Intensity I_{650}	log I_{650}
100 : 0	952.9	2.97905	2733	3.43664
90 : 10	395.0	2.59660	1781	3.25066
80 : 20	154.9	2.19005	1143	3.05805
70 : 30	71.9	1.85673	515.6	2.71231
60 : 40	36.9	1.56703	433.5	2.63699
50 : 50	7.8	0.89209	199.5	2.29994
40 : 60	4.5	0.65321	154.7	2.18949
30 : 70	2.3	0.36173	92.1	1.96426
20 : 80	1.5	0.17609	63.53	1.80298
10 : 90	0.9	-0.04576	60.87	1.78440
0 : 100	0.6	-0.22185	44.18	1.64523

Fig.S4. Fluorescence intensity I_{655} (I_{650} representing probe fluorescence intensity at 650 nm) of 10 μM **GX-VS** in different glycerol / MeOH volume ratio solutions which have different viscosity value η. λ_{ex}= 545 nm.

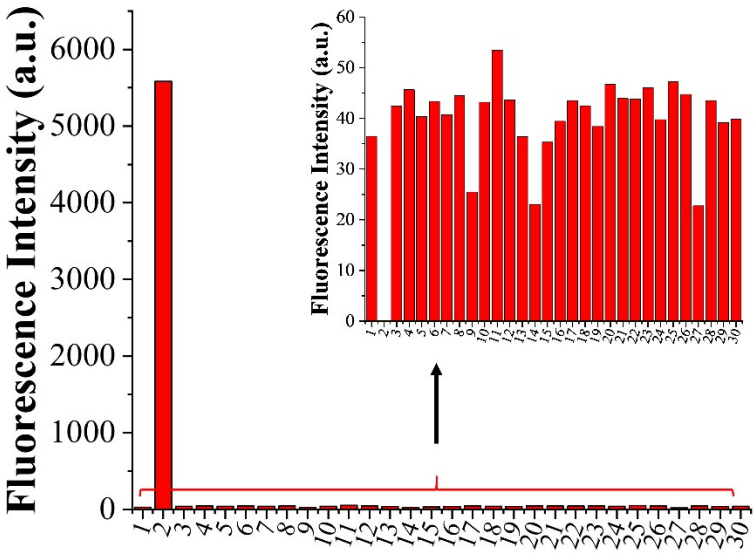

Fig.S5. Fluorescence intensity of **GX-VS** (10 mM) in 99% glycerol and in PBS buffer with the presence of various analytes (100 mM). 1. Blank; 2. 99% Glycerol; 3. L- Cysteine; 4. L- Alanine; 5. L- Methionine; 6. L- Arginine; 7. Ascorbic acid; 8. L- Lysine; 9. L- Leucine; 10. L- Proline; 11. L- Tryptophan; 12. L- Serine; 13. L- Phenylalanine; 14. Glutathione; 15. $MgCl_2$; 16. $ZnCl_2$; 17. $CaCl_2$; 18. $CuSO_4$; 19. KI; 20. $FeCl_3$; 21. Na_2CO_3; 22. $NaHSO_3$; 23. NaHS; 24. Na_2S; 25. H_2O_2; 26. NaClO; 27. Cytochrome c; 28. NADH-Na_2; 29. riboflavin; 30. FAD-Na_2. λ_{ex}: 545 nm, slit width: $d_{ex} = d_{em} = 10$ nm. The inline figure represents the results not including the glycerol solvent test.

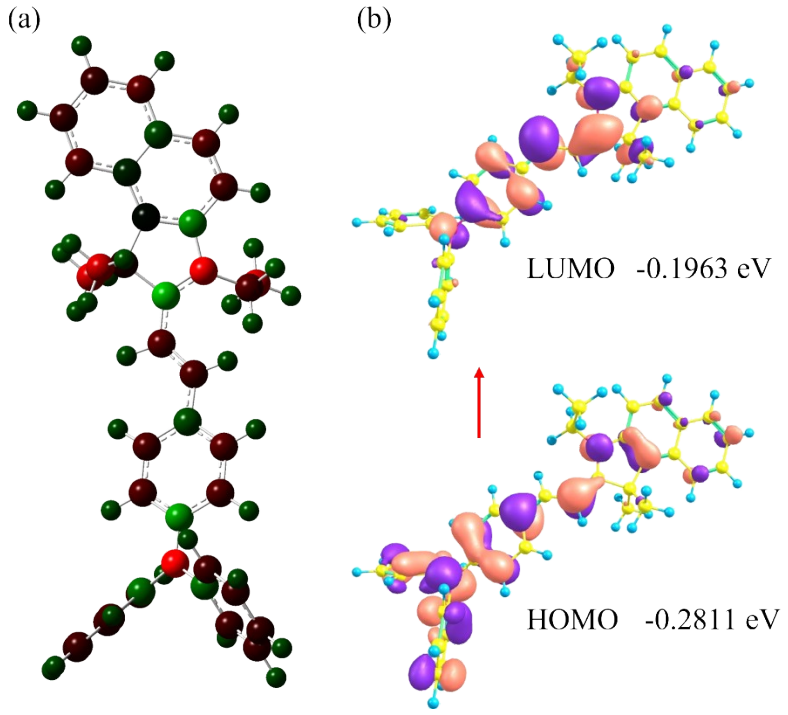

Fig.S6. (a) Charge density geometry of probe **GX-VS** in gas phase; (b) HOMO and LUMO

profiles of the probe computed in gas phase.

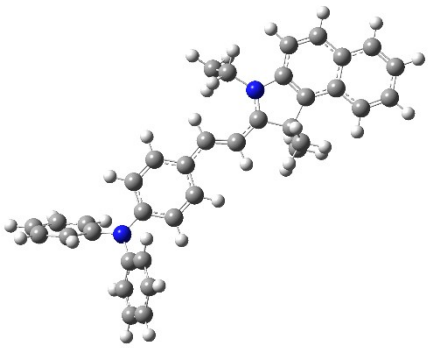

Fig.S7. Optimized conformation of the probe **GX-VS** in ground state using Gaussian 16 program by DFT B3LYP/6-31G (d) methods. (Angle between the plane of one benzene ring in triphenylamine and the plane of the indole analogue was 18.72°)

Dihedral angle	LUMO (eV)	HOMO (eV)	$\Delta_{HOMO-LUMO}$ (eV)	*f*
0°	-3.537482	-5.254521	-1.717039	1.8996
90°	-1.883029	-6.462708	-4.579679	0.8255

Fig.S8. Energy profile of the probe **GX-VS** molecular orbitals.

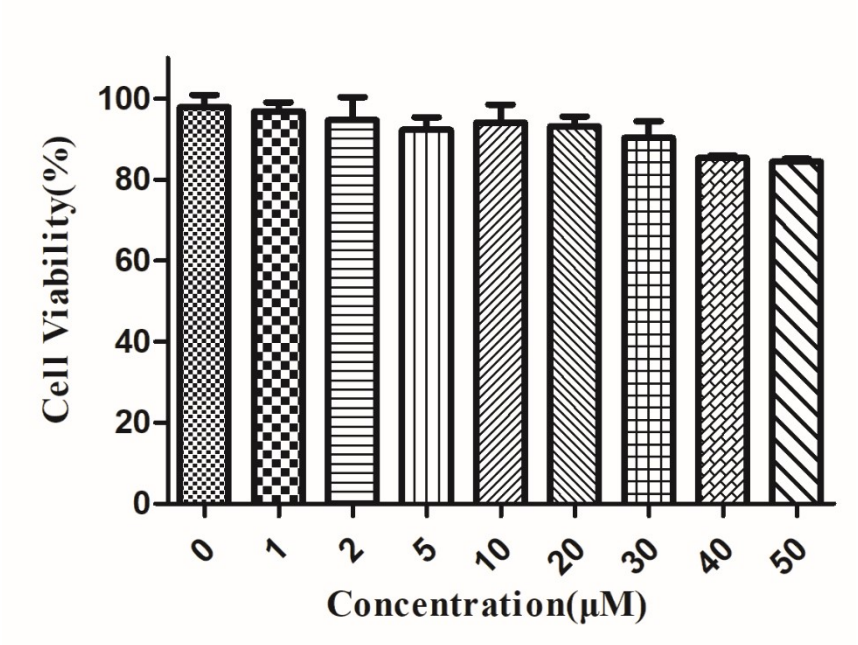

Fig.S9. Cytotoxicity assays of **GX-VS** at different concentrations for HeLa cells

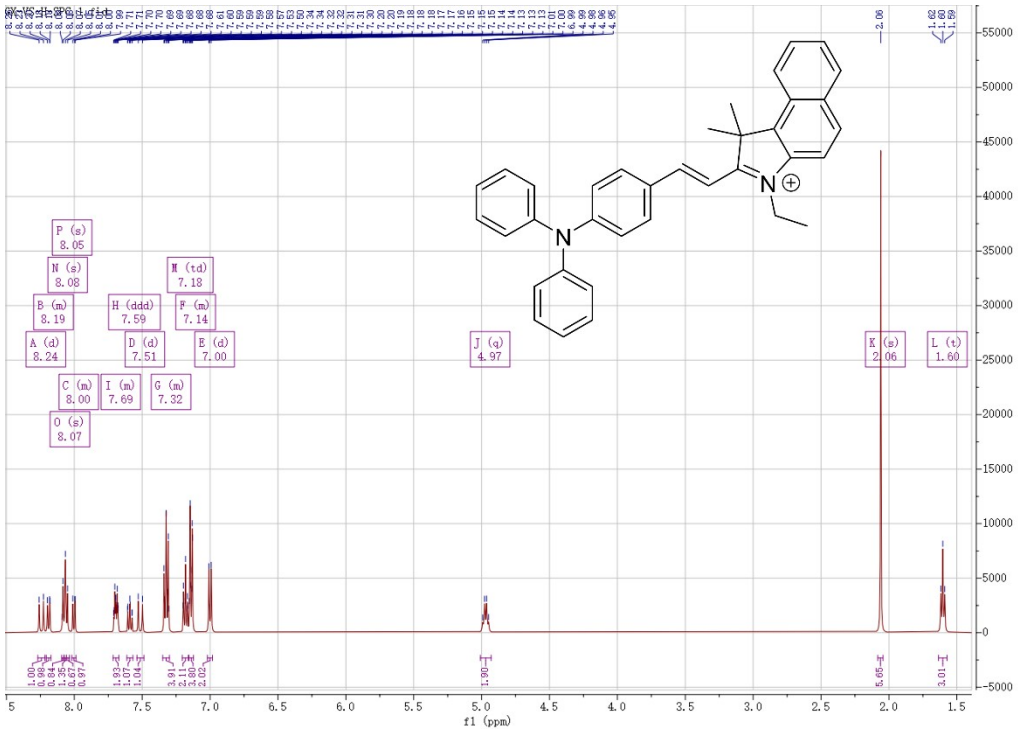

Fig.S10. ^{1}H NMR spectra of **GX-VS** in $CDCl_3$

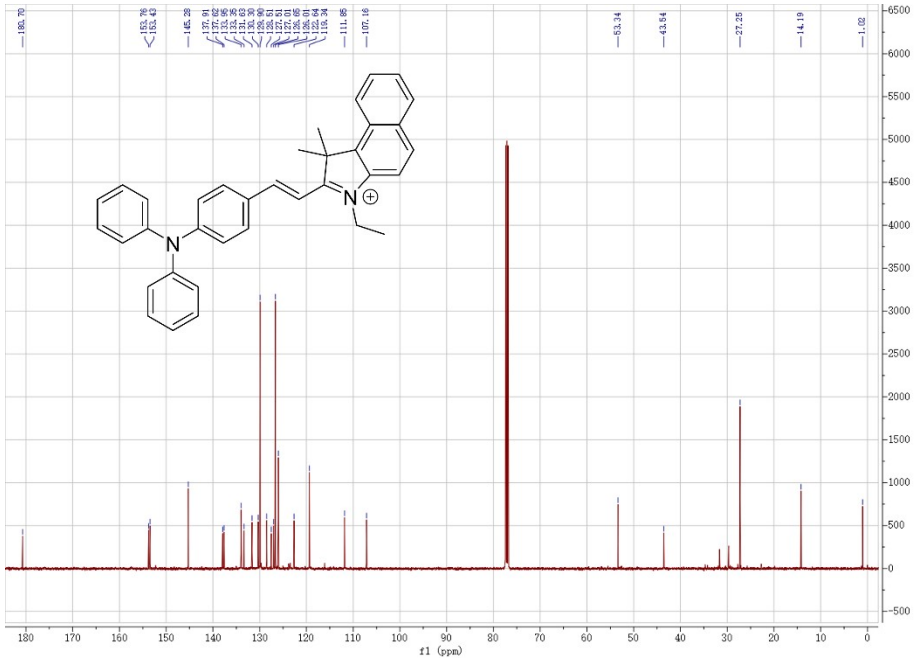

Fig.S11. ^{13}C NMR spectrum of **GX-VS** in $CDCl_3$

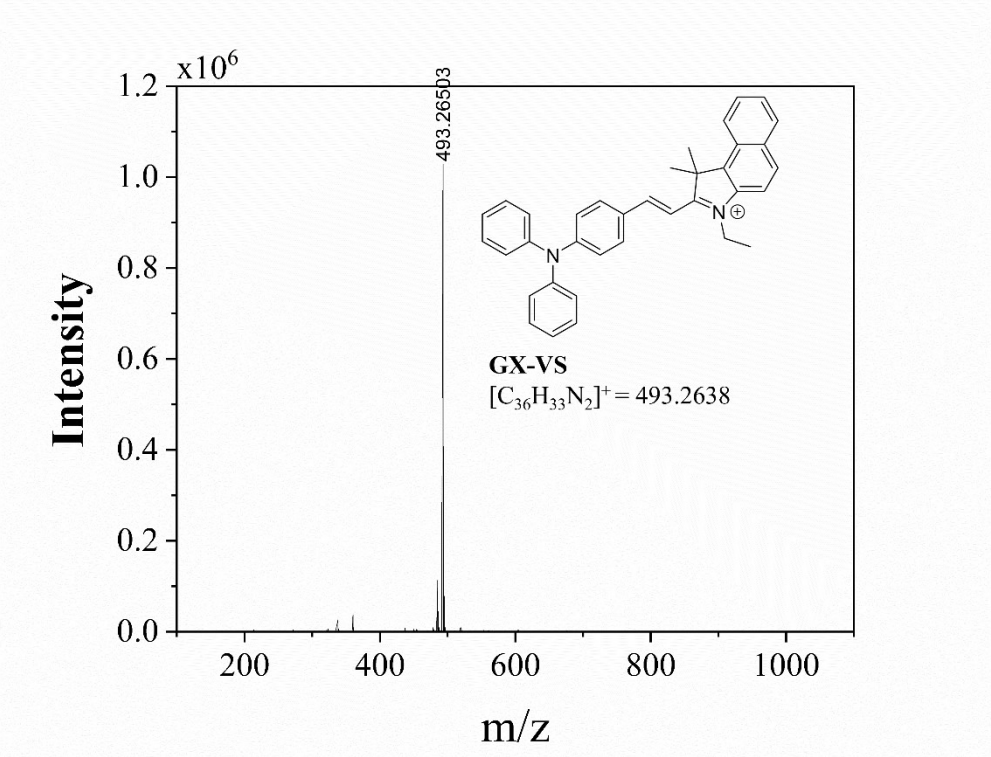

Fig.S12. HRMS of **GX-VS**