

## **Probing Mitochondrial Health with Near-Infrared Fluorescence: Xylan-Based Probe for Unveiling Cellular Viscosity**

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## 1. Materials and Instruments

All chemical reagents such as xylan (Shanghai Maclin Biotechnology Co., LTD.), 4-acetylene benzaldehyde (Shanghai Maclin Biotechnology Co., LTD.), anhydrous copper sulfate (Shanghai Maclin Biotechnology Co., LTD.), sodium ascorbate (Shanghai Maclin Biotechnology Co., LTD.) are purchased from suppliers without further purification. Organic solvents used in the experiment: methylene chloride, methanol, N, n-dimethylformamide, petroleum ether, glacial acetic acid, Dulbecco modified Eagle's culture-medium (DMEM), fetal bovine serum (FBS), streptomycin, penicillin and so on were purchased from suppliers and dried according to standard methods. The experimental treatment was double distilled water and all the reactions were carried out in nitrogen atmosphere. The hydrogen spectrum was recorded using deuterium-dimethyl sulfoxide ( $\text{DMSO-D}_6$ ) deuterium-oxide ( $\text{D}_2\text{O}$ ) as solvent on Brooke Dalton spectrometer (400Hz). The high-resolution mass spectrometry (HR-MS) spectra were recorded on a maxis ultrahigh resolution-time-of-flight (TOF) instrument (Bruker Co., Ltd., Germany). Fourier-transform infrared (FT-IR) spectra were performed on a Bruker TENSOR27 spectrometer in the range of  $4000 - 400 \text{ cm}^{-1}$  by pressing samples into pure potassium bromide pellets. UV-Vis spectra were measured on a Shimadzu UV-2600 spectrometer. Fluorescence spectra were performed on a Hitachi F-7100 spectrofluorometer at room temperature. The scanning electron microscopy (SEM) images was collected on Hitachi S4700 instrument. The X-ray photoelectron spectroscopy (XPS) spectra was collected on thermo fisher scientific

ESCALAB Xi+ instrument. The 3-(4, 5-Dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide (MTT) assays were recorded using a Multiskan FC microplate reader (Thermal Fisher). The fluorescence images of the cells and zebrafish were performed on a TCS SP8 confocal laser scanning microscope (Leica Co., Ltd., Germany).

## 2. Fourier transform infrared spectroscopy (FTIR)

The sample is dried at 80°C overnight. It is important to note that no new content has been added to the original text. After drying, it is mixed with a specific amount of KBr, ground with agate, and hand-pressed into a transparent sheet. Fourier transform infrared spectroscopy was performed in transmission mode. The wavelength range of 400  $\text{cm}^{-1}$  to 4000  $\text{cm}^{-1}$  was scanned 16 times with a resolution of 4  $\text{cm}^{-1}$ .

## 3. X-ray photoelectron spectroscopy

The sample is dried and ground, attached to the tin foil, and the surface is pressed by hand until smooth. Then the content of each element was analyzed.

## 4. Scanning electron microscopy (SEM)

The sample was dispersed evenly in ethanol at a concentration of 0.1% and then dropped onto the copper sheet until the ethanol completely volatilized. After being sprayed with gold for five minutes, the sample's morphology was observed using a scanning electron microscope at an accelerated voltage of 15 kV.

## 5. General procedure for fluorescence detection

NI-XylIV and RV solutions were added to detection solutions with different viscosities consisting of glycerol and phosphate buffered saline (PBS, pH 7.4) to detect changes in fluorescence intensity. The fluorescence spectra were obtained on a HITACHI F-7100 spectrophotometer (Japan). Fluorescence spectra of NI-XylIV and RV were tested at room temperature (titration experiment, pH stability, polarity stability and ion selectivity). The ion selectivity of NI-XylIV was evaluated by adding the same amount of different common small molecule ions to 2ml phosphate buffered saline (PBS, pH 7.4). For glycerol, cysteine (Cys),  $S_2O_3^{2-}$ ,  $ClO^-$ ,  $NO_2^-$ ,  $HSO_3^-$ ,  $NO_3^-$ ,  $SO_3^{2-}$ ,  $SO_4^{2-}$ ,  $S^{2-}$ ,  $Vc^-$ , tryptophan (Trp),  $Fe^{3+}$ ,  $Mg^{2+}$ ,  $Cu^{2+}$ ,  $Ca^{2+}$ ,  $Zn^{2+}$ ,  $Al^{3+}$ , leucine (Leu), Glutamic (Glu), and  $H_2O_2$  were tested, and the detection solution was continuously shaken at room temperature for 5 hours and then spectroscopically determined. In order to ensure data consistency, all spectral test procedures are consistent.

## 6. The cytotoxicity assay experiment

For cytotoxicity assay, HepG2 cells were seeded in a 96-well plate at an initial density of  $1 \times 10^4$  cells/well in 100  $\mu$ L of complete DMEM medium. The 96-well plate of inoculated cells was placed in a 37 °C constant temperature incubator containing 5 %  $CO_2$  and 95 % air for 24 h, and the old DMEM culture medium was aspirated, and the probes NI-XylIV and RV were configured with different concentrations of probes and DMEM were added to the 96-well plate, respectively. Incubation continued for 24 h in

37 °C constant temperature incubator with 5 % CO<sub>2</sub> and 95 % air. Then the old culture medium was sucked out and 100 µL DMEM mixed MTT (methylthiazoltetrazole, 5 mg/mL PBS) was added to each well. After incubation for 4 h, the old culture medium mixed with MTT was sucked out, and the purple precipitate was completely dissolved by adding DMSO solution of 100 µL into each well. After the absorbance of each well at 492 nm was measured by enzyme marker, and the cell survival rate was calculated using the following data.

$$Cell\ survival\ rate\ (\%) = \frac{OD_{492}sample - OD_{492}blank}{OD_{492}control - OD_{492}blank} \times 100\%$$

In which *OD<sub>492</sub> blank*, *OD<sub>492</sub> control* and *OD<sub>492</sub> sample* respectively represent the optical density of cells at the wavelength of 490nm with or without probes in the culture medium.

All experiments were performed in compliance with the relevant laws and institutional guidelines of the Animal Ethical Experimentation Committee of Qilu University of Technology (Shandong Academy of Sciences), and in accordance with the requirements of the National Law on the Use of Laboratory Animals (China).

## 7. Cell imaging experiment

To detect viscosity changes in living cells, HepG2 cells were pretreated with monensin (10 µM) for 45 minutes to induce cell viscosity changes. NI-XylV (20 µg/mL) was added and incubated for 45 minutes. During incubation, fluorescence imaging was performed at 15, 30 and 45 min after NI-XylV was added. Before fluorescence imaging, the culture solution was sucked away and cleaned twice with

PBS before imaging under the microscope. In fluorescence imaging, the green channel is excited at 488 nm, and the red channel is excited at 588 nm.

To measure the endogenous viscosity, change of cells, the cells were subjected to starvation culture four hours in advance to induce the viscosity change, and then 20 µg/ml NI-XylV was added to the confocal laser imaging dish for 30 min for fluorescence imaging. In fluorescence imaging, the green channel is excited at 488 nm, and the red channel is excited at 588 nm.

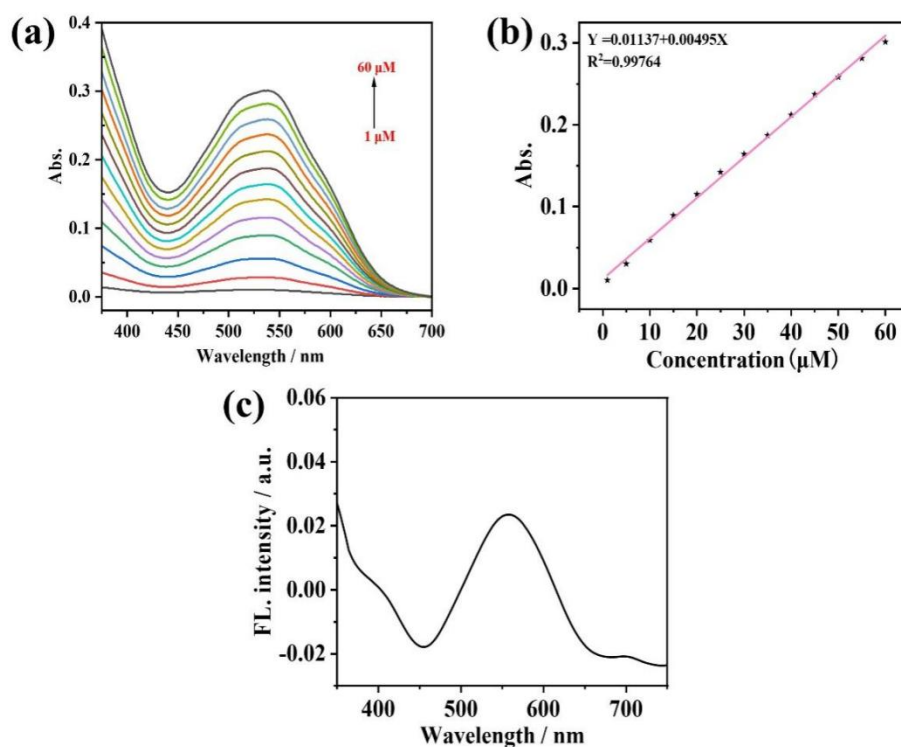
#### 8. Zebrafish imaging experiment

20 µg/ml NI-XylV was added to the zebrafish nutrient solution for incubation for 60 min, and then 10 µM monensin and 10 µM nystatin were added for further incubation for 60 min. Afterward, residual probes were rinsed with PBS and zebrafish were fluorescently imaged. In fluorescence imaging, the green channel is excited at 488 nm, and the red channel is excited at 588 nm.

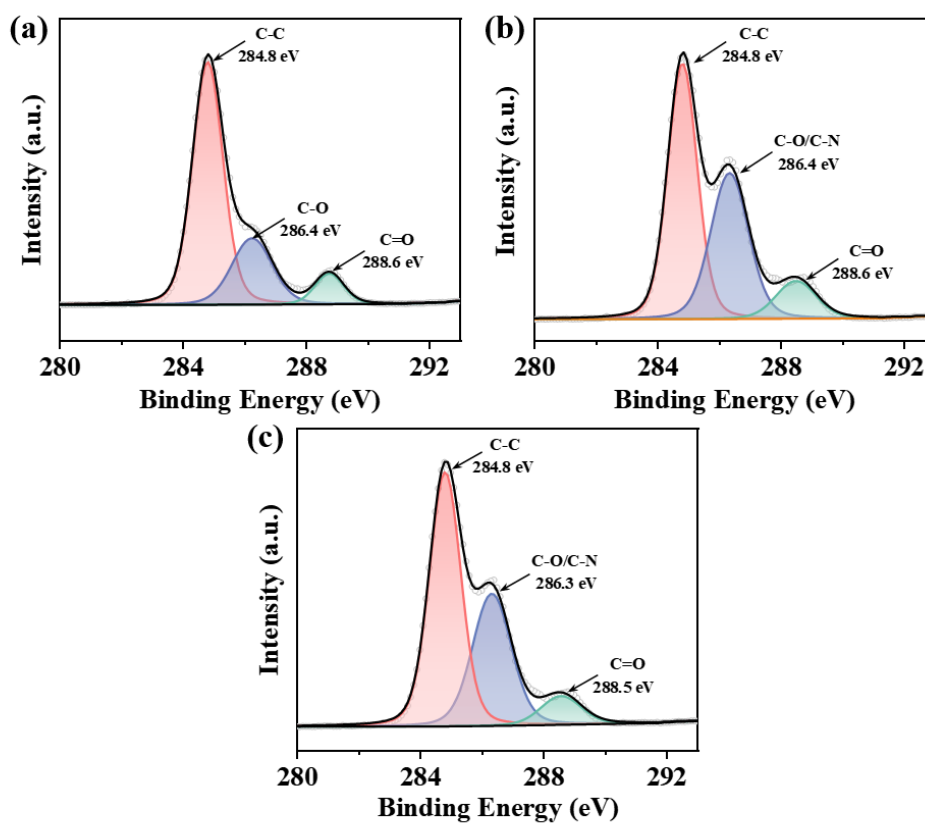
#### 9. Calculation of NI-XylV grafting rates

$$\text{Grafting rate (\%)} = \frac{C_{\text{NI-XylV}} \times V_{\text{eq}}}{n_{\text{RV}}} \times 100\%$$

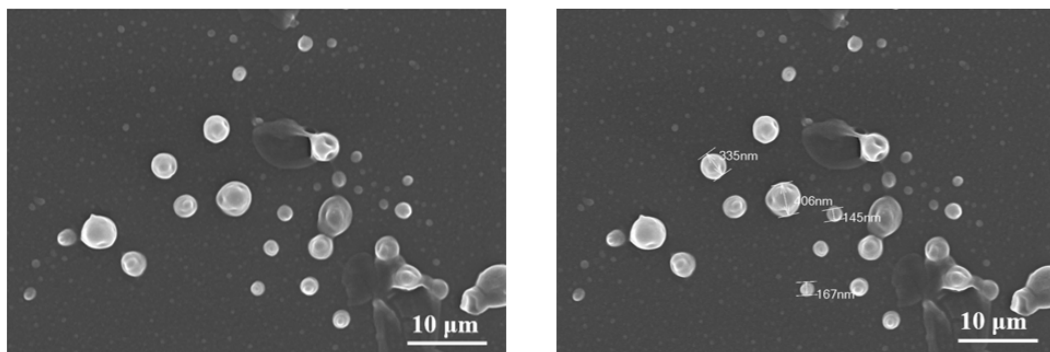
$C_{\text{NI-XylV}}$  is obtained by substituting the UV absorption value of NI-XylV into a standard curve of small molecule compounds at different concentrations to obtain the corresponding concentration;  $V_{\text{eq}}$  is the volume of NI-XylV probe solution;  $n_{\text{RV}}$  is the amount of substance said to be added to the small molecule RV when it is reacted with xylan.



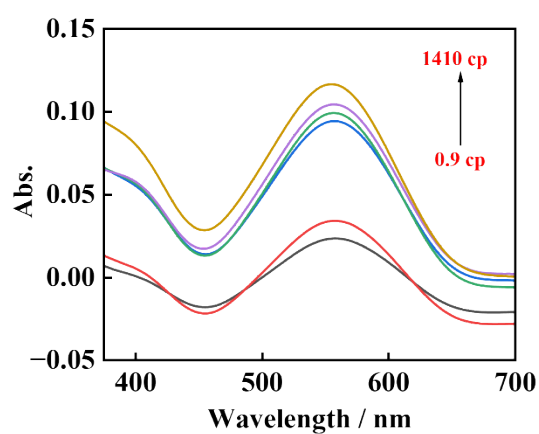
**Fig. S1** (a) UV-Vis spectra of RV in DMSO at concentration range from 1 to 60  $\mu\text{M}$ ; (b) Dependence of absorbance at 555 nm on concentration of RV; (c) UV-Vis spectrum of NI-XylIV (1 mg/mL).



**Fig. S2** High resolution XPS C1s scan of Xyl-N<sub>3</sub> and NI-XylIV.

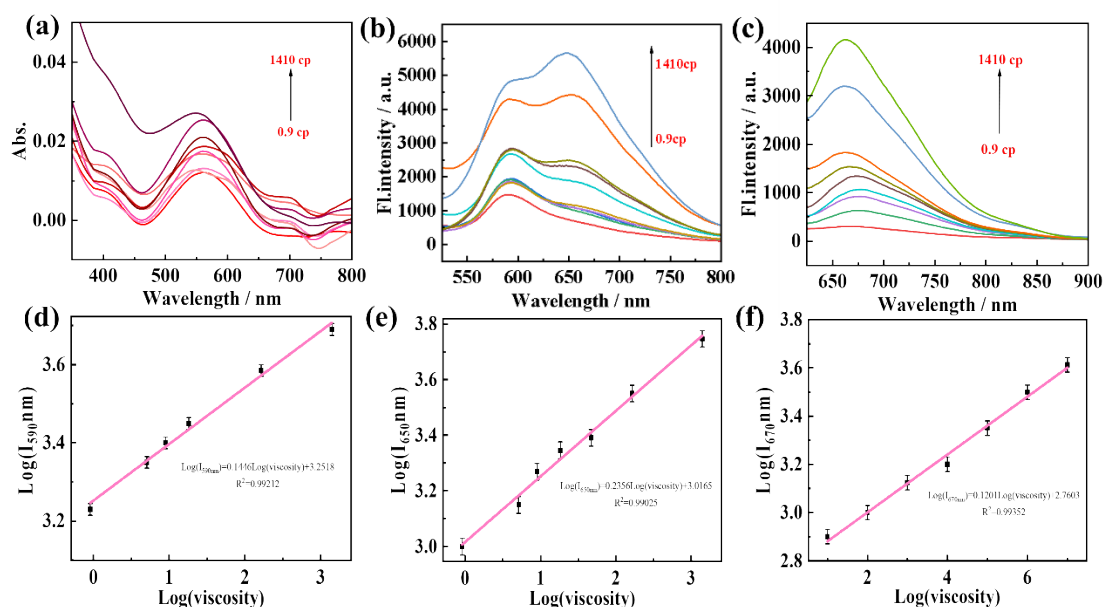


**Fig. S3** SEM images of dilute NI-XylIV in PBS and sample were prepared on mica by freezing-drying scale bar: 10  $\mu\text{m}$ .

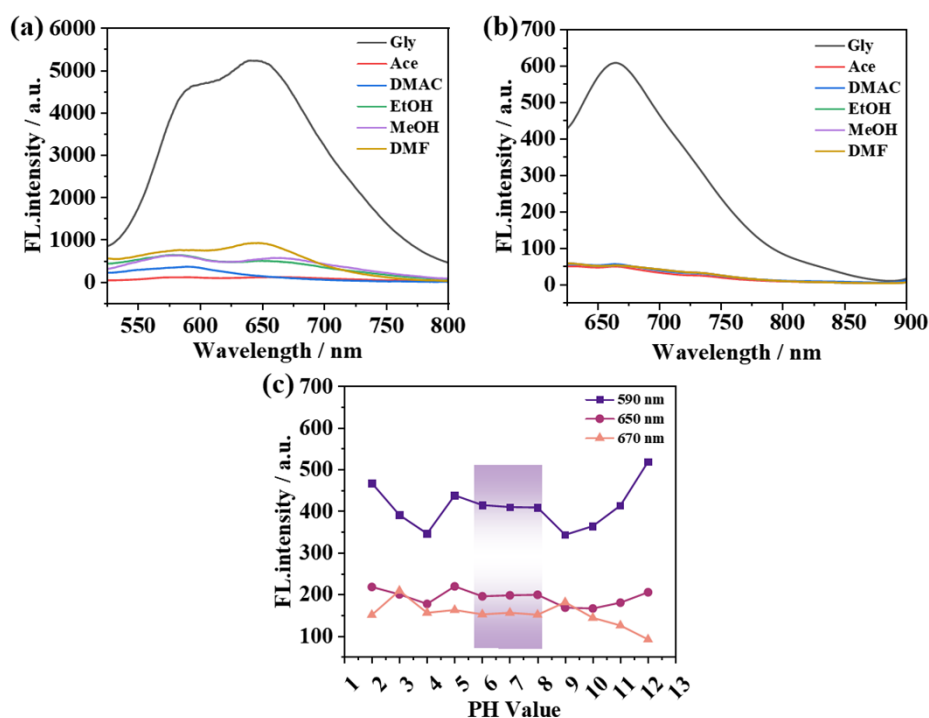


**Fig. S4** UV-Vis spectra changes of 10  $\mu\text{g/mL}$  NI-XylIV in the glycerol-PBS mixture as the glycerol content increased from 0 to 100%.

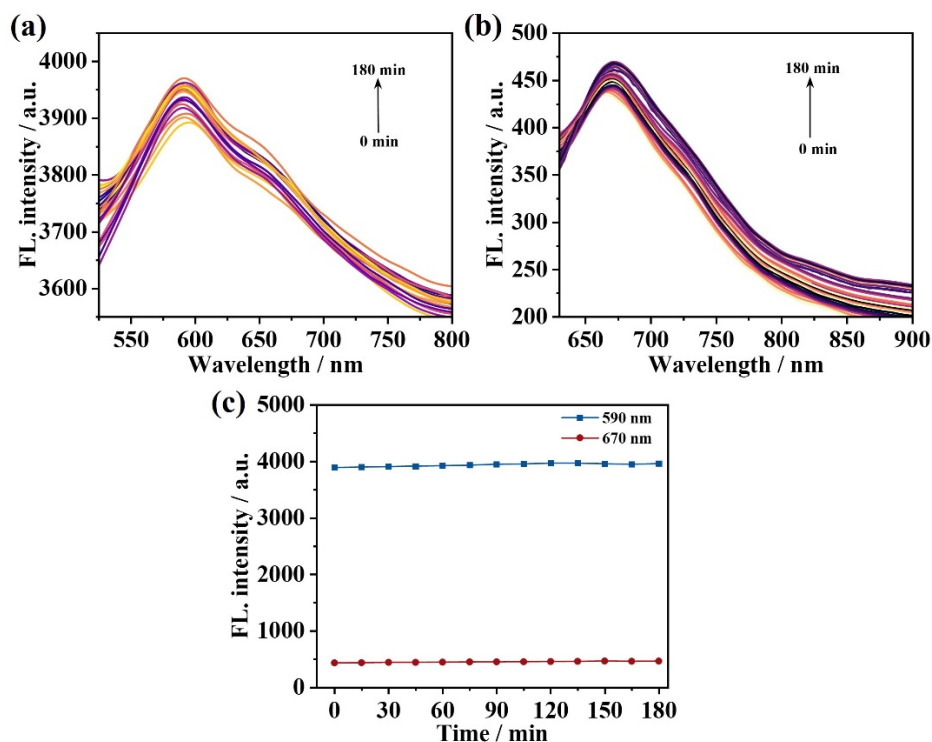




**Fig. S5** The UV-Vis absorption spectra of RV (a) under different viscosity solvents condition; (b-c) Fluorescence spectra changes of RV with viscosity; (d-f) there was 10  $\mu\text{M}$  RV in the glycerol-PBS mixture as the glycerol content increased from 0 to 100%;  $\lambda_{\text{ex}} = 500 \text{ nm}$ ,  $\lambda_{\text{ex}} = 600 \text{ nm}$ .

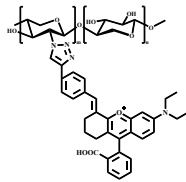
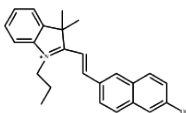


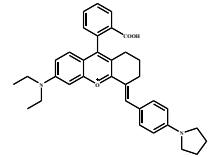
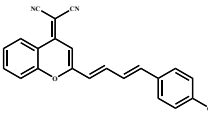
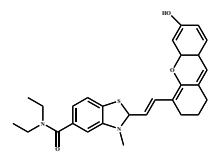
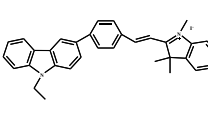
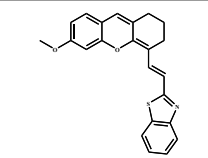
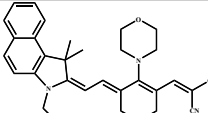
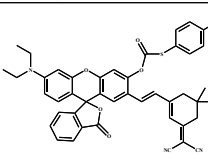
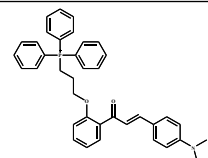
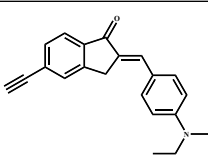
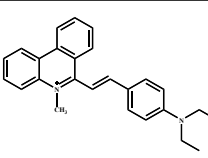
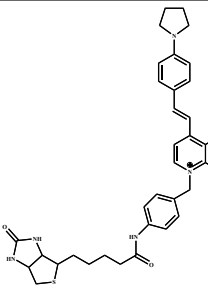
**Fig. S6** (a-b) Fluorescence spectrum changes of NI-XylIV probe in different polar solvents and glycerol; (c) Fluorescence spectrum changes of NI-XylIV probes in solvents with different PH values.

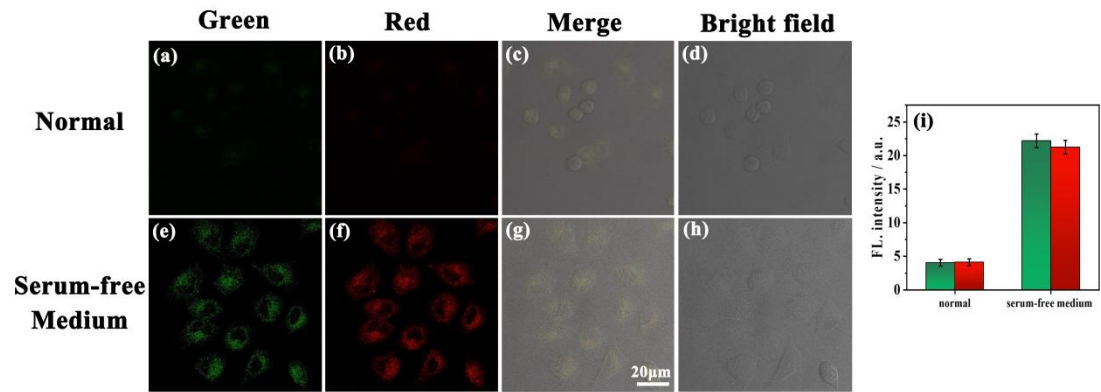


**Fig. S7** (a-b) NI-XylIV probe time-responsive fluorescence changes in 80% glycerol assay solution;  $\lambda_{\text{ex}} = 500 \text{ nm}$ ,  $\lambda_{\text{ex}} = 600 \text{ nm}$ . (c) Line graphs of fluorescence intensity at 590 nm and 670 nm over a 180 min period.

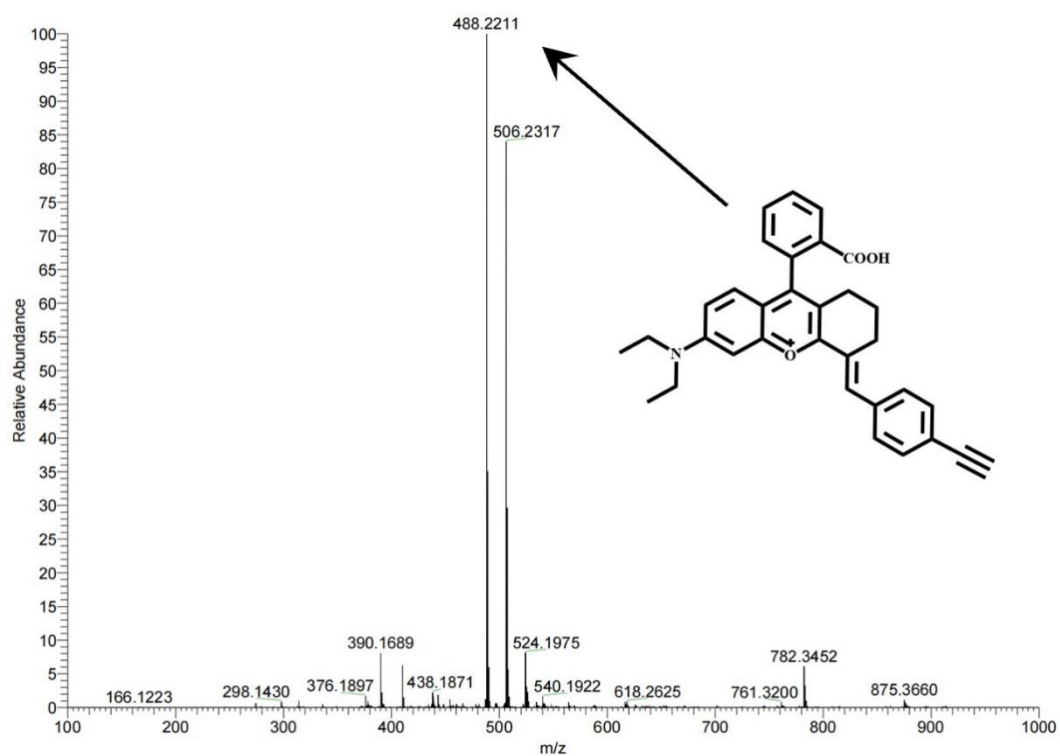
**Table S1** Comparison of NI-XylIV with previously reported fluorescent probes for the detection of Viscosity.

Probes	$\lambda_{\text{ex}} / \text{nm}$	$\lambda_{\text{em}} / \text{nm}$	Switching Type	Probe solvents	Application system	Reference
	500 nm, 600 nm	590 nm, 670 nm	TICT	H <sub>2</sub> O	Cell, zebrafish	This work
	450 nm	586 nm	TICT	DMSO	Cell, zebrafish	Spectrochim. Acta, Part A 2024,315,124246

	680 nm	740 nm	TICT	DMSO	Cell, Mouse imaging	Chem. Eng. J. 2022,445,136448
	480 nm	640 nm	TICT	DMSO	Cell, Mouse imaging	Sci. Rep. 2024,14,1336
	600 nm	670 nm	TICT	DMSO	Cell	Spectrochim. Acta, Part A. 2024,318,124486
	560 nm	620 nm	TICT	DMSO	Cell, Mouse imaging	Anal. Chem. 2019,91,8414-8421
	460 nm	640 nm	TICT	DMSO	Cell	Dyes Pigm. 2024,229,112305
	630 nm	723 nm	TICT	DMSO	Cell, Mouse imaging	Talanta 2024,272,125825
	480 nm	550 nm	TICT	DMSO	Cell	Spectrochim. Acta, Part A. 2024,315,124270
	430 nm	533 nm	TICT	DMSO	Cell	Dyes Pigm. 2021,194,109593
	488 nm	616 nm	TICT	DMSO	Cell	Anal. Chim. Acta 2022,1232,340454
	480 nm	666 nm	TICT	DMSO	Cell	Dyes Pigm. 2025,232,112479
	610 nm	680 nm	TICT	DMSO	Cell, Mouse imaging	Anal. Chem. 2024,96,13447-13454



**Fig. S8** Fluorescence imaging of mitochondria viscosity during mitophagy induced by the serum-free medium. (a-d) HepG2 cells were cultured in normal medium for 24 h and incubated with NI-XylV for 30 min. (e-h) HepG2 cells cultured in fetal bovine serum-free medium for 4 h and incubated with NI-XylV for 30 min. (i) Fluorescence intensity change of HepG2 cells under normal treatment and starvation treatment. (Error bar:  $n = 3$ ). green channel ( $\lambda_{\text{ex}} = 488 \text{ nm}$ ;  $\lambda_{\text{em}} = 500 - 600 \text{ nm}$ ), red channel ( $\lambda_{\text{ex}} = 588 \text{ nm}$ ;  $\lambda_{\text{em}} = 600 - 700 \text{ nm}$ ). Scale bar:  $20 \mu\text{m}$



**Fig. S9** HR-MS spectrum of **RV**.

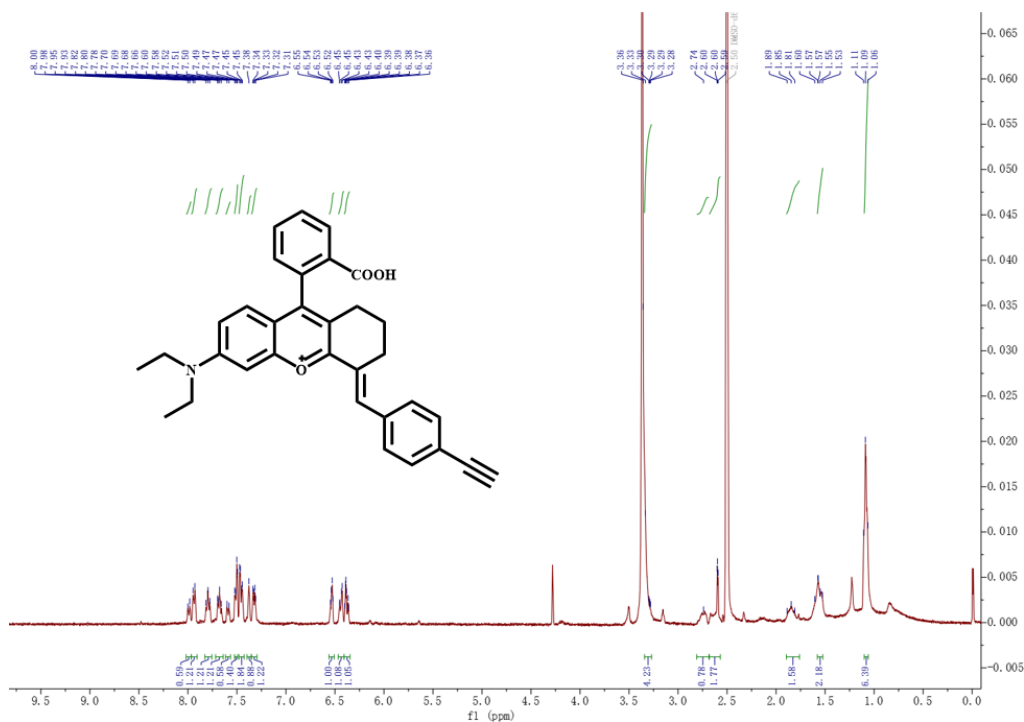


Fig. S10  $^1\text{H}$  NMR spectrum of RV in DMSO- $\text{d}_6$ .

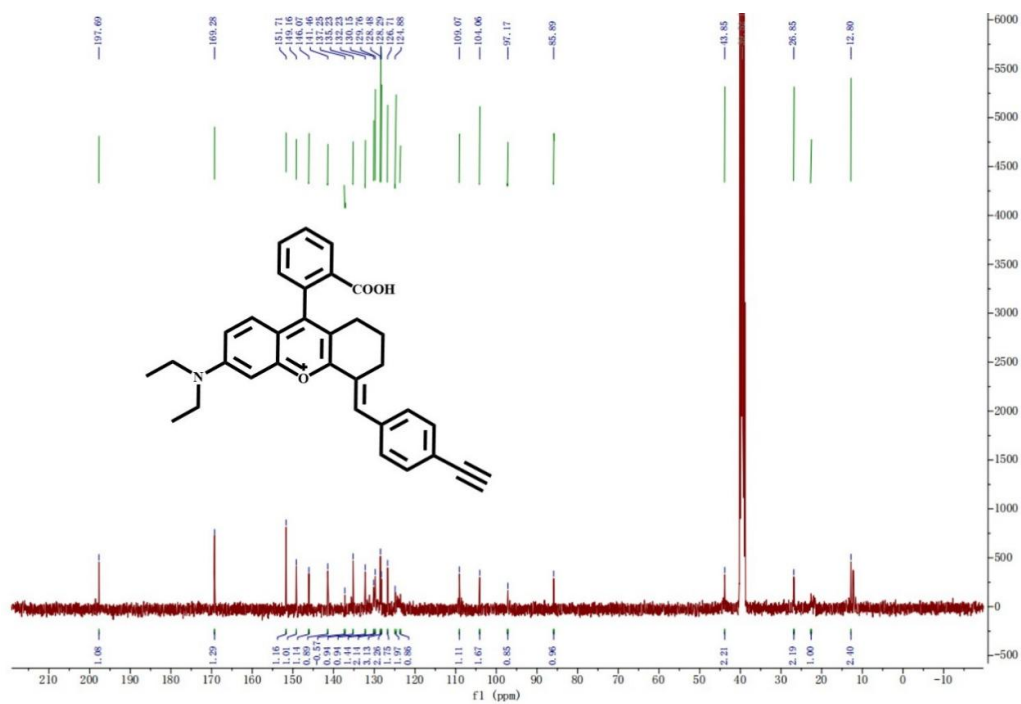
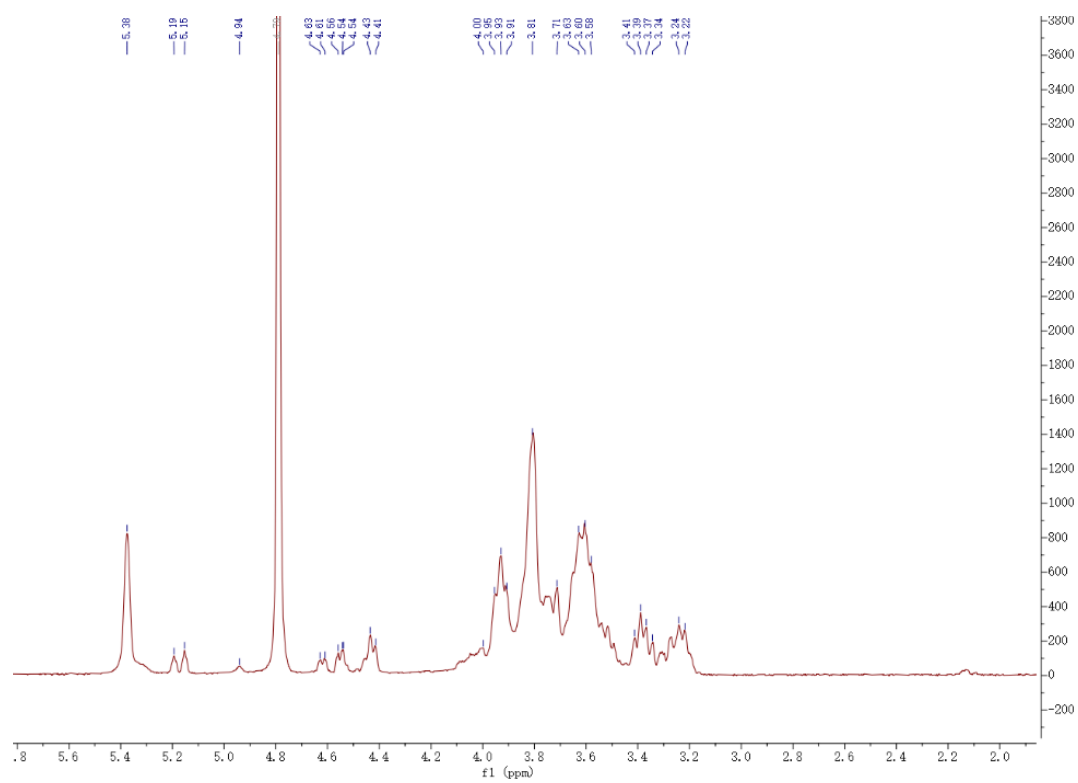


Fig. S11  $^{13}\text{C}$  NMR spectrum of RV in DMSO- $\text{d}_6$



**Fig. S12**  $^1\text{H}$  NMR spectrum of Xyl in  $\text{D}_2\text{O}$ .





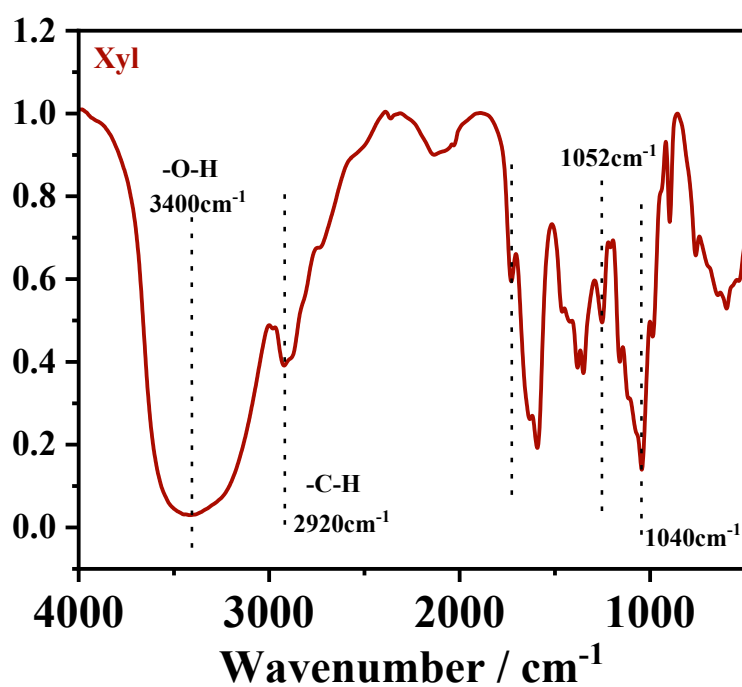


Fig. S15 FT-IR spectra of Xyl.

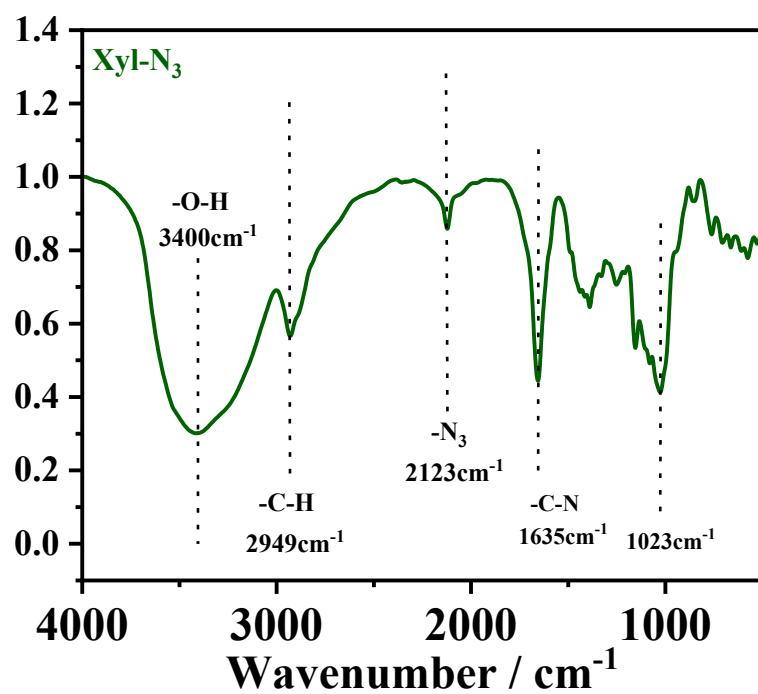
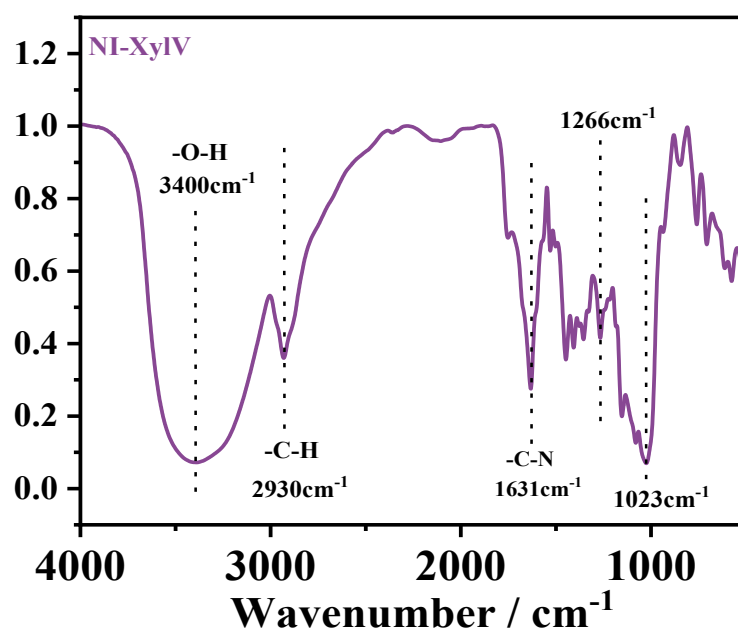


Fig. S16 FT-IR spectra of Xyl- $\text{N}_3$ .



**Fig. S17** FT-IR spectra of NI-XylIV.