Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.
This journal is © The Royal Society of Chemistry 2023

Supporting Information

Solvatochromic Sensors Detect Proteome Aggregation in Stressed Liver Tissues with Hepatic Cancer and Cirrhosis

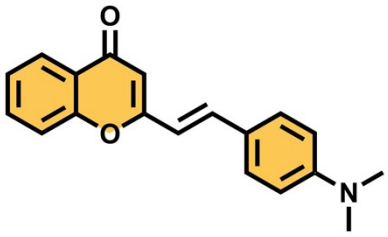
Biao Jing[a,b], ‡, Junpeng Li[a,b] ‡, Kun Guo[a], Lianggang Zeng[b], Jidong Sui[a], Zhenduo Zhang[a,b], Zhiming Wang[a, b], Hao Jin[a,b], Jialu Sun[b], Zhao Xue[a], Qi Zhao[a,b], Wang Wan*[b], Xuepeng Dong*[a]

a. The Second Hospital of Dalian Medical University, 467 Zhongshan Road, Dalian, 116023, China.

b. CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China.

‡. These authors contributed equally to this work

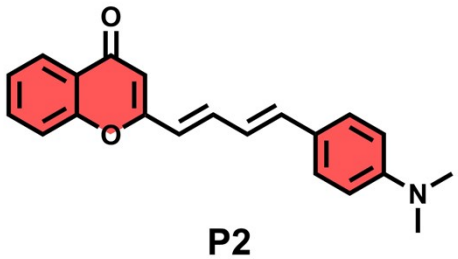
1. Supplementary Figures



P1

	solvent	dielectric constant	λ_{abs} (nm)	λ_{em} (nm)	$\Delta\lambda$ (nm)
1	Glycerol	46.50	447	607	160
2	Glycol	37.00	440	610	170
3	MeOH	32.70	427	598	171
4	EtOH	24.50	427	588	161
5	i-PrOH	19.90	424	575	151
6	n-BuOH	17.10	428	581	153
7	t-BuOH	11.40	418	555	137
8	DCM	8.90	418	532	114
9	THF	7.60	410	521	111
10	EA	6.02	406	519	113
11	Toluene	2.40	408	504	96
12	Dioxane	2.25	409	509	100
13	CCl4	2.24	408	500	92

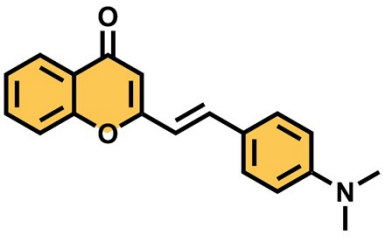
Figure S1. Photophysical properties of P1 across different solvents. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in NEST 96-Well flat bottom transparent plates. Measurements in different solvents were described in **Experimental Methods 2.2**. All measurements were repeated for three times.



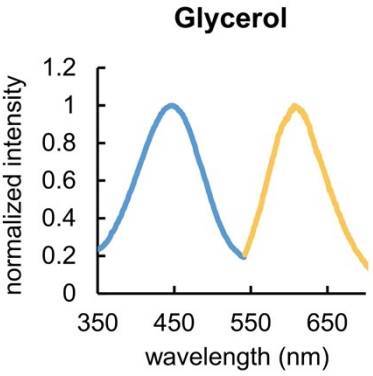
P2

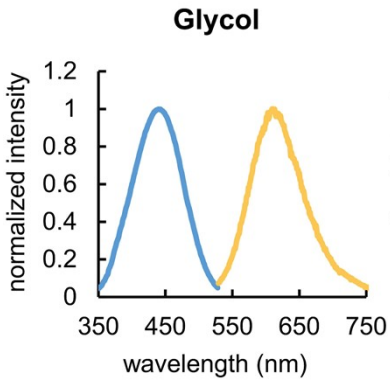
	solvent	dielectric constant	λ_{abs} (nm)	λ_{em} (nm)	$\Delta\lambda$ (nm)
1	Glycerol	46.5	465	689	224
2	Glycol	37	459	697	238
3	MeOH	32.7	443	682	239
4	EtOH	24.5	444	670	226
5	i-PrOH	19.9	442	662	220
6	n-BuOH	17.1	446	666	220
7	t-BuOH	11.4	439	624	185
8	DCM	8.9	433	613	180
9	THF	7.6	430	584	154
10	EA	6.02	424	583	159
11	Toluene	2.4	429	558	129
12	Dioxane	2.2	427	565	138
13	CCl_4	2.2	430	549	119

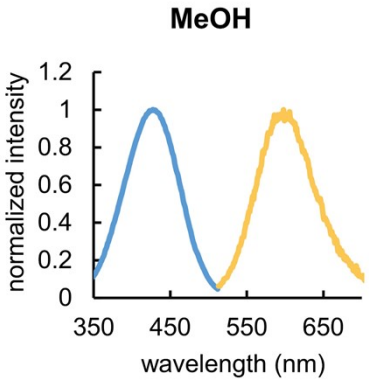
Figure S2. Photophysical properties of P2 across different solvents. All measurements were carried out using a Tecan Spark Fluorescence Plate Reader in NEST 96-Well flat bottom transparent plates. Measurements in different solvents were described in **Experimental Methods 2.2**. All measurements were repeated for three times.

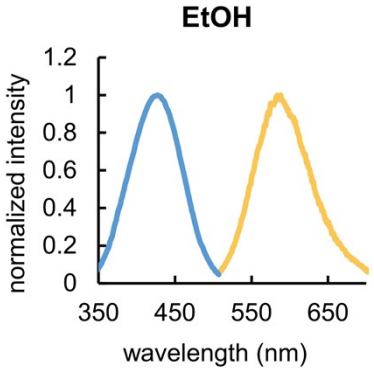

O
O
N

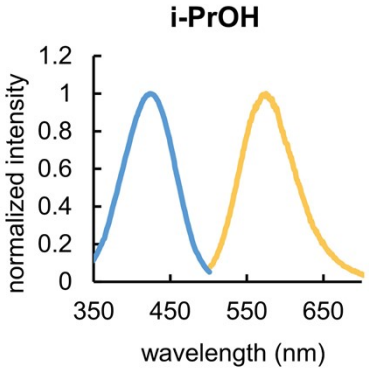
P1

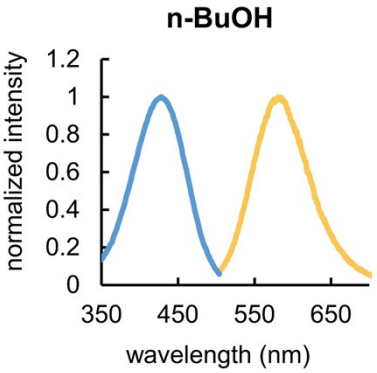

Glycerol
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350
450
550
650
wavelength (nm)

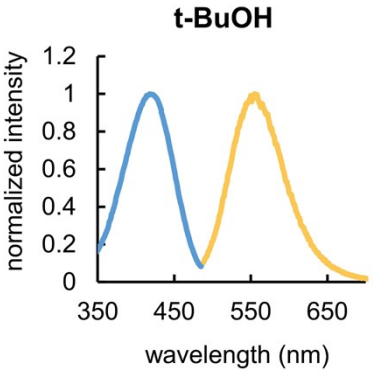

Glycol
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350
450
550
650
750
wavelength (nm)


MeOH
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350
450
550
650
wavelength (nm)


EtOH
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350
450
550
650
wavelength (nm)


i-PrOH
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350
450
550
650
wavelength (nm)


n-BuOH
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350
450
550
650
wavelength (nm)


t-BuOH
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350
450
550
650
wavelength (nm)

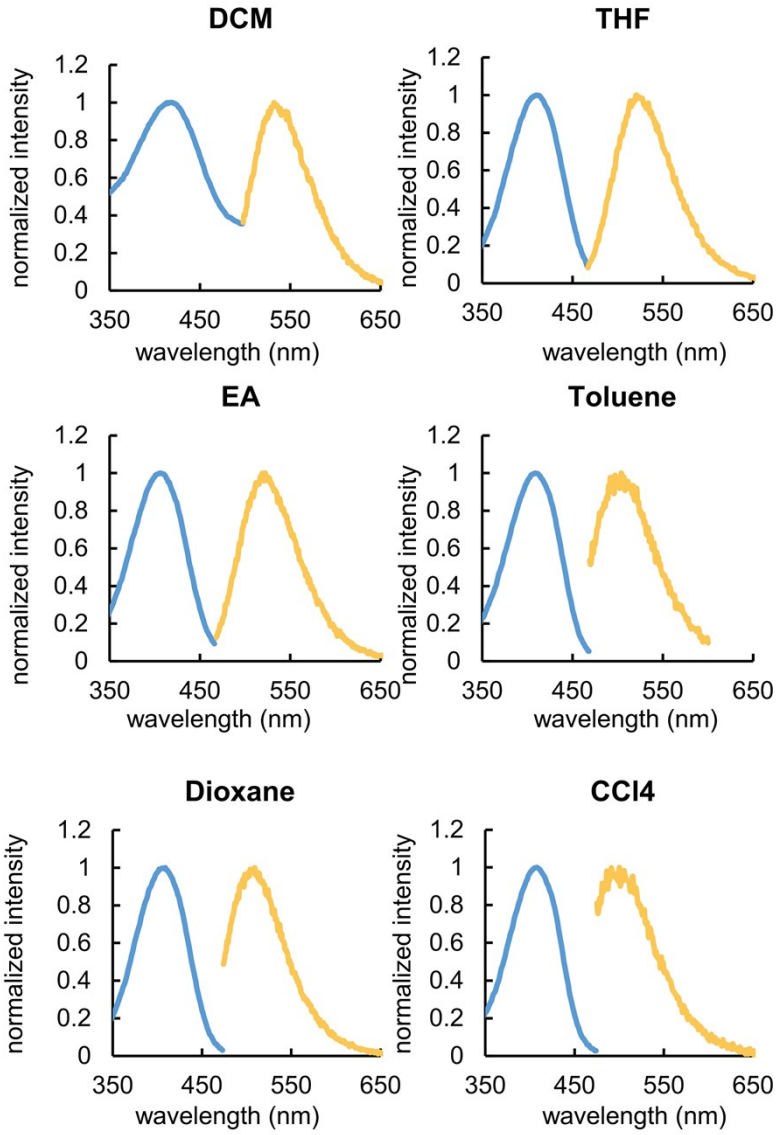
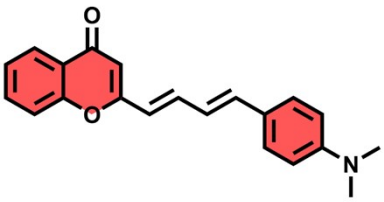
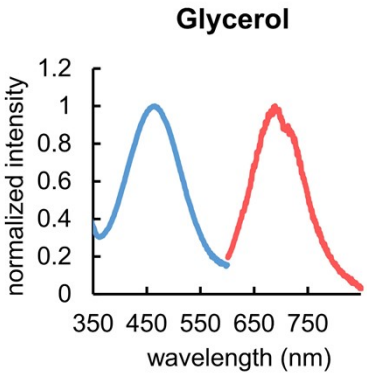
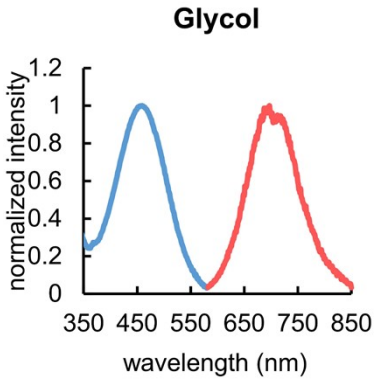


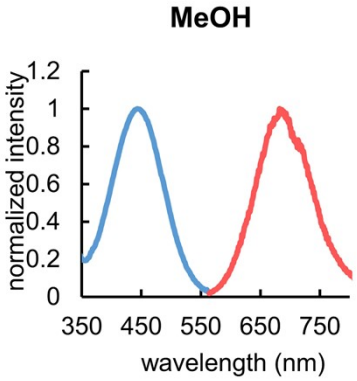
Figure S3. Normalized fluorescence excitation and emission spectra of P1 across the tested solvents. P1 was prepared to 20 μM for excitation scan and emission measurements. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in NEST 96-Well flat bottom transparent plates. The experimental procedure followed **Experimental Methods 2.2**. All measurements were repeated for three times.

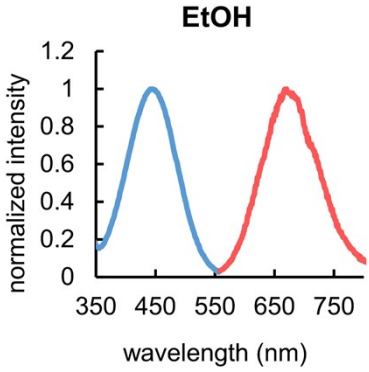

O
O
N

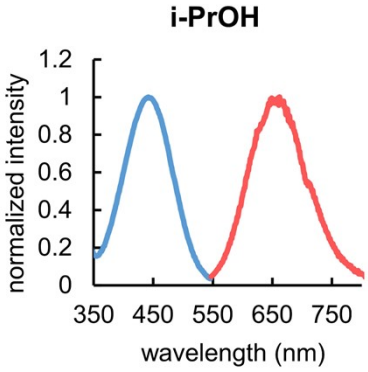
P2

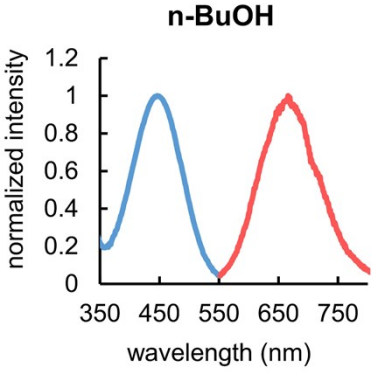

Glycerol
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350 450 550 650 750
wavelength (nm)

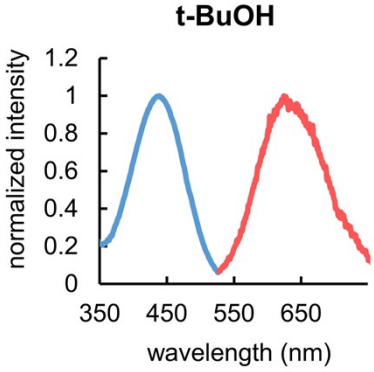

Glycol
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350 450 550 650 750 850
wavelength (nm)


MeOH
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350 450 550 650 750
wavelength (nm)


EtOH
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350 450 550 650 750
wavelength (nm)


i-PrOH
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350 450 550 650 750
wavelength (nm)


n-BuOH
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350 450 550 650 750
wavelength (nm)


t-BuOH
normalized intensity
1.2
1
0.8
0.6
0.4
0.2
0
350 450 550 650
wavelength (nm)

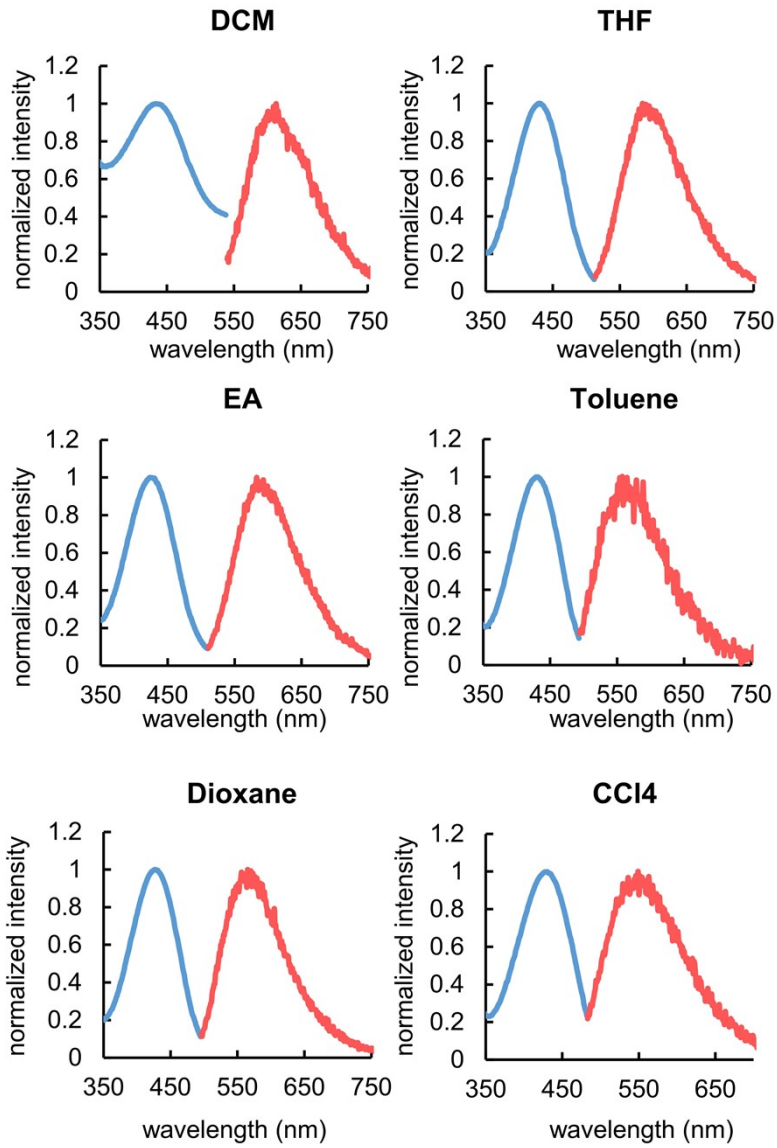


Figure S4. Normalized fluorescence excitation and emission spectra of P2 across the tested solvents. P2 was prepared to 20 μM for excitation scan and emission measurements. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in NEST 96-Well flat bottom transparent plates. The experimental procedure followed **Experimental Methods 2.2**. All measurements were repeated for three times.

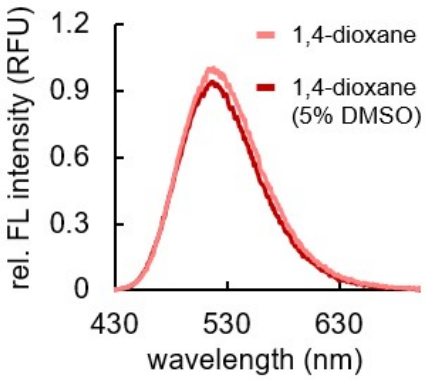


Figure S5. The fluorescence emission of P1 in pure 1,4-dioxane and 1,4-dioxane containing 5 % percent of DMSO. As shown in figure, the 5% of DMSO from stock solution have subtle influence on the fluorescence property of testing sample. The experiment was conducted according to **Experimental Methods section 2.2**.

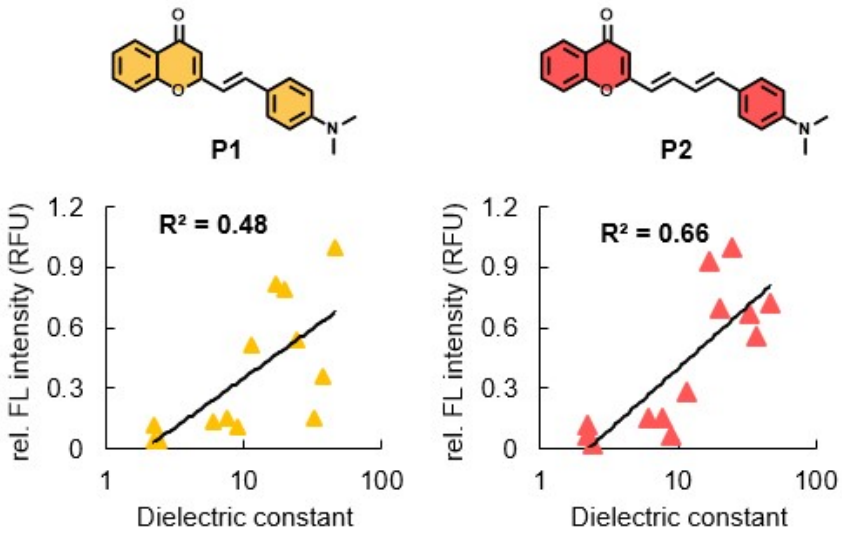


Figure S6. The relative fluorescence emission intensity of P1 and P2 in solvents bearing different dielectric constant. Compared to the emission wavelength versus dielectric constant in **Fig. 2**, the emission intensity of P1 and P2 displayed no obvious correlation with dielectric constant.

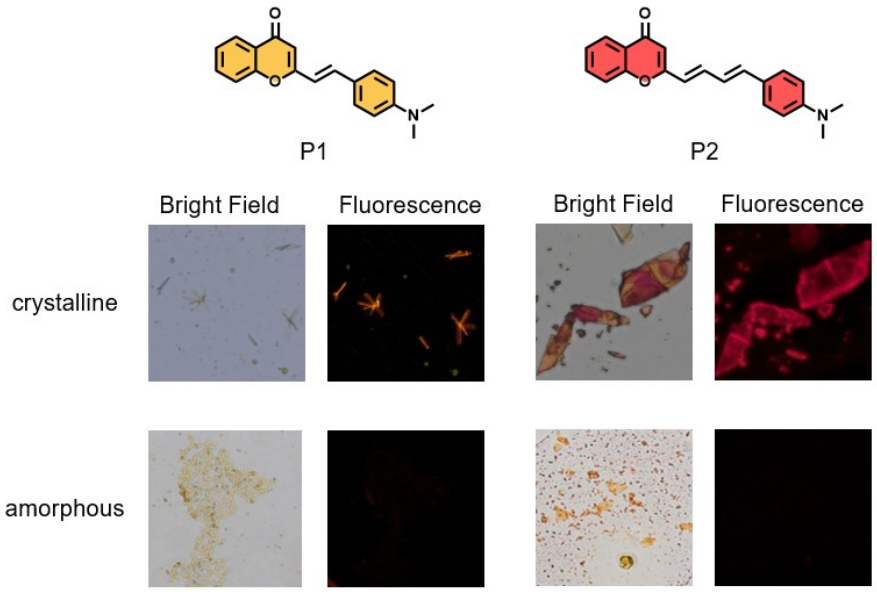


Figure S7. The P1 and P2 are not fluorescent as amorphous solids that were fast precipitated out from THF/H_2O mixed solvent. Amorphous solid was prepared through fast precipitation of P1 by diluting a 10 mM stock solution to 200 μM in H_2O; crystalline solid was prepared through slow evaporation of solvent from a 200 μM DCM solution. Images were collected using Olympus® IX73 Research Inverted Microscope under bright field and fluorescence mode excited by blue light (BP 340-390 nm).

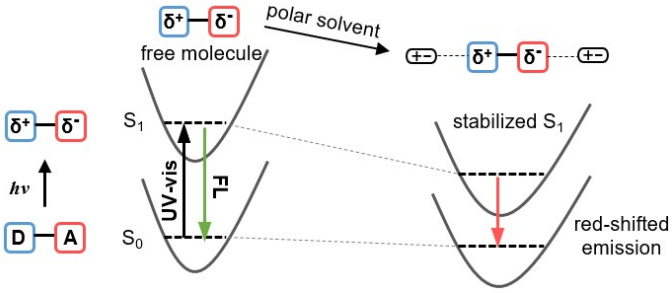


Figure S8. Intramolecular charge transfer (ICT) leads to fluorescence emission and bathochromic shift in polar solvent. Polar solvent molecules interact with the highly polarized fluorophore molecule at to stabilize the energy of excited state.

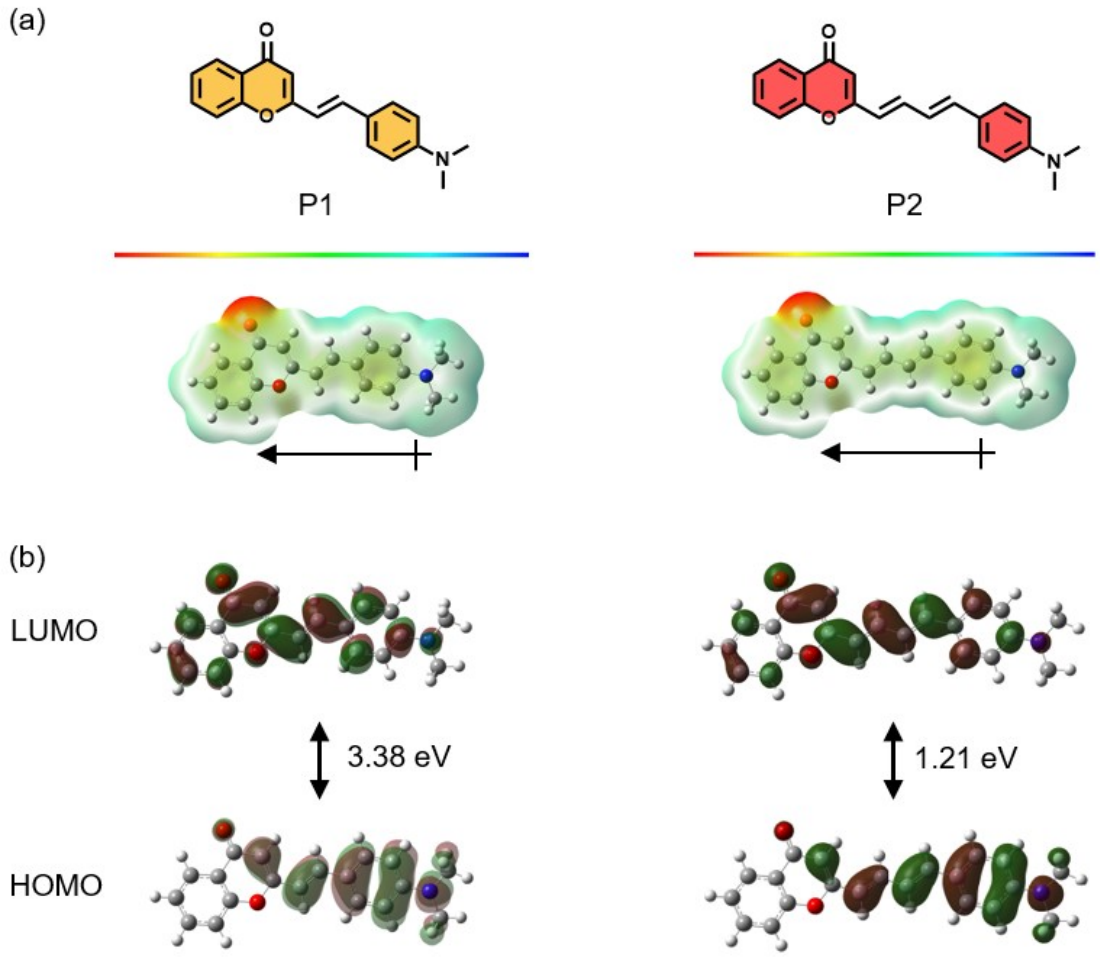


Figure S9. The charge separation of polar P1/P2 molecules allows the presence of intramolecular charge separation. (a) Electrostatic potential maps based on the electron density of P1 and P2, indicating the charge separation inside molecules. (b) DFT calculated energy gap between the HOMO and LUMO of P1/P2.

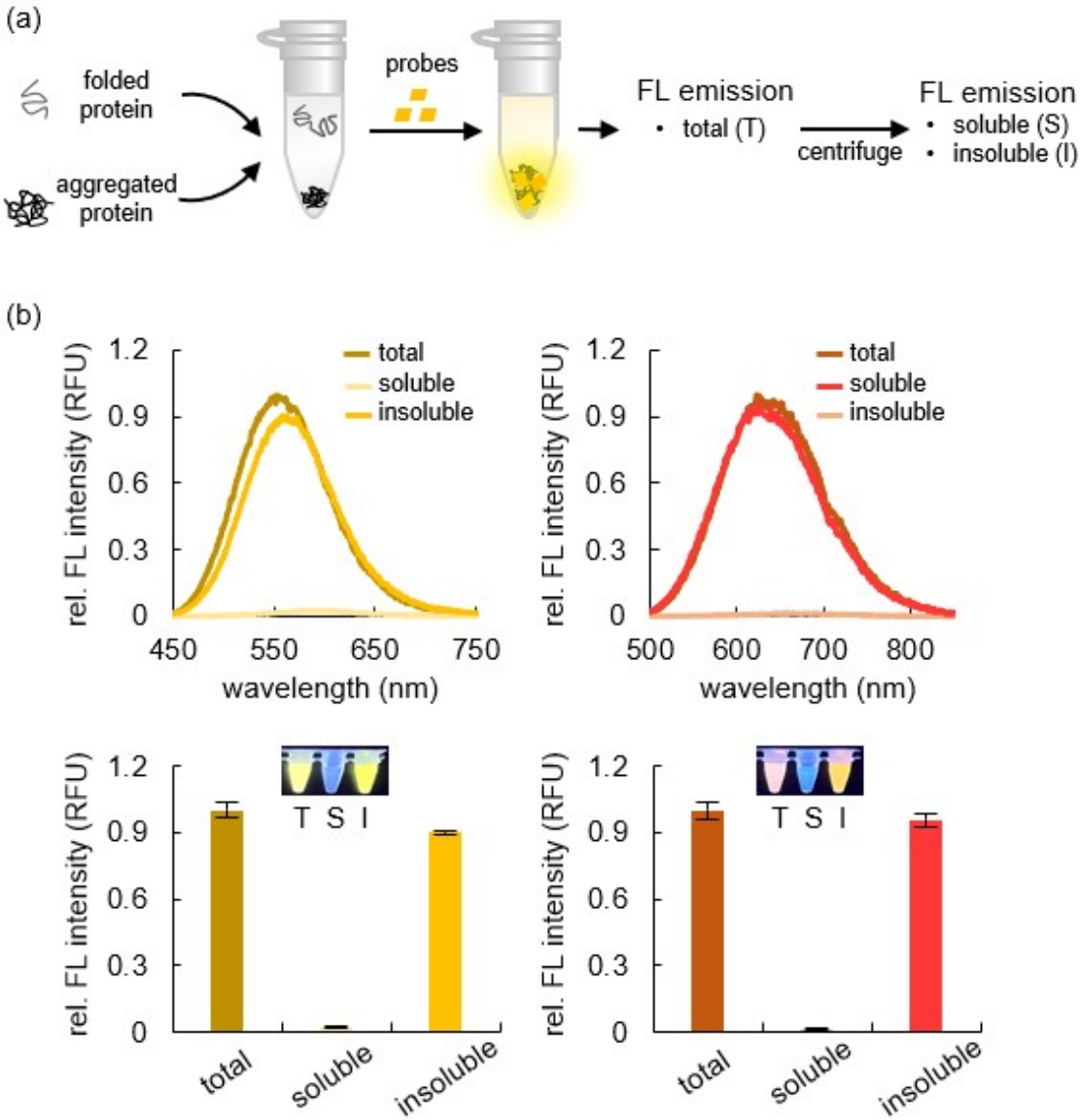


Figure S10. P1 and P2 have binding selectivity toward aggregated protein. (a) 60 μL folded DHFR protein (100 μM) mixed with 60 μL suspension of aggregated DHFR (100 μM, incubated in acidic aggregation buffer at 65 °C for 5 min), then added P1 or P2 (20 μM) to incubate at ambient temperature for another 5 min. The fluorescence emission of abovementioned mixture was measured as total fluorescence (**T**). The mixture was centrifuged (14,000 rmp) at 4 °C for 5 min. The supernatant containing folded protein was pipetted out, and the emission was measured as soluble fluorescence (**S**). The residual insoluble precipitate was re-suspended in 120 μL PBS buffer and the emission was measured as insoluble fluorescence (**I**). (b) The normalized fluorescence emission intensity of each fraction revealed P1 and P2 tended to retain in aggregated protein.

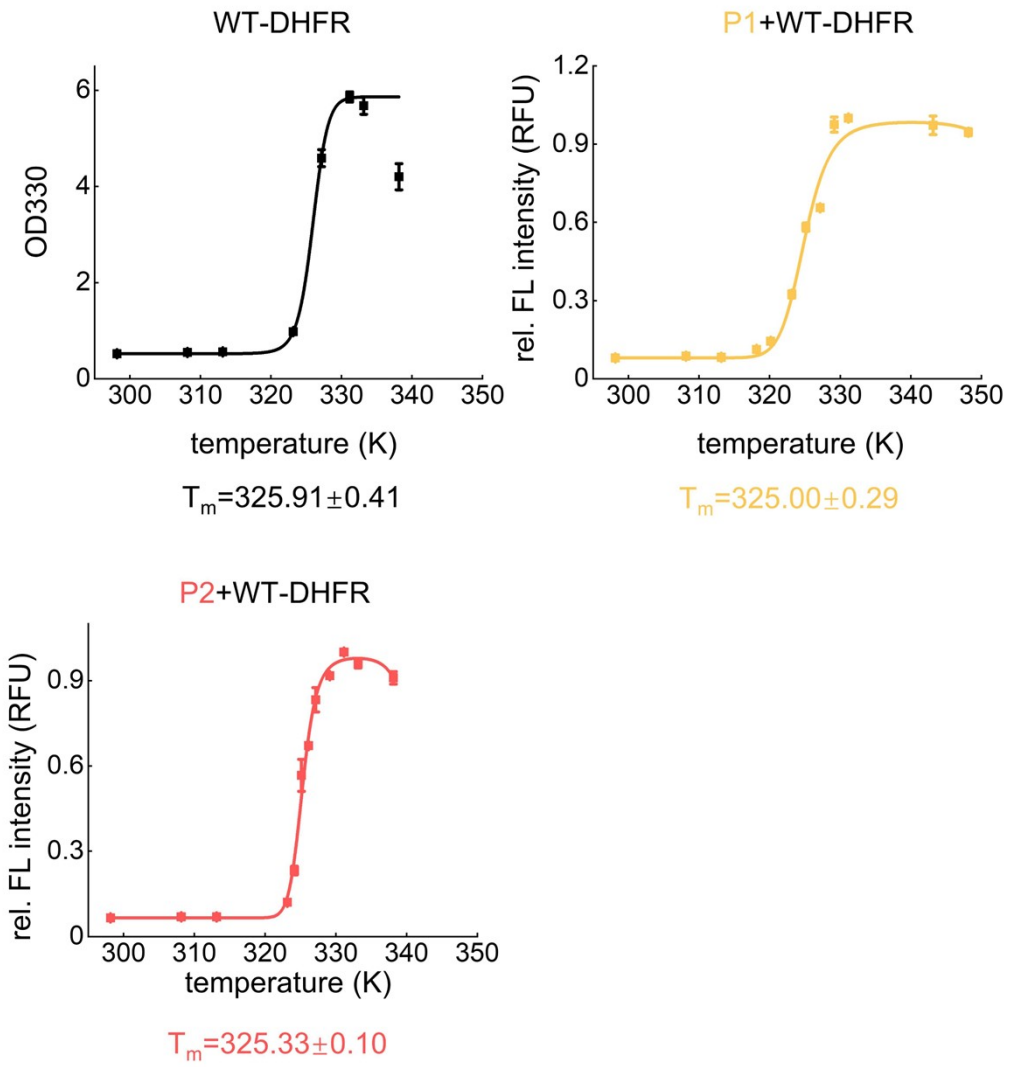


Figure S11. Thermal shift analysis emphasizes that the fluorescence of P1 and P2 occurs at lower temperature than the OD_{330} turbidity signal which measures the aggregation process of WT-DHFR. The experimental procedure followed **Experimental Methods 2.6**.

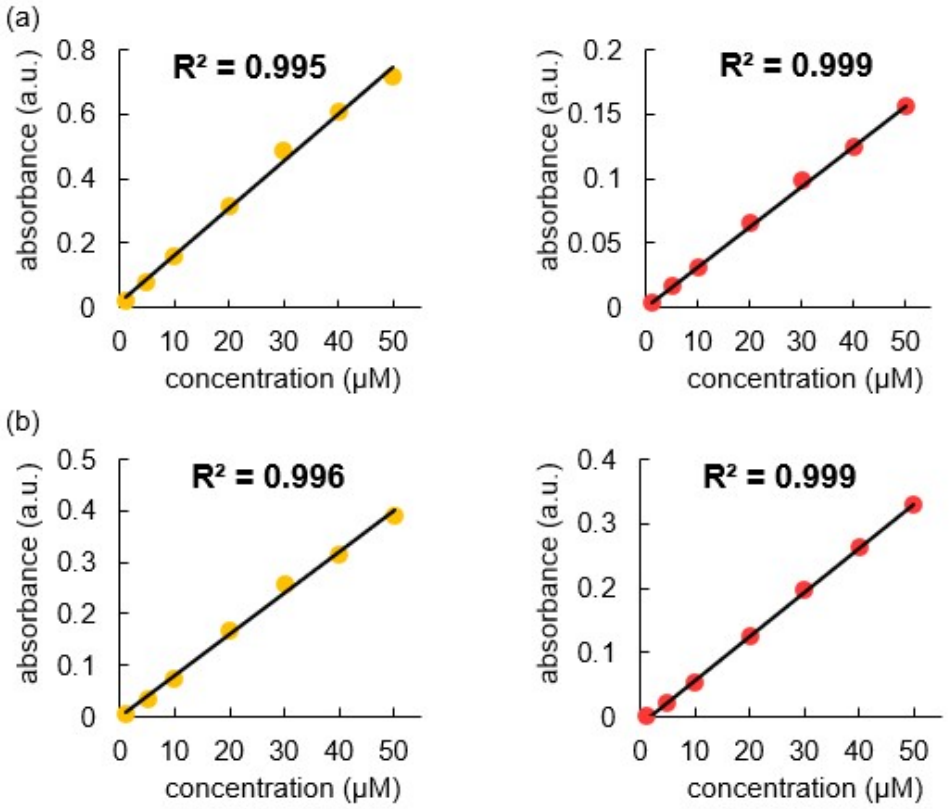


Figure S12. The Linear range of P1 and P2 in PBS buffer. The P1 and P2 was diluted from 1mM stock solution to prepare different concentrations in PBS buffer. Both P1 and P2 exhibited same linear range up to 50 μM in aqueous condition.

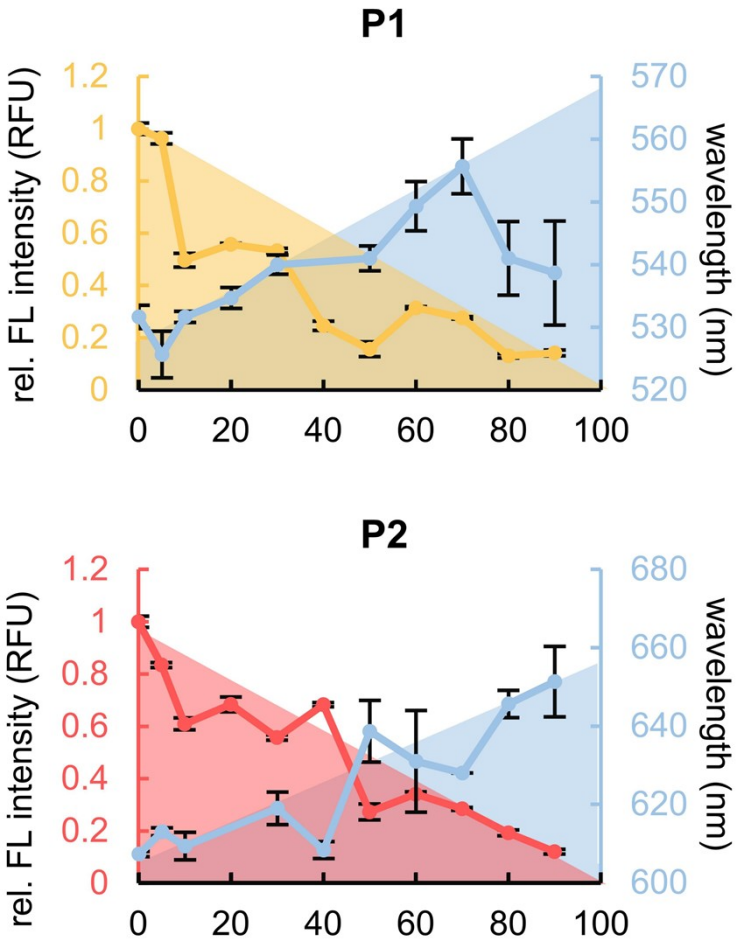


Figure S13. Proteolytic resistance of aggregated WT-DHFR measured by P1 or P2. To measure the fluorescence decrease kinetics profile upon proteolysis, 6 μL proteinase K (50 μg·mL^{-1}) was introduced to 120 μL aggregated protein suspension (50 μM WT-DHFR, 5 μM P1 or P2, incubated at 65 °C). The reaction mixture was incubated at room temperature for indicated durations. The suspension was centrifuged, and the supernatant was discarded, then added equal volume of aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) to finely mixed. The experiment followed the **Experimental Methods 2.7**.

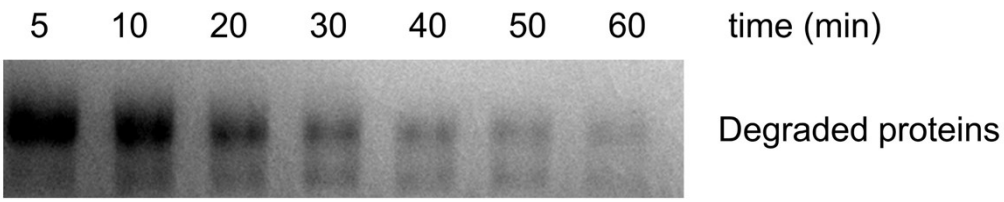


Figure S14. Proteolytic resistance of aggregated WT-DHFR measured by SDS-PAGE gel. The WT-DHFR (50 μM) was incubated in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) at 65 °C for 5 min to form aggregates. Proteinase K (2 μg·mL-1) was introduced to aggregated WT-DHFR for enzymatic hydrolysis. At 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, the digested samples were visualized by Coomassie bright blue stained SDS-PAGE gel. The experiment followed the **Experimental Methods 2.7**.

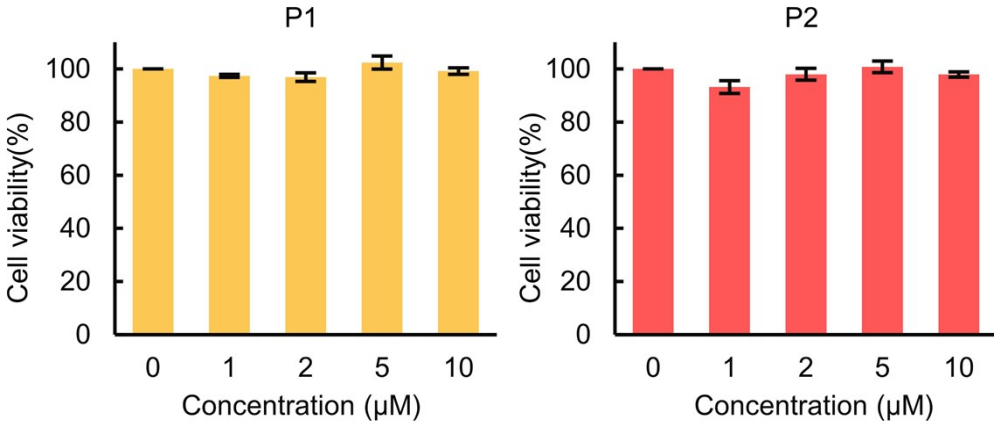


Figure S15. P1 and P2 exhibit negligible cytotoxicity in HepG2 cells up to 10 μM concentration. The experiment was conducted in accordance with **Experimental Methods section 2.9**.

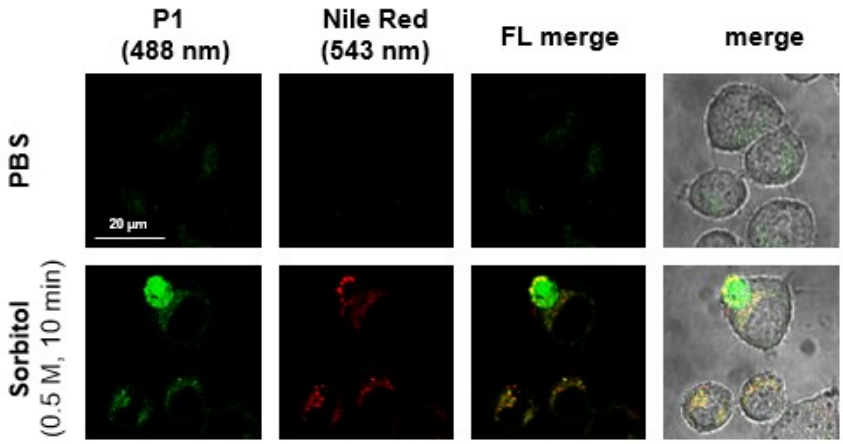


Figure S16. Confocal images of HepG2 cells treated with PBS buffer and sorbitol (0.5 M, 10 min) stained by Nile red (2 μM) and P1 (5 μM). Fluorescence colocalization analysis of fluorescent region, and Pearson correlation coefficients is 0.22, indicating no significant correlation between P1 and Nile red signal. Green: P1. Red: Nile red. Scale bar: 20 μm. Fluorescence of P1 was visualized using Ar laser (488 nm). Fluorescence of Nile red was visualized using green HeNe laser (543 nm).

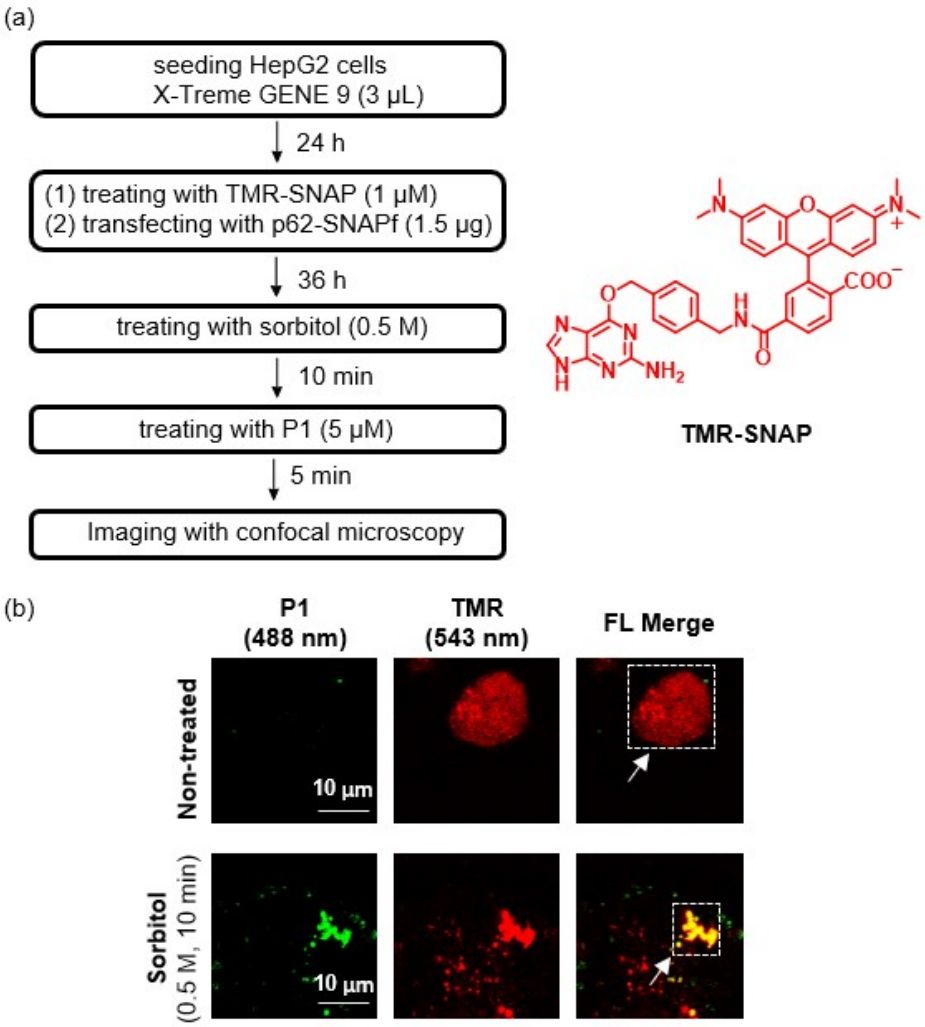
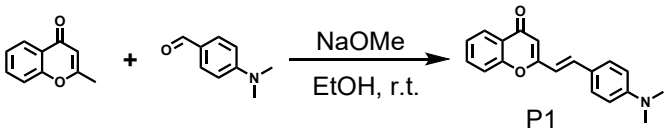


Figure S17. p62 formed aggregates under stress induced by osmotic stressor sorbitol. (a) Workflow of experimental procedures. The HepG2 cell was seeded in culture dish until 60 % confluency. The cell was replaced with RPMI 1640 medium bearing 1 μM TMR-SNAP probe. OPTI-MEM (50 μL) and X-tremeGENE 9 (3 μL) were finely mixed in dark, and then added p62-SNAPf (1 μg) as transfection reagent. To the cell was added 25 μL pre-mixed transfection reagent. The cell was incubated for another 36 h and replaced with RPMI 1640 containing 0.5 M sorbitol, and then added P1 (μM) for 10 min incubation. The fluorescence imaging was carried out by fluorescence confocal microscopy after replaced with fresh RPMI 1640 medium. (b) Confocal images of HepG2 cells expressing p62-SNAPf in the presence of TMR-SNAP (1.0 μM) with (bottom panel) or without (top panel) sorbitol (0.5 M). Co-localization indicated that P1 stained aggregated protein under sorbitol stressing (Pearson correlation coefficients was 0.71). Fluorescence of P1 was visualized using Ar laser (488 nm). Fluorescence of Nile red was visualized using green HeNe laser (543 nm).

2. Experimental Methods

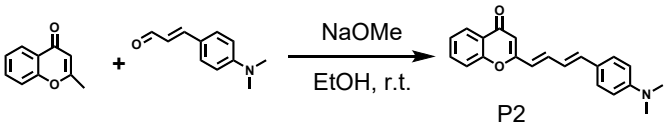
2.1 Synthetic Procedures

All synthesis followed literature reported methods [1].



P1

P1: (E)-2-(4-(dimethylamino) styryl)-4H-chromen-4-one (P1): In a heavy wall pressure vessel, 2-methyl-4-chromone (480 mg, 3.0 mmol) and 4-dimethylamino-benzaldehyde (675 mg, 4.5 mmol) were dissolved in 5 mL anhydrous ethanol, followed by adding sodium methoxide (NaOMe) (405 mg, 7.5 mmol), and the tube was sealed with a Teflon screw cap equipped with a Viton O-ring. The mixture was stirred at room temperature overnight, and turned dark-red. The reaction was quenched with water and then extracted with 50 mL ethyl acetate three times. The organic phase was combined and washed with saturated brine, and then dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure and the product was purified through flash silica gel chromatography (PE/EA = 4:1) to yield orange crystals. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.18 (dd, J = 8.0 Hz, 1.6 Hz, 1H), 7.66 (td, *J* = 16.0 Hz, 1.6 Hz, 1H), 7.47 - 7.57 (m, 4H), 7.37 (t, *J* = 8.0 Hz, 1H), 6.71 (d, *J* = 12.0 Hz, 2H), 6.56 (d, *J* = 16.0 Hz, 1H), 6.26 (s, 1H), 3.04 (s, 6H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$) δ 178.3, 163.0, 151.1, 137.5, 133.3, 129.4, 126.7, 124.7, 124.2, 122.9, 117.1, 114.8, 112.0, 108.9. 40.2 ppm. HRMS (m/z) Anal. Calc'd for C19H17NO2 $(M+H)^+$: 292.1332, Found $(M+H)^+$: 292.1326.



P2

P2: 2-((1E,3E)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4H-chromen-4-one (P2): In a heavy wall pressure vessel, 2-methyl-4-chromone (320 mg, 2.0 mmol) and 4-dimethylaminociamaldehyde (386 mg, 2.2 mmol) were dissolved in 5 mL anhydrous ethanol, followed by adding sodium ethoxide (NaOEt) (544 mg, 8.0 mmol), and the tube was sealed with a Teflon screw cap equipped with a Viton O-ring. The mixture was stirred at room temperature overnight, and turned dark-red. The reaction was quenched with water and then extracted with 50 mL ethyl acetate three times. The combined organic phase was washed with saturated brine, and then dried over anhydrous Na2SO4. Solvent was removed under reduced pressure and the product was purified through flash silica gel chromatography (PE/EA = 7:3) to yield dark-red crystals. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.17 (d, *J* = 8.0 Hz, 1H), 7.64 (t, *J* = 8.0 Hz, 1H), 7.34-7.48 (m, 5H), 6.64 -6.92 (m, 4H), 6.24 (dd, *J* = 20.0 Hz, 4 Hz, 2H), 3.01 (s, 6H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$) δ 178.3, 162.5, 156.0, 139.9, 138.6, 133.4, 128.6, 125.6, 124.8, 124.2, 120.7, 117.7, 112.4, 109.4, 51.6, 40.4 ppm. HRMS (m/z) Anal. Calc'd for $C_{21}H_{19}NO_2$ $(M+H)^+$: 318.1489, Found $(M+H)^+$: 318.1502.

2.2 Fluorescence spectra and solvatochromism measurement

For the measurement of solvatochromism, 6 μL P1 or P2 (1 mM DMSO) was diluted in 114 μL solvents to prepare 20 μM testing samples (5 % DMSO in each sample). 100 μL of each sample was pipetted into a BeyoGold96-Well Black Opaque plate to measure the fluorescence intensity. Fluorescence spectra were collected using a Tecan Spark Fluorescence Plate Reader. Each spectrum was normalized against its maximal fluorescence intensity.

In order to exclude the interference of DMSO, the stock solution of control set was prepared in 1,4-dioxane. 6 μL of P1 or P2 (1 mM 1,4-dioxane) was diluted in 114 μL 1,4-dioxane). 100 μL of each sample was pipetted into a BeyoGold 96-Well Black Opaque plate to measure the fluorescence intensity.

2.3 Viscosity dependence measurement and calculation

To minimize the impact of solvent polarity on the fluorescence emission intensity, glycerol/ethylene glycol mixture was chosen for the measurement of viscosity sensitivity due to their similar polarity (dielectric constants of glycerol = 46.5, dielectric constants of ethylene glycol = 37.0). Mixed solutions having different viscosity were prepared by changing the percentage of glycerol in ethylene glycol as 0%, 20%, 40%, 60%, 80%, and 100% (volume fraction). Viscosity of the mixtures was calculated according to the previously reported method.[1] The mixture viscosity (η_{mix}) can be calculated using Equation S1 (viscosity of glycerol =1500 mPa·S, viscosity of ethylene glycol =25.66 mPa·S).

$$ln\eta_{m_{ix}} = \Sigma_{i=1}^{n} w_i \cdot \ln \eta_{mix} \quad \text{(S1)}$$

All tested sample solutions were diluted from 1 mM DMSO stock solution to 20 μM. Fluorescence emission spectra were collected using a Tecan Spark Fluorescence Plate Reader in BeyoGold™ 96-Well Black Opaque plates. Excitation wavelength was 427 nm for P1, 447 nm for P2.

The viscosity-dependent curve was plotted using logarithm of solvent viscosity as x-axis and logarithm of emission intensity as y-axis, and the viscosity dependence parameter x was determined based on the Förster-Hoffmann equation (Equation S2).

$$\log I = x\log \eta + c \quad \text{(S2)}$$

in which *I* is the fluorescence intensity, *η* is viscosity, *x* is the viscosity sensitivity, *C* is a constant and *x* represents the sensitivity of the fluorescent probe to the viscosity. Error bar: standard error (n = 3).

2.4 Plasmids construction and protein purifications

E. coli wild type DHFR (WT-DHFR), mutant-DHFR (M42T: H114R) and sortase genes were codon optimized, synthesized, and sub-cloned into pET- 29b (+) vectors. To facilitate purification, all proteins were cloned with His-tag at the C-termini. Wild type TTR (WT-TTR) genes were codon optimized, synthesized by GenScript in Nanjing, China, and sub-cloned into pET-29b (+) vectors without His-tag to minimize amyloid formation interference. Human Immunoglobulin G (Human Ig) was purchased from HUALAN Biological Engineering, Inc directly.

BL21(DE3) *E. coli* cells were transformed with WT-DHFR, mut-DHFR (M42T: H114R), and sortase protein plasmids. Cells were grown to OD_{600} of 0.6-0.8 before being stimulated by IPTG (0.5 mM) at 37 °C for 4 h (WT-DHFR), 30 °C for 4 h (mut-DHFR (M42T: H114R)), 18 °C for 16 h (sortase). Cultured cells were resuspended in resuspension buffer (50 mM Tris•HCl, 100 mM NaCl, pH = 8.00). Cells expressing recombinant proteins were thawed and lysed by sonication at 4 °C. Lysed cells were centrifuged at 12,000 rpm for 30 min. The supernatant was collected and loaded onto a 10 mL Ni-NTA column, which was then washed with buffer (50 mM Tris•HCl, 100 mM NaCl, pH = 8.00). Proteins were then eluted by gradient increase of imidazole buffer (50 mM Tris·HCl, 100 mM NaCl, 500 mM imidazole, pH = 8.00). The protein fractions were identified by SDS-PAGE analysis, pooled, and concentrated. Proteins purified by Ni-NTA column were further purified using 120 mL Superdex 200 size-exclusion column in phosphate buffer (10 mM sodium phosphates, 100 mM KCl, 1 mM EDTA, acidified by HCl to pH = 7.40). Fractions containing target proteins were identified by SDS-PAGE gel analysis, pooled, and concentrated. Based on SDS-PAGE gel, the purity was estimated at > 95% with no significant impurities identified.

WT-TTR was expressed in *E. coli* BL21 DE3 cells and purified using a 4-step method including a salt-cut step and three chromatographic steps described in previous publication [3]. A pET29b (+) vector encoding the TTR-L55P sequence was transformed into BL21 DE3 cells and plated onto LB-agar plates with kanamycin. When cell growth monitored by OD_{600} reached 0.6-0.8, isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) was introduced into culture to induce the overexpression of WT-TTR, and after induction of 5 h, the cells were harvested by centrifugation (10 min at 13700 × g). WT-TTR was purified at room temperature. Fresh cell pellets were re-suspended in Buffer A (50 mM Tris, 150 mM NaCl, pH 7.5; 15 mL buffer/L of culture). Re-suspended cells were thawed and lysed by sonication at 4 °C. The sonication was carried out using 80% of the output power on a mode with 2 seconds on and 2 seconds off for 1 min. This process was repeated 6 times. Lysed cell suspension was centrifuged for 30 min at 16,000 × g and supernatant was collected for following step. To precipitate the majority of the cellular proteins, ammonium sulfate (242 $g \cdot L^{-1}$) was slowly added into the supernatant with rigorous stirring at 4 °C for 15 min. The solution was then centrifuged at 12,000 × g for 15 min at 4 °C. The pellet (mostly proteins other than WT-TTR) was discarded, and the supernatant containing WT-TTR was supplemented with additional ammonium sulfate to a final concentration of 365 $g \cdot L^{-1}$ with rigorous stirring at 4 °C for 15 min. The solution was centrifuged at 12,000 × g for another 15 min at 4 °C. The pellet was resuspended in 20−30 mL of anion exchange buffer A (25 mM Tris and 1 mM EDTA, pH 8.0) and dialyzed against 4 L of buffer A at 4 °C overnight. After dialysis, the protein solution was filtered through 0.45 μm film and purified by 50 mL Source 15Q anion exchange column equilibrated with buffer A. WT-TTR was eluted using a linear gradient of NaCl (160 mL; 50−350mM) followed by a NaCl wash (50 mL, 350 mM). Fractions containing WT-TTR was further purified using a 120 mL Superdex 75 gel filtration column in SEC buffer (10 mM sodium phosphate, 100 mM KCl, and 1 mM EDTA, pH = 7.6) to

remove contaminants and other soluble aggregates. The above steps together yielded WT-TTR in SEC buffer. Based on SDS-PAGE gel, the purity was estimated > 98% without significant impurities identified.

2.5 Fractionation experiment of aggregated DHFR

WT-DHFR (50 μM) and P1 or P2 (15 μM) were mixed in aggregation buffer (200 mM NaOAc, 100 mM KCl, acidified by AcOH to pH = 6.23) and heated at 65 °C for 5 min. 6 trials were prepared in parallel (120 μL). After proteins were fully aggregated, 100 μL of the total aggregated sample (T) in three sample was utilized for fluorescence reading and the rest was for electrophoresis. The other aggregated sample was centrifuged for 15 min at 4 °C to yield soluble supernatant (S) and insoluble precipitation (I) fractions. The insoluble fraction (I) was resuspended in an equal volume of aggregation buffer after the soluble fraction (S) was carefully decanted and saved for fluorescence measurement and electrophoresis analyses.

(1) After proteins fully aggregated, 100 μL of total aggregated sample (T), soluble fraction (S), and resuspended insoluble fraction (I) were pipetted into BeyoGold96-Well Black Opaque plates to collect fluorescence emission intensity by using 450 nm as excitation wavelength at a Tecan Spark Fluorescence Plate Reader. Error bars: standard error (n = 3); (2) In addition, fluorescence image of these fractions was taken under 365 nm UV light.

2.6 Thermal shift assay to quantitatively analyze protein thermodynamic stability.

Thermal shift assay by P1 or P2:

Freshly purified WT-DHFR protein (50 μM) and P1 or P2 (5 μM) were mixed in acidic aggregation buffer (200 mM NaOAc, 100 mM KCl, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 65 °C for 5 min. The incubated mixture (100 μL) was transferred into a BeyoGold96-Well Black Opaque plates to collect fluorescence emission intensity using Tecan Spark Fluorescence Plate Reader. Excitation wavelength was 427 nm for P1 and 447 nm for P2. Error bars: standard error (n = 3).

Thermal shift assay by OD_{330}:

Freshly purified WT-DHFR protein in acidic aggregation buffer (200 mM NaOAc, 100 mM KCl, acidified by AcOH to pH = 6.23) under different temperatures for 5 min. The incubated mixture was pipetted into quartz cuvette to measure optical density at 330 nm (OD_{330}) **3**.

The melting temperatures (T_m) were obtained through non-linear curve fitting of optical density readings at different incubation temperatures using the program OriginPro 2018:

$$F(T) = F(post) + \frac{[F(pre) - F(post)]}{1 + exp\{\frac{-\triangle H_u}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right) + \frac{\triangle C_{pu}}{R}\left[\ln\left(\frac{T}{T_m}\right) + \frac{T}{T_m} - 1\right]\}}$$

in which F(T) is the OD_{330} signal at temperature T; T_m is the melting temperature of the protein aggregation transition, F(pre) and F(post) are the initial and ending

transitional OD_{330} signal, respectively, R is the gas constant, ΔH_u is the enthalpy of protein aggregation, and ΔC_{pu} is the heat capacity change upon protein aggregation.

2.7 Proteolytic resistance.

Proteinase K was utilized in the proteolytic resistance experiment for aggregated WT-DHFR. Proteinase K (50 $\mu g \cdot mL^{-1}$) was introduced to 120 μL aggregated protein mixture, and 7 trials of mixture were prepared for time-dependent experiments. At 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min incubation time, 80 μL digested samples were mixed with 20 μL 5× loading buffer and incubated at 95 °C for 5 min. Samples were loaded onto an 18% acrylamide SDS-PAGE gel for electrophoresis analysis. To visualize the presence of degraded proteins, the gel was stained with Coomassie brilliant blue. BeyoTime Protein Marker served as the protein molecular weight ladder on SDS-PAGE gel.

To measure the fluorescence decrease kinetics profile upon proteolysis, proteinase K (50 μg·mL-1) was introduced to 120 μL aggregated protein solution (50 μM WT-DHFR, 5 μM P1 or P2). The reaction mixture was incubated at room temperature for indicated time. The suspension was centrifuged and the supernatant was aspirated, then equal volume of aggregation buffer (200 mM NaOAc, 100 mM KCl, acidified by AcOH to pH = 6.23) was added to resuspend. Fluorescent spectra were collected from 0 min to 90 min with 5-min intervals.

2.8 Aggregation conditions for different recombinant proteins

For WT-DHFR, mutant-DHFR (M42T:H114R), sortase, Lysate (E. coli) and human immunoglobulin:

In acidic aggregation buffer (200 mM NaOAc, 100 mM KCl, acidified by AcOH to pH = 6.23) freshly purified protein (50 μM) and P1 or P2 (15 μM) were mixed, and incubated different temperatures for 5 min (65 °C for WT-DHFR, Lysate (*E. coli*) and mutant-DHFR, 70 °C for sortase, 95 °C for human immunoglobulin). The incubated mixture (100 μL) was homogenized and pipetted into a BeyoGold™ 96-Well Black Opaque plates to collect fluorescence emission using Tecan Spark Fluorescence Plate Reader. Excitation wavelength was 427 nm for P1 and 447 nm for P2. Error bars: standard error (n = 3).

For WT-TTR:

In acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 4.4), freshly purified WT-TTR (3.6 μM) and P1 or P2 probe (15 μM) were mixed and incubated at 95 °C for 10 min. The incubated mixture (100 μL) was pipetted into BeyoGold™ 96-Well Black Opaque plates to collect fluorescence emission using Tecan Spark Fluorescence Plate Reader. Excitation wavelength was 427 nm for P1 and 447 nm for P2. Error bars: standard error (n = 3).

2.9 CCK8 assay for P1 and P2.

HepG2 cells (hepatocellular carcinoma) were grown in RPMI 1640 medium with 10 % FBS and 1 % antibiotics (penicillin-streptomycin) at 37 °C in a humidified environment of 5 % CO_2. Then, the cells were harvested and seeded in 96-well

plates (1 × 10^4 /well in 200 μL media) and incubated at 60 % confluency. Subsequently, the medium was replaced with the fresh medium containing different concentrations of P1 or P2. After further incubation for 24 h, the medium was replaced with 100 μL fresh RPMI 1640 media (containing 10% CCK-8) and then incubated for another 1 h in dark. Next, the absorbance of the products was measured at 450 nm by the Tecan Spark Fluorescence Plate Reader. The control set was identical as above-mentioned steps without addition of fluorescent sensors. The relative cell viability was calculated according to the following formula: Cell viability (%) = (OD_{sample} - $ODb_{ackground}$)/($OD_{control}$ - $OD_{background}$) × 100%.

2.10 Confocal imaging of aggregated proteome in stressed cells

The HepG2 cells were seeded at 70 % confluency 12 h prior to addition of fluorescence sensors in 20 mm NEST glass bottom cell culture dishes (polystyrene, non-pyrogenic, sterile). Cells were grown in RPMI-1640 media supplemented with 10 % FBS and 1 % Penicillin-Streptomycin antibiotics until they reached 70 % confluency.

Experimental set: 0.5 M sorbitol and P1 or P2 (5 μM) were introduced into the media and incubated for 10 min. Next, replaced with fresh medium with Hoechst 33342 and incubated for 30min.

Negative control: P1 or P2 (5 μM) was added into cells for 10 min incubation. Next, replaced with fresh medium and incubated for 30 min.

Fluorescence images were taken using Olympus FV1000 FluoView™ confocal microscope. Nuclear staining fluorescence was visualized using ultroviolet light laser (405 nm). The fluorescence of aggregated proteome in cellular milieu was visualized using green laser (488 nm).

2.11 Confocal λ-imaging and quantitative image analysis

Cell culture for λ-imaging followed the same as above-mentioned method. For P1 λ-deconvolution: Excitation wavelength was 488 nm, and emission spectra were collected in 490 nm – 600 nm with a 2 nm step-size and 5 nm band width. For P2 λ-deconvolution: Excitation wavelength was 488 nm, and emission spectra were collected in 500 nm – 650 nm with a 2 nm step-size and 5 nm band width. Images were collected using Olympus FV1000MPE.

2.12 Establishment of a Liver Injury Animal Model.

Female Balb/C mice, aged 6-8 weeks, were obtained from Liaoning Changsheng Biotechnology Co., Ltd. An overdose of acetaminophen (APAP, 500 $mg \cdot kg^{-1}$) was administered *via* intraperitoneal injection. Mouse were sacrificed at 4 or 12hours post-injection, followed by dissection of the liver and embedding in optimal cutting temperature (OCT) compound.

The authors declare that all animal studies were approved by the Institute Animal Care and Use Committee (IACUC) of Dalian Medical University (No. AAE21110) and carried out in accordance with established institutional guidelines and approved protocols.

2.13 Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assay.

The measurement of blood concentration of AST or ALT was performed using AST/GOT or ALT/GOT kit (Nanjing Jiancheng Bioeng. Inst.). The blood was collected from tail vein. The blood samples were placed into promoting coagulating tube, and then centrifuged (1,500 × g) for 15 min. The supernatant was separated as serum.

Control set: 0.5 mL AST or ALT reagent solutions were incubated at 37 °C for 30 min. 0.5 mL 2, 4-dinitrophenylhydrazine and 0.1 mL serum were added into testing sample, and then the obtained testing sample was incubated at 37 °C for 20 min. Next, 5.0 mL NaOH (0.4 M) was introduced and kept under ambient temperature for 5 min. The absorption at 505 nm (OD505) was measured as background.

Experimental set: 0.1 mL serum and 0.5 mL 37 °C pre-heated AST or ALT reagent solutions were mixed, and then incubated at 37 °C for 30 min. 0.5 mL 2, 4-dinitrophenylhydrazine was added into testing sample and incubated at 37 °C for another 20 min. Next, 5.0 mL NaOH (0.4 M) was introduced and kept under ambient temperature for 5 min. The absorption at 505 nm (OD_{505}) was measured and then deducted OD_{505} of control set as the optical density of the colour generated by deamination (ΔOD). AST/GOT and ALT/GOT values were calculated from standard curve using ΔOD values of experimental sets.

2.14 Histological analysis of liver tissue sections with different degrees of hepatic stress.

Freshly dissected liver tissues were embedded in optimal cutting temperature compound (OCT) compound and subsequently sectioned into 6 μm slices using an EICA CM1950 clinic cryostat. The prepared tissue sections were fixed in pre-cooled acetone at 4°C for 10 minutes and then stored at -20°C. When needed, tissue sections were sequentially thawed in a 4°C refrigerator and at room temperature for 30 minutes each. After thawing, frozen sections were washed three times in PBS buffer for 5 minutes. Tissue sections were removed from PBS, and tissue area was circumscribed with a PAP pen. Target areas were covered with either P1 or P2 solution (5.0 μM) and incubated in the dark for 1 hour, and then soaked with PBS buffer for 5 minutes. Sections were further stained with Hoechst 33342 (2.0 $\mu g \cdot mL^{-1}$) for 15 minutes then washed by PBS. Finally, stained tissue sections were mounted with an anti-fading mounting medium. Fluorescent images of tissue sections were acquired using an Olympus FV1000 FluoView™ confocal microscope. Nuclear staining fluorescence was observed with a violet laser (405 nm), aggregated proteome stained with the P1 or P2 probe in target areas of tissue was observed with a green laser (488 nm).

2.15 Confocal λ-imaging and quantitative image analysis

Preparation of tissue sections for λ-imaging followed the same operation as above-mentioned **2.14** method. For P1 λ- deconvolution: Excitation wavelength was 488 nm, emission spectra were collected in 490 nm – 600 nm with a 2 nm step-size

and 5 nm band width. For P2 λ- deconvolution: Excitation wavelength was 488 nm, emission spectra were collected in 500 nm – 650 nm with a 2 nm step-size and 5 nm band width. Images were collected using Olympus FV1000MPE.

3. Reference

[1] Wan, W.; Zeng, L; Jin, W.; Chen, X.; Shen, D.; Huang, Y.; Wang, M.; Bai, Y.; Lyu, H.; Dong, X.; Gao, Z.; Wang, L.; Liu, X.; Liu, Y.*; A Solvatochromic Fluorescent Probe Reveals Polarity Heterogeneity upon Protein Aggregation in Cells. *Angew. Chem. Int. Ed*. **2021**, *60*, 25865-25871.