Electronic Supplementary Material (ESI) for Analytical Methods.

Supporting Information

A rapid and highly selective paper-based device for high-throughput detection of cysteine with red fluorescent emission and large Stokes shift

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Materials and methods

Materials and instruments

All metal salts were derived from their nitrate or chloride salts. All reagents and chemicals were purchased from commercial suppliers and used without further purification, except when specified. ^{1}H NMR spectra were recorded at 300 and 75 MHz for ^{13}C NMR using solvent peak as internal reference at 25°C. Absorption and fluorescence spectra were recorded on an UV-vis spectrometer and on a spectro fluorometer at room temperature. The Fluorescence paper images were captured by an OmegaLum W Multicolor fluorescence, chemiluminescence, and visible light gel imaging system (Aplegen, America) and a Nikon Eclipse TE2000U inverted fluorescence microscope equipped with a cooled CCD camera (Nikon, Japan).

*Synthesis of **TCF***

A mixture of malononitrile (3.96 g, 60 mmol), 3-hydroxy-3-methyl-2-butonone (2.04 g, 20 mmol), sodium ethoxide (204 mg, 3.0 mmol), and ethanol (15 mL) was stirred at 80^{o}C for 2 h. This is cooled in a refrigerator and the solid filter, washed with cold EtOH, then dried and obtained target product as light yellow crystal (yield 71%). ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ = 2.36 (s, 3 H), 1.62 (s, 6 H).

*Synthesis of **TCFP***

Two drops of piperidine were added to a mixture of 4-hydroxybenzaldehyde (0.122 g, 1.0 mmol) and **TCF** (0.228 g, 1.15 mmol) in EtOH (8 mL). The reaction mixture was heated in the microwave for 15 min at 100^{o}C, which was then cooled to room temperature. The solid precipitate was filtered off to afford the compound as an orange solid (yield 65%). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ = 7.91 (d, *J* = 15 Hz, 1 H), 7.80 (d, *J* = 5 Hz, 1 H), 7.01 (d, *J* = 20 Hz, 2 H), 6.88 (d, *J* = 5 Hz, 2 H), 1.77 (s, 6 H).

*Synthesis of **TCFP-cys***

Acryloyl chloride (54 μL, 0.6 mmol) was added dropwise to a solution of **TCFP** (90 mg, 0.3 mmol) and Et_3N (210 μL, 1.5 mmol) in anhydrous CH_2Cl_2 (10 mL) at 0°C. Stirring was continued at this temperature for 30 min, and then the resulting mixture was further stirred overnight at room temperature. Water (10 mL) was added to the mixture, followed by extraction with CH_2Cl_2. The combined organic phase was dried by anhydrous Na_2SO_4. The solvent was removed by

evaporation, and the residue was purified by flash column chromatography to afford **TCFP-cys** as a solid (yield 43%). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ = 8.01 (d, *J* = 10 Hz, 2 H), 7.95 (d, *J* = 15 Hz, 1 H), 7.36 (d, *J* = 5 Hz, 2 H), 7.24 (d, *J* = 15 Hz, 1 H), 6.58 (d, *J* = 5 Hz, 1 H), 6.42 (m, 1 H), 6.20 (d, *J* = 10 Hz, 1 H), 1.80 (s, 6 H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ = 177.52, 175.51, 164.24, 153.47, 146.61, 134.61, 132.65, 131.31, 127.85, 123.15, 116.0, 113.09, 112.26, 111.24, 100.14, 99.92, 55.06, 25.53.

*Synthesis of **TCFN***

Two drops of piperidine were added to a mixture of 6-hydroxy-2-naphthaldehyde (189.2 mg，1.1 mmol) and **TCF** (199 mg, 1.0 mmol) in EtOH (8 mL). The reaction mixture was heated in the microwave for 15 min at 100^{o}C, which was then cooled to room temperature. The solid precipitate was filtered off to afford the compound as an orange solid (yield 58%). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ = 8.31 (s, 1 H), 8.10 (d, *J* = 16 Hz, 1 H), 7.96 (d, *J* = 8.5 Hz, 1 H), 7.91 (d, *J* = 8.5 Hz, 1 H), 7.79 (d, *J* = 8.5 Hz, 1 H), 7.25 (d, *J* = 16.0 Hz, 1 H), 7.17 (m, 2 H), 1.82 (s, 6 H).

*Synthesis of **TCFN-cys***

Acryloyl chloride (36 μL, 0.4 mmol) was added dropwise to a solution of **TCFN** (70 mg, 0.2 mmol) and Et_3N (210 μL, 1.5 mmol) in anhyrous CH_2Cl_2 (10 mL) at 0°C. Stirring was continued at this temperature for 30 min, and then the resulting mixture was further stirred overnight at room temperature. Water (10 mL) was added to the mixture, followed by extraction with CH_2Cl_2. The combined organic phase was dried by anhydrous Na_2SO_4. The solvent was removed by evaporation, and the residue was purified by flash column chromatography to afford **TCFP-cys** as a solid (yield, 51%). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ = 8.48 (s, 1 H), 8.10 (m, 3 H), 8.02 (d, *J* = 9.0 Hz, 1 H), 7.82 (d, *J* = 1.5 Hz, 1 H), 7.46 (m, 1 H), 7.37 (d, *J* = 16.5 Hz, 1 H), 6.62 (d, *J* = 27.0 Hz, 1 H), 6.49 (m, 1 H), 6.22 (d, *J* = 10.5 Hz, 1 H),1.83 (s, 6 H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ = 177.61, 175.47, 164.63, 150.29, 147.53, 135.52, 134.56, 132.60, 132.49, 131.31, 131.25, 129.10, 127.96, 125.08, 123.15, 119.36, 116.18, 113.15, 112.32, 111.42, 99.97, 99.80, 54.95, 25.53. HRMS (*m*/*z*): calcd $[M]^+$ for $C_{25}H_{17}N_3O_3$: 406.1270; found, 406.1167.

Optical studies

Stock solution of **TCFP-cys** and **TCFN-cys** (1 mM) was prepared in analytical grade DMSO. The stock solution of amino acids and metal ions was prepared in deionized water, and the concentration was 10 mM. The stock solutions were used freshly, a **TCFP-cys** or **TCFN-cys** (5

μM) solution in DMSO/H_2O (9:1, v/v) was prepared for the typical optical study. For the time-dependent fluorescence spectra, the probe solution was added with desired Cys. The fluorescence spectra were recorded immediately with the excitation of 480 nm.

Fabrication of paper-based device

An array device with 8 rows × 12 columns working zones was constructed in Whatman chromatography paper 3#. This array was comprised of one layer with the detection area. The paper's hydrophobization detection area was designed by the wax. This wax patterned paper contains 96 working zones (1.2 mm in diameter) independently. The patterns of hydrophobic barriers on a white background were designed with probe for detect copper. The wax patterns were printed on paper sheet in bulk using the wax printer (FUJI XEROX Phaser 8580 DN, America). The wax-printed paper sheet was baked at 150^{o}C for 5 min, and the wax melted and penetrated through the thickness of the porous-structured paper to form the hydrophobic patterns. The patterned paper sheet was ready for use after removing the paper sheet from the hot plate and allowing it to cool to room temperature.

Preparation of test paper and analytical application.

To demonstrate the feasibility method for *in vitro* detection, our work chose the HepG2 cells lysis buffer, urine (mouse) and bovine serum for quantifying the Cys content. After dealing with these samples, there were diluted in incubation buffer solution. And then the fluorescent probe (2 μL, respectively) was applied to each paper zone and incubated at room temperature. After that, a series of different doses of samples were dropped into the center zone until dry off. Finally, the fluorescence was measured under excitation at 460 nm.

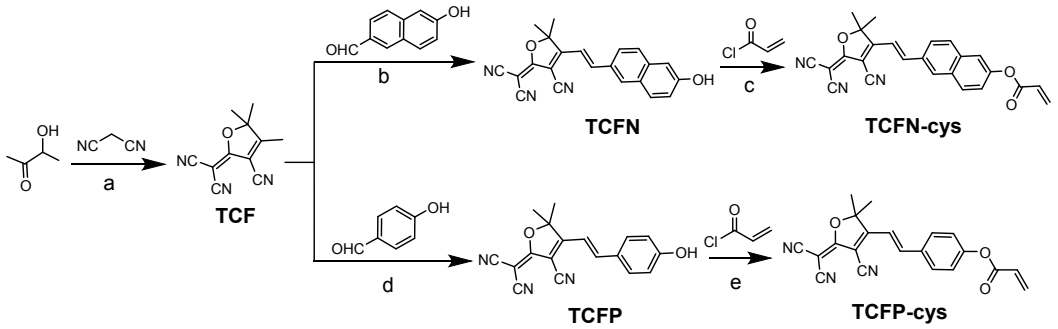
Cell culture and cytotoxicity activity assay

HepG2 cells were purchased from Shanghai Sangon, which were cultured in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% FBS, 100.0 mg/L streptomycin, and 100 IU/mL penicillin. HepG2 cells were seeded in glass-bottom dishes (Mattek) and grown to 70-80% confluency. The cytotoxicity activities of the probes were determined using an MTT colorimetric cell proliferation kit (Roche), following the manufacturer guidelines. Briefly, different cells were grown to 20-30% confluency (they would reach 80-90% confluency within 48-72 h in the absence of compounds) in 96-well plates under the conditions described above. The medium was aspirated, washed with PBS, and then treated, in duplicate, with 0.1 mL of the medium containing different

concentrations of **TCFN-cys**. Probe was applied from DMSO stocks, whereby DMSO never exceeded 1% in the final solution. The same volume of DMSO was used as a negative control, while the same volume of staurosporine (STS, 200 nM) was used as a positive control. After a total treatment time of 24 h, proliferation was assayed using the MTT colorimetric cell proliferation kit (Roche), following manufacturer guidelines.

Confocal microscopy imaging

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) including 10% FBS (Fetal Bovine Serum), 100 mg/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2, water saturated incubator at 37°C. Cells were plated in 96-well plates and incubated with probe **TCFN-cys** concentration of 20 μM for 24 h at 37°C. Before observing the cells with the help of the confocal laser scanning microscopy, the cells were washed with PBS buffer solution three times (collected at 480-720 nm upon the excitation at 460 nm).



Scheme S1. The synthesis route of probes. Reagents and conditions. a) NaOEt, EtOH, 80°C, 2 h, 71%; b) piperidine, EtOH, microwave, 100°C, 15 min, 58%; c) CH_2Cl_2, TEA, overnight, room temperature, 51%; d) piperidine, EtOH, microwave, 100°C, 15 min, 65%; e) CH_2Cl_2, TEA, overnight, room temperature, 43%.

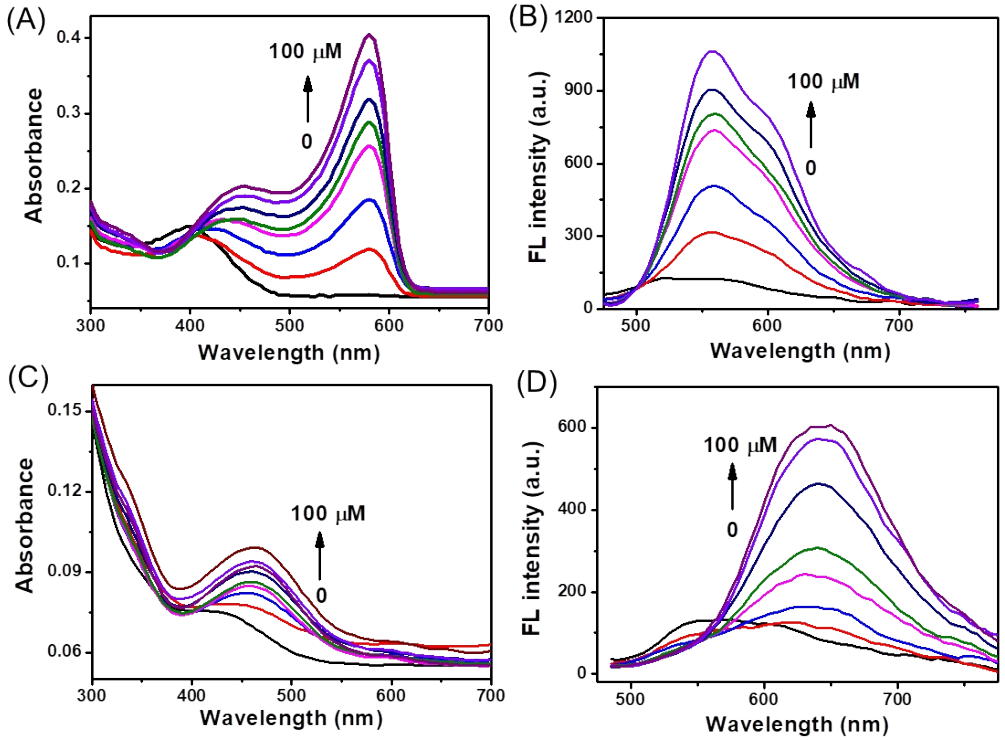


Fig. S1. (A) UV-Vis spectra and (B) fluorescence spectra of **TCFP-cys** (5 μM) incubated with different concentrations of Cys for 10 min in PBS buffer (10 mM, pH 7.4, 10% DMSO); (C) UV-Vis spectra and (D) fluorescence spectra of **TCFN-cys** (5 μM) incubated with different concentrations of Cys for 10 min in PBS buffer (10 mM, pH 7.4, 10% DMSO).

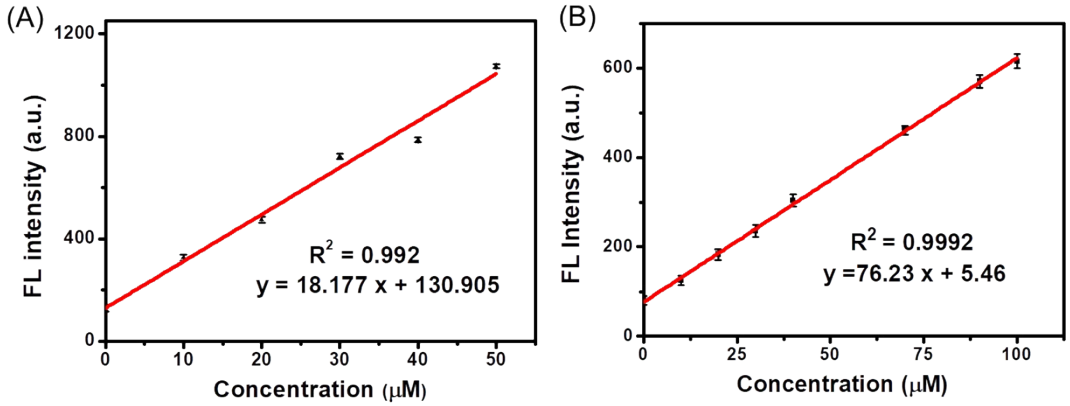


Fig. S2. (A) Fluorescence intensity of **TCFP-cys** and **TCFN-cys** (5 μM) incubated with different concentration of Cys for 5 min in PBS buffer (10 mM, pH 7.4, 10% DMSO).

The method for determining the limit of detection (LOD)

First the calibration curve was obtained from the plot of fluorescence intensity at 650 nm, as a function of the Cys concentration. The regression curve equation was then obtained for the lower concentration part.

The detection limit = 3 × S.D. / k

Where k is the slope of the curve equation, and S.D. represents the standard deviation for the probe solution's fluorescence intensity in the absence of Cys.[1]

For determining the LOD of the **TCFN-cys**

I_{650} = 5.46 + 76.23[CYS] (R^2 = 0.9992)

LOD = 3 × 6.60 / 76.23 = 0.26 μM

For determining the LOD of the **TCFP-cys**

I_{550} = 130.905 + 18.177[CYS] (R^2 = 0.992)

LOD = 3 × 30.49 / 18.177 = 11.20 μM

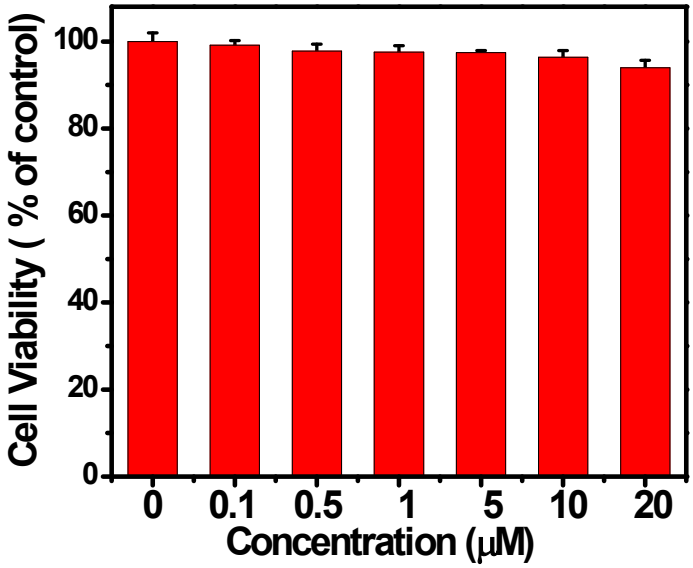


Fig. S3. MTT assay of HepG2 cells treated with **TCFN-cys** at different concentrations for 24 h.

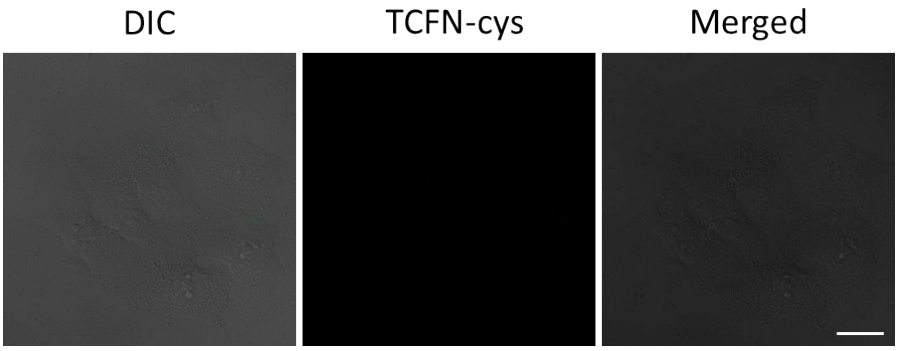


Fig. S4. Confocal fluorescence images of HepG2 cells without probe treatment.

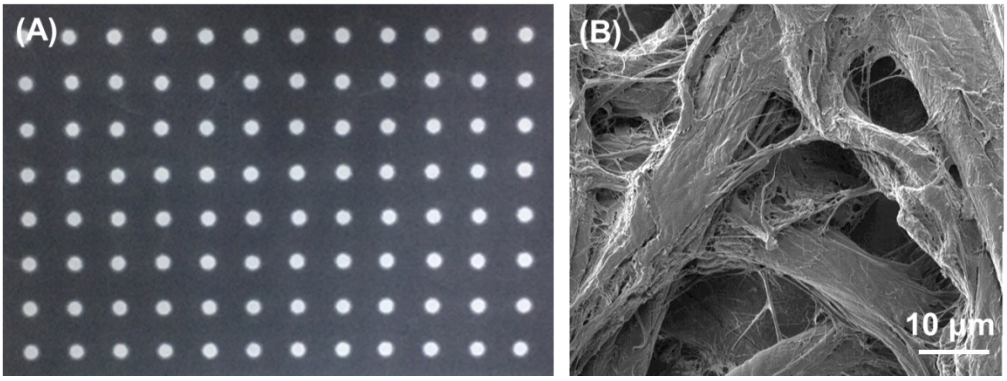


Fig. S5. (A) The photographs of 96-well paper-based device. The diameter of the microzone is 1.2 mm; (B) SEM images of paper-based device. Scale bar = 10 μm.

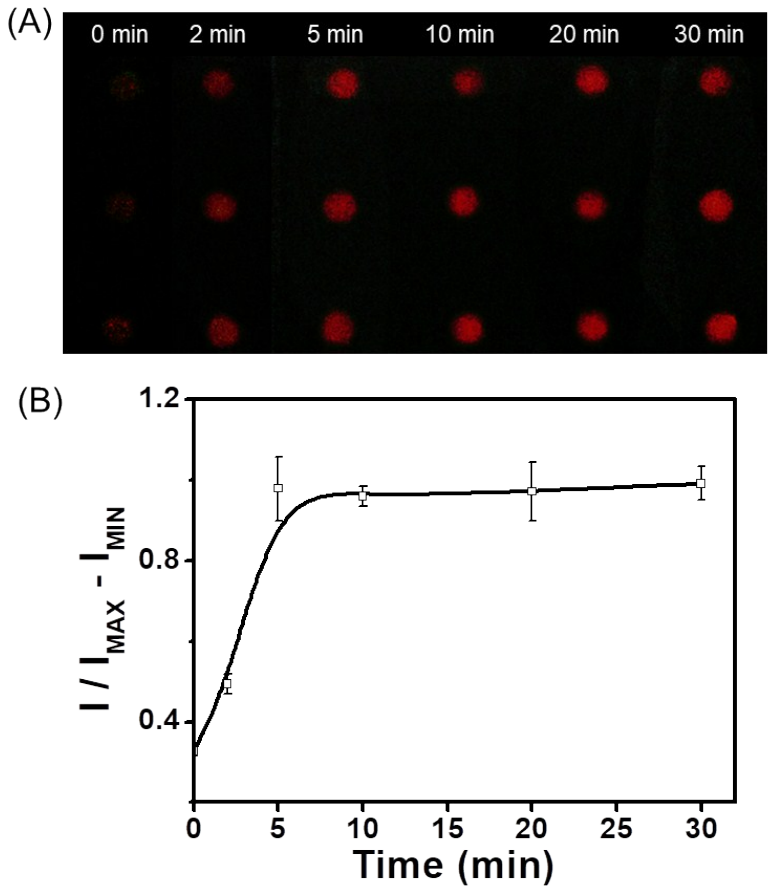


Fig. S6. The fluorescence images (A) and relative fluorescence intensity (B) of the **TCFN-cys** incubated with Cys in the paper-based device for different time. λ_{ex} = 460 nm.

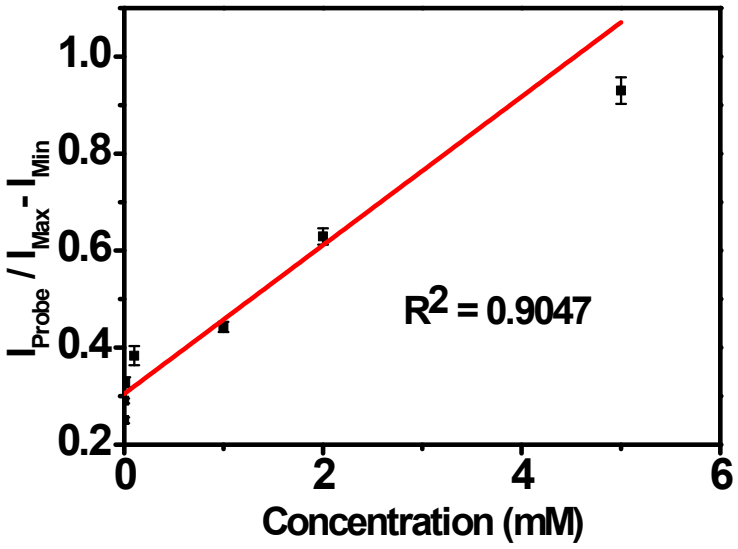


Fig. S7. The linear relationship between the fluorescence intensity and Cys concentration (0-5 mM) on the paper-based device.

The method for determining the limit of detection (LOD)

The LOD of the paper-based device was calculated according to the probe **TCFN-cys** in the solution.

The detection limit = 3 × S.D. / k

Where k is the slope of the curve equation, and S.D. represents the standard deviation for the paper-based device's fluorescence intensity in the absence of Cys.

For determining the LOD of the paper-based device

$I_{probe} / I_{max} - I_{min} = 0.6221 + 0.00369[CYS]$ ($R^2 = 0.9047$)

LOD = 3 × 0.015 / 0.00369 = 12.4 nM

Table S1. The determination of Cys with the paper-based device in real samples.

Samples	Detected (μM)	Added (μM)	Founded (μM)	Recovery
HepG2 (2%)	10	20	19.46±1.96	103%
		30	28.63±1.09	110%
		40	41.25±2.98	112%
serum	2.5	20	19.57±2.85	112%
		30	30.63±1.34	107%
		40	39.87±1.79	104%
urine	1.36	20	20.32±2.13	123%
		30	29.54±1.96	105%
		40	40.95±1.48	106%

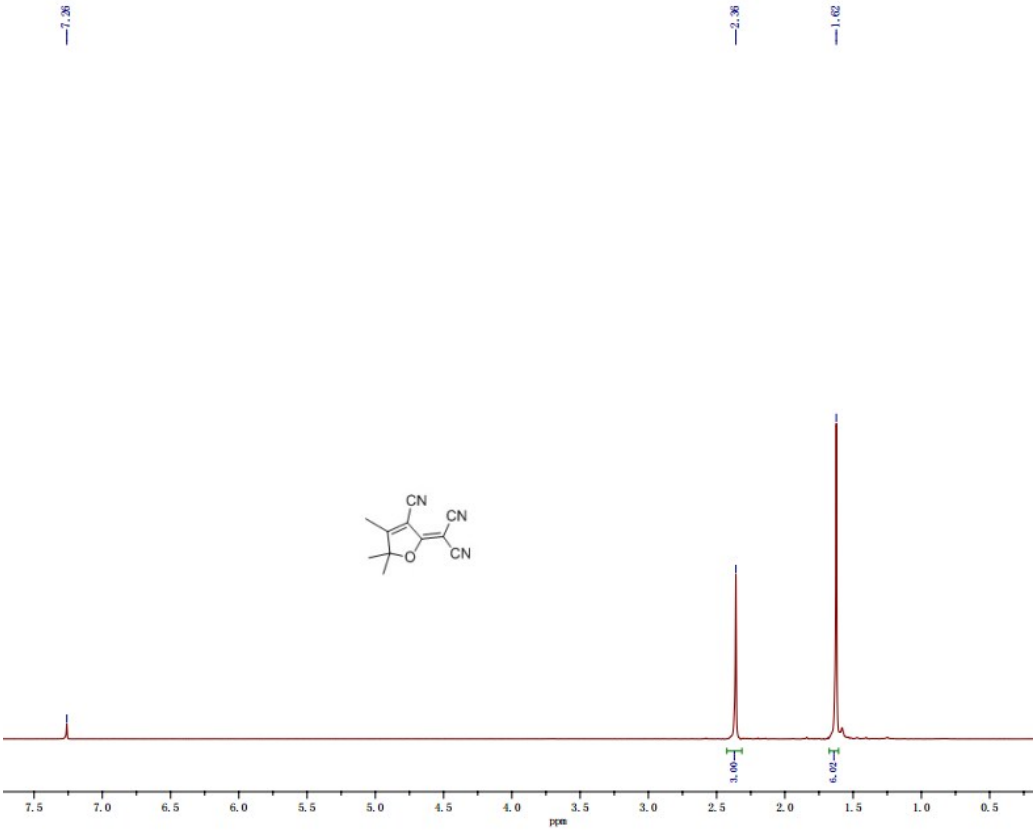


Fig. S8. [1]H MNR spectrum of **TCF**

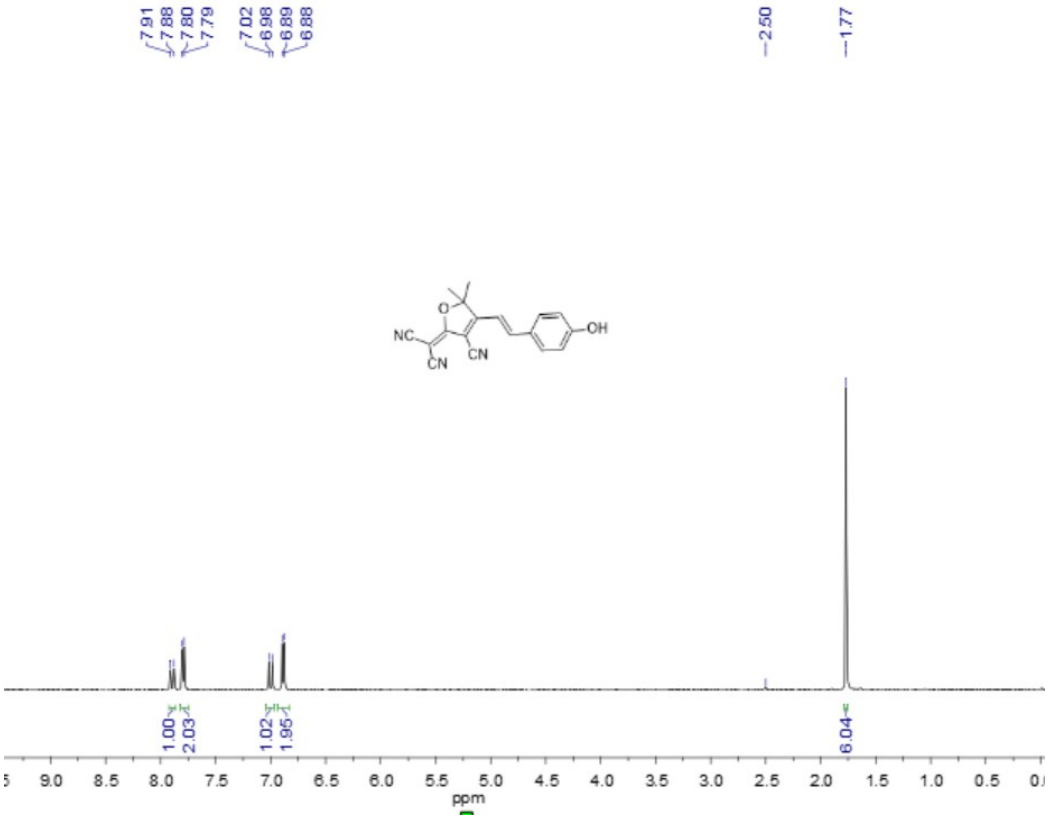


Fig. S9. [1]H MNR spectrum of **TCFP**

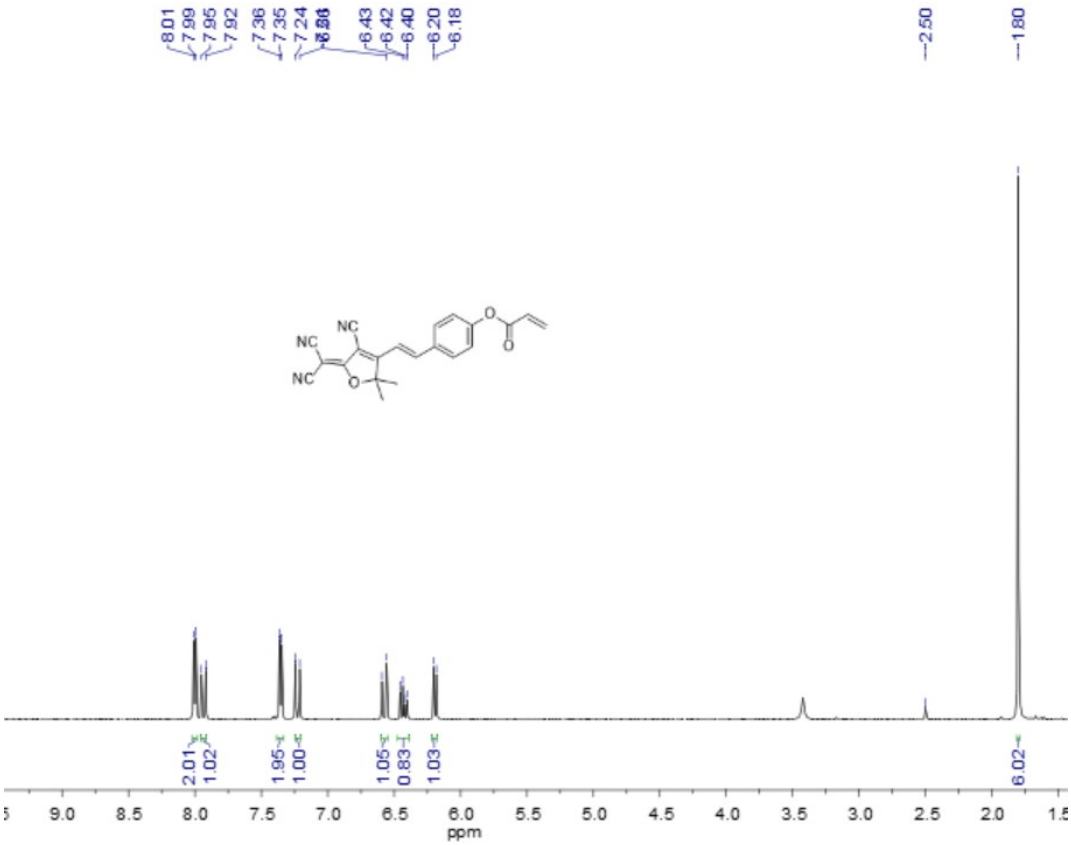


Fig. S10. ^{1}H MNR spectrum of **TCFP-cys**

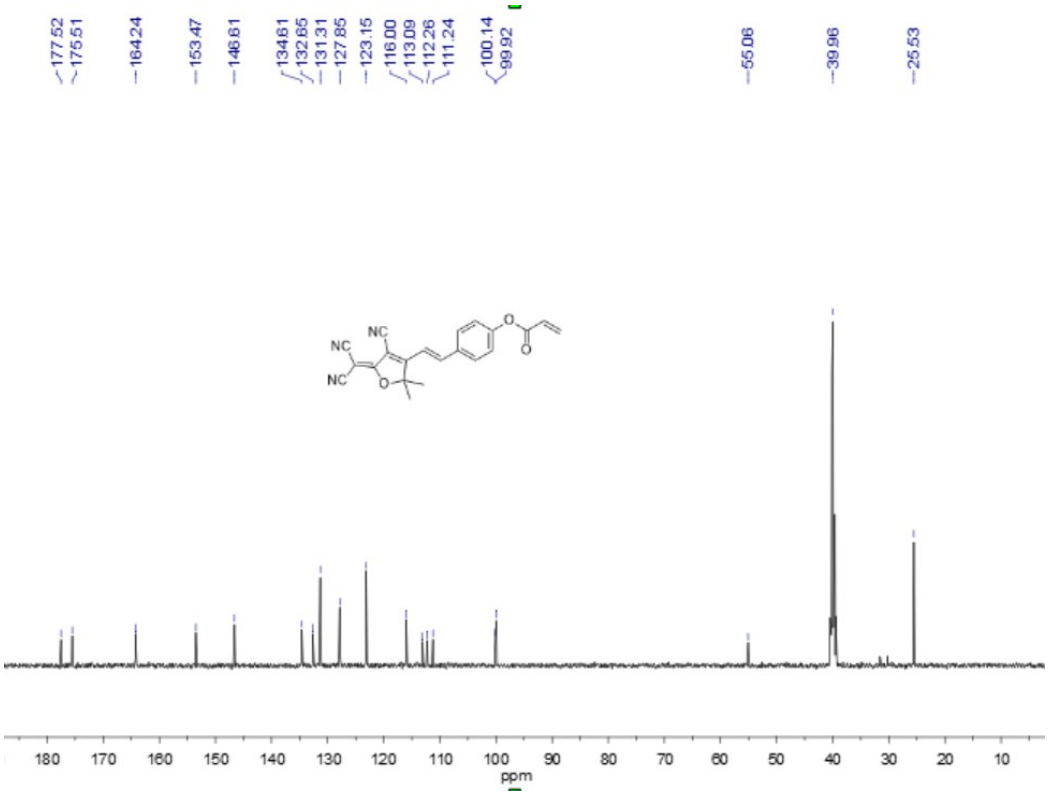


Fig. S11. ^{13}C MNR spectrum of **TCFP-cys**

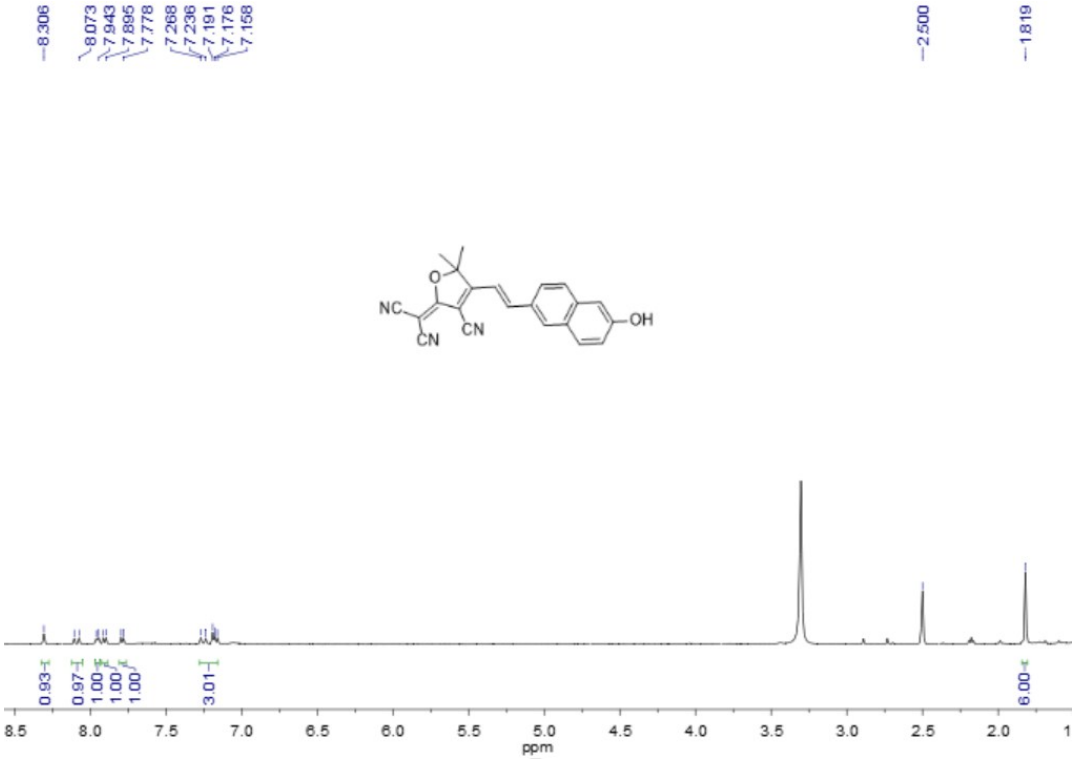


Fig. S12. [1]H MNR spectrum of **TCFN**

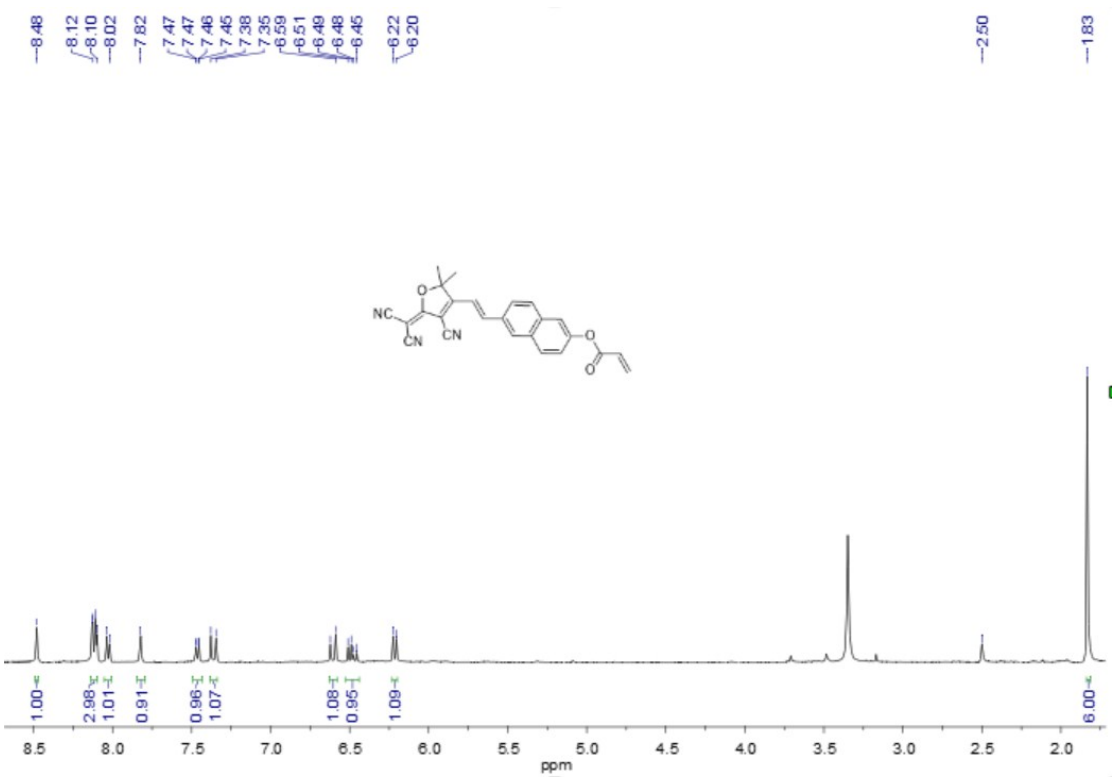


Fig. S13. [1]H MNR spectrum of **TCFN-cys**

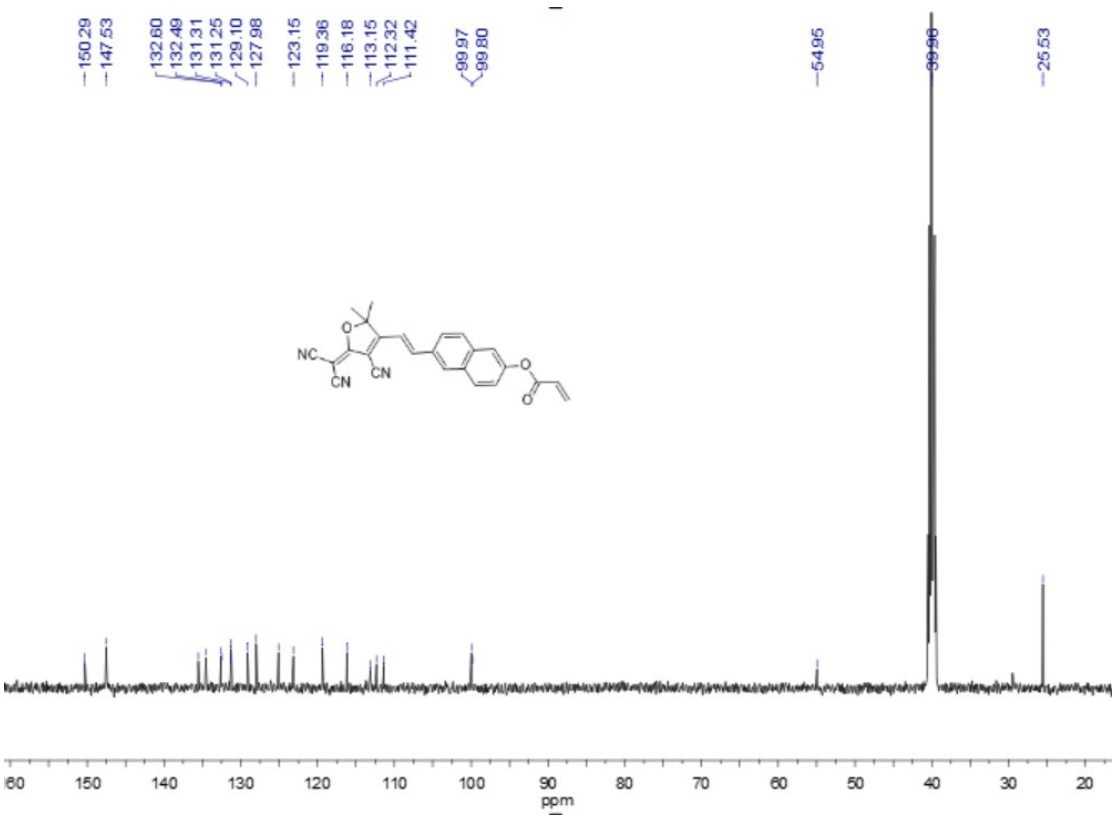


Fig. S14. ^{13}C MNR spectrum of **TCFN-cys**

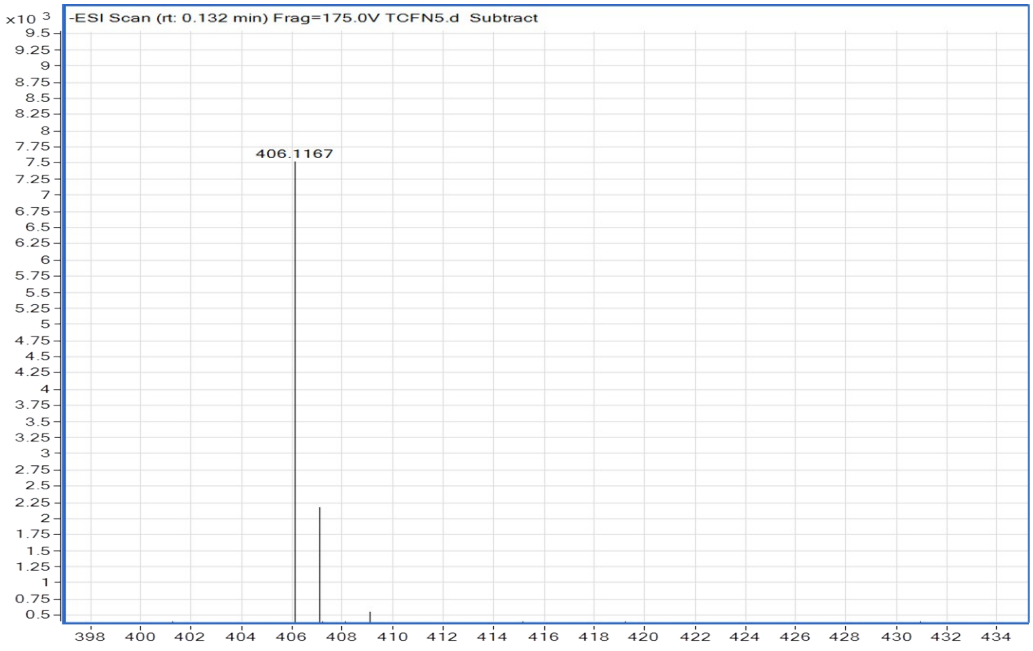


Fig. S15. HR Mass spectrum of **TCFN-cys**

[1] V. Thomsen, D. Schatzlein, and D. Mercuro, *Spectroscopy* 2003, 18, 112-114.