Supporting Information for

A long-lived ruthenium(II) complex-based time-gated luminescent probe for background-free detection of hydrazine hydrate

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Contents

1. General information

1.1 Materials and physical measurements

All chemicals were used without any purification. Compound 1 and compound 4 were synthesized according to the literature method.[1, 2] Compound 2, H_2SO_4, CH_2Cl_2, CH_3OH, CH_3CH_2OH, CH_3CN, KNO_3, NH_4PF_6, hydrazine hydrate, were purchased from Chemical Reagents Company. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin sulfate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Life Technologies. The PBS buffer at pH 7.4 consisting of 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 was prepared in our laboratory. Deionized distilled water was used throughout.

^1H NMR and ^{13}C NMR spectra (600 MHz for ^1H NMR; 150 MHz for ^{13}C NMR) were determined on a nuclear magnetic resonance spectrometer from Japan Electronics Co., Ltd. Elemental analysis was performed on a Vario-EL analyser. High-resolution mass spectrometry (HRMS) was performed on an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray ionization source. Absorption spectra were recorded on a Perkin-Elmer Lambda 35 UV-vis spectrometer. Luminescence spectra, luminescence lifetimes, and time-gated luminescence spectra were measured on an Edinburgh FS5 spectrometer. All the spectra were measured in 10 mM PBS buffer (pH 7.4, 10% DMSO).

The luminescence imaging experiments of cells were performed on Leica confocal microscope. Under the confocal microscope, the Ru(II) complex was excited at 488 nm, and the emission in 570-660 nm was collected. The luminescence intensities and colocalization of images were analyzed by using Image J software version 1.44p.

1.2 TGL response of Ru-COU to hydrazine hydrate

TGL measurements for **Ru-COU** (10 μM) in response to hydrazine hydrate were conducted in PBS buffer (10 mM, pH 7.4, 10% DMSO) at room temperature (approximately 25 °C). After the probe was incubated with different concentrations of

hydrazine hydrate at room temperature for 30 minutes, the TGL signal was recorded with a delay time of 20 ns. λ_{ex} = 450 nm, λ_{em} = 625 nm.

1.3 Determination of the detection limit

The detection limit was calculated based on time-gated luminescence titration. The time-gated luminescence spectra of free **Ru-COU** were measured by five times and its standard deviation was obtained. To gain the slope, the time-gated luminescence intensities at 625 nm was plotted as the increasing concentrations of the corresponding hydrazine hydrate. So, the detection limit was calculated with the following equation (1):

$$\text{Detection limit} = 3\sigma/k \quad \text{(1)}$$

Where σ is the standard deviation of blank measurement, k is the slope between the luminescence intensities versus the concentrations of hydrazine hydrate.

1.4 TGL detection of hydrazine hydrate in drug, food and water samples

The drug, food, and water samples were diluted with PBS buffer (10 mM, pH 7.4). Subsequently, **Ru-COU** was added to the solution to a final concentration of 10 μM. After a 30-minute reaction at room temperature, the TGL intensity at 625 nm was measured with a delay time of 20 ns. λ_{ex} = 450 nm.

1.5 Cytotoxicity

The cytotoxicity of **Ru-COU** to 4T1 cells was examined by MTT assay method.[3,4] 4T1 cells were seeded at a density of 5×10^4 cells/mL in a 96-well micro-assay culture plate. After growth at 37 °C in a 5% CO_2 incubator for 24 h, the cells were cultured with the freshly prepared medium containing different concentrations of **Ru-COU**. The group with the addition of culture medium only was employed as the control, and the wells containing culture media without cells were used as blanks. After incubation for 24 h in dark, cell culture medium was removed, then 100 μL PBS solution of MTT (0.5 mg/mL, pH 7.4) was added into each well for further incubation for 4 h. Then, the excess MTT solution was carefully removed, and the formed formazan was dissolved in 100 μL dimethyl sulfoxide (DMSO). The absorbance at 540 nm was measured in an

Infinite M200 Pro Microplate Reader. The results from the five individual experiments were averaged.

The following formula was used to calculate the viability of cell growth: Vialibity (%) = (mean of absorbance value of treatment group-blank)/(mean absorbance value of control-blank) × 100.

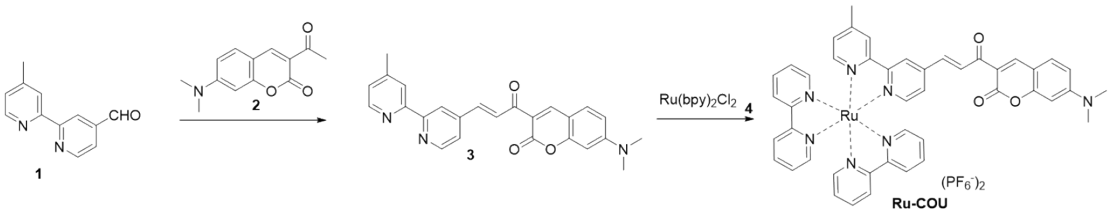
1.6 Imaging hydrazine hydrate in cells

Following culture in confocal dishes, 4T1 cells were treated with hydrazine hydrate (0, 10, 20, 30, and 50 μM) for 30 minutes. After an additional 2-hour incubation with **Ru-COU** (10 μM), the cells were subjected to confocal microscopy. λ_{ex} = 488 nm, λ_{em} = 570-660 nm.

1.7 Statistical analysis

Each bar in this paper represents the mean ± S.D. of at least three experiments. Statistical comparison between two groups was determined by using Sidak's multiple comparison test. All statistical analyses were conducted with Excel (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). A value of $p < 0.05$ was considered statistically significant.

2. Synthesis and characterization



Scheme S1. Reaction pathway for the synthesis of **Ru-COU**

Synthesis of Compound 3. Compound 1 (198.1 mg, 1 mmol) and compound 2 (231.1 mg, 1 mmol) were dissolved in 10 mL of H_2SO_4, stirring at 100°C for 12 hours. After the reaction completed, the solvent was cooled to room temperature. 10 mL of ice water was added to obtain the crude product. Then, the crude product was purified by column chromatography on silica gel (SiO_2, 200-300 mesh) eluted with CH_2Cl_2/CH_3OH (30/1, v/v) to obtain compound 3 as red solid. (289.2 mg, 70.3% yield). HRMS: 412.4437 $(M+H)^+$. ^1H NMR (600 MHz, $CDCl_3$): δ (ppm) = 8.71 (d, J = 6.0 Hz, 1H), 8.64-8.54 (m, 3H), 8.34 (d, J = 18.6 Hz, 1H), 8.25 (s, 1H), 7.80 (d, J = 18.6 Hz, 1H), 7.56 (dd, J_1 = 6.0 Hz, J_2 = 1.8 Hz, 1H), 7.47 (d, J = 10.8 Hz, 1H), 7.16 (d, J = 6.0 Hz, 1H), 6.67 (dd, J_1 = 10.8 Hz, J_2 = 3.0 Hz, 1H), 6.51 (d, J = 3.0 Hz, 1H), 3.15 (s, 6H), 2.46 (s, 3H).

Synthesis of Ru-COU. Compound 3 (41.1 mg, 0.1 mmol) and compound 4 (48.3 mg, 0.1 mmol) were dissolved in 5 mL of CH_3CH_2OH, stirring at 80°C for 12 hours. After the reaction completed, the solvent was cooled to room temperature. The crude product was purified by column chromatography on silica gel (SiO_2, 200-300 mesh) with a gradient of CH_3CN and a saturated aqueous solution of KNO_3 as the eluent. The targeted fractions were collected, and redissolved in H_2O. NH_4PF_6 solution was added to obtain **Ru-COU** as deep red solid (86.9 mg, 77.9% yield). HRMS: 970.1620 $(M-PF_6^-)^+$, 412.5975 $(M-2PF_6^-)^{2+}$. ^1H NMR (600 MHz, CD_3CN): δ (ppm) = 8.65 (s, 1H), 8.57-8.46 (m, 6H), 8.26 (d, J = 24.0 Hz, 1H), 8.07 (t, J = 12.0 Hz, 4H), 7.80 (d, J = 8.4 Hz, 1H), 7.77-7.66 (m, 5H), 7.61-7.54 (m, 3H), 7.46-7.38 (m, 4H), 7.28 (d, J = 9.0 Hz, 1H), 6.84-6.78 (m, 1H), 6.58-6.53 (m, 1H), 3.13 (s, 6H), 2.57 (s, 3H). ^{13}C NMR (150 MHz, CD_3CN): δ (ppm) = 185.75, 160.61, 158.62, 157.88, 157.06, 157.03, 156.97, 156.88, 156.22, 155.75, 152.00, 151.77, 151.73, 151.66, 151.63, 150.82, 150.66, 148.98,

144.32, 137.88, 137.83, 136.25, 132.08, 131.90, 128.62, 127.63, 125.37, 124.95, 124.30, 122.59, 116.08, 110.66, 108.60, 96.63, 39.77, 20.37. Elemental analysis calcd. (%) for $C_{45}H_{37}F_{12}RuN_7O_3P_2$: C 48.48, H 3.35, N 8.79; found (%): C 48.41, H 3.34, N 8.74.

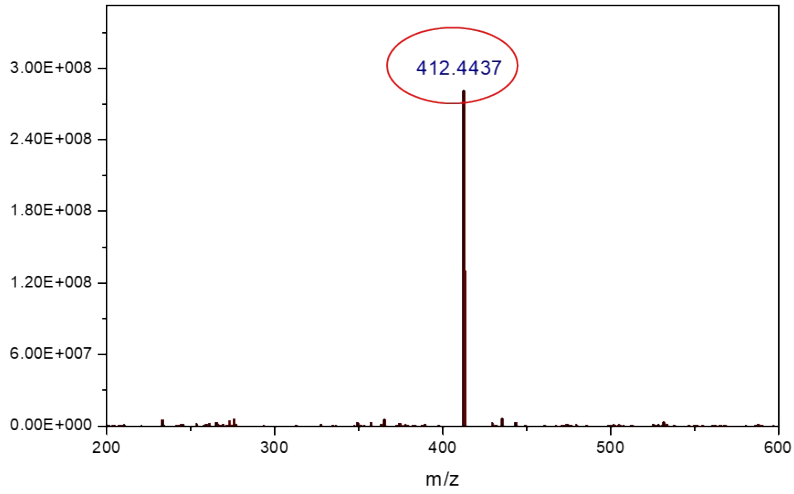


Fig. S1. HRMS (positive mode) of compound 3.

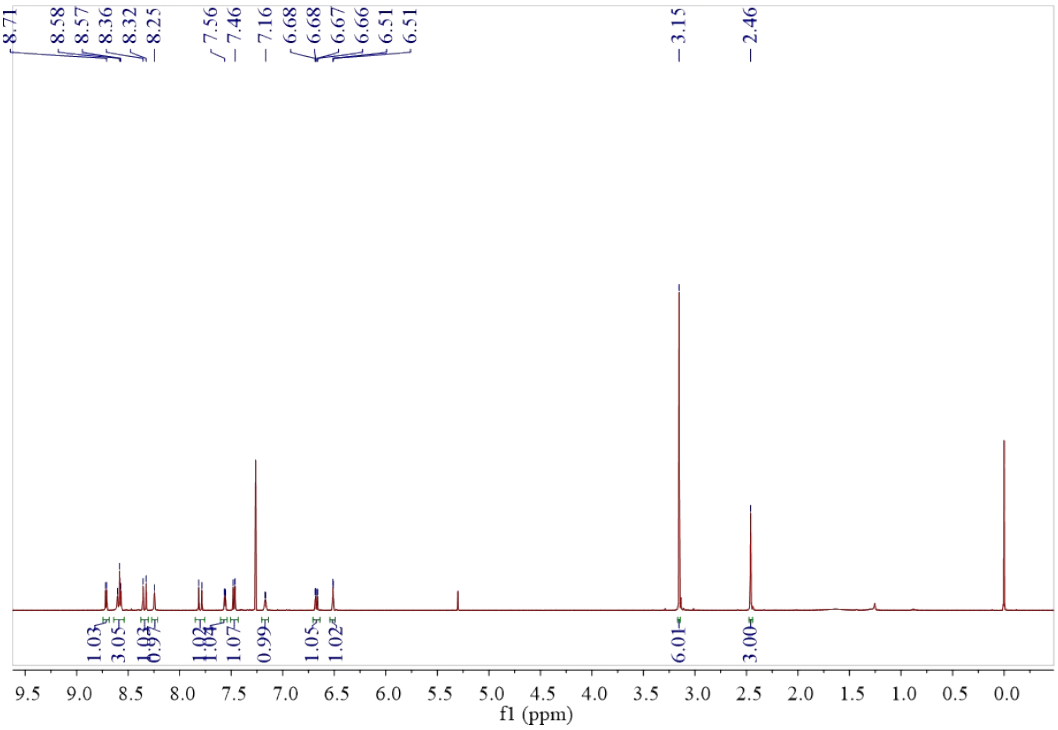


Fig. S2. ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of compound 3.

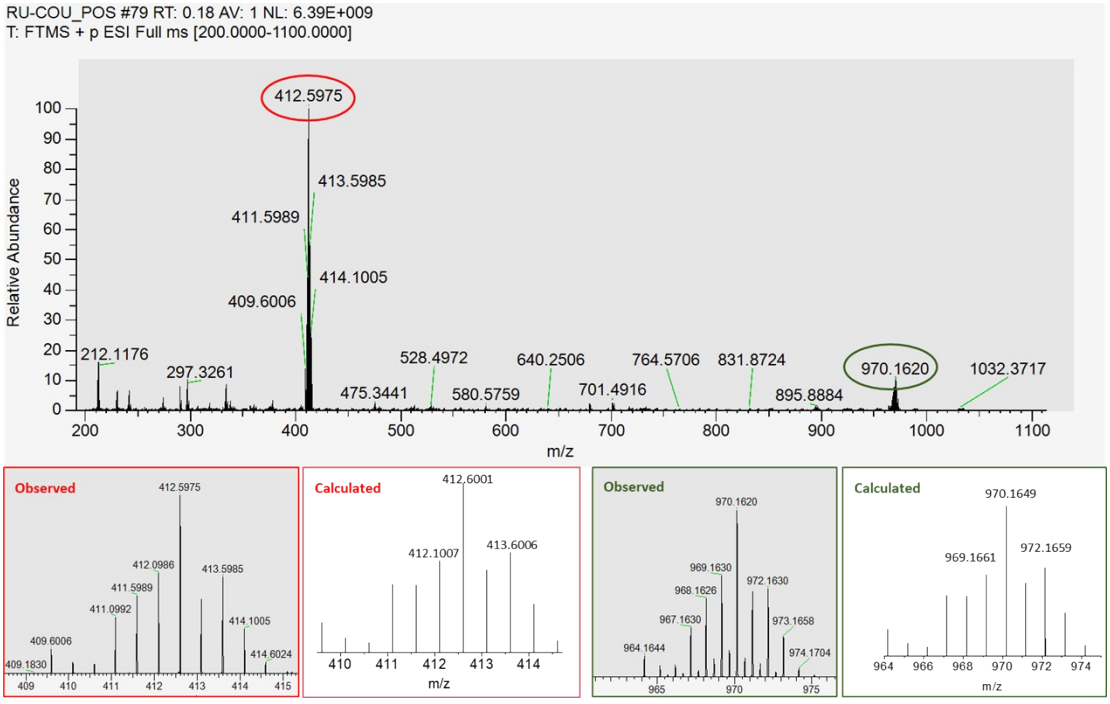


Fig. S3. HRMS (positive mode) of **Ru-COU**.

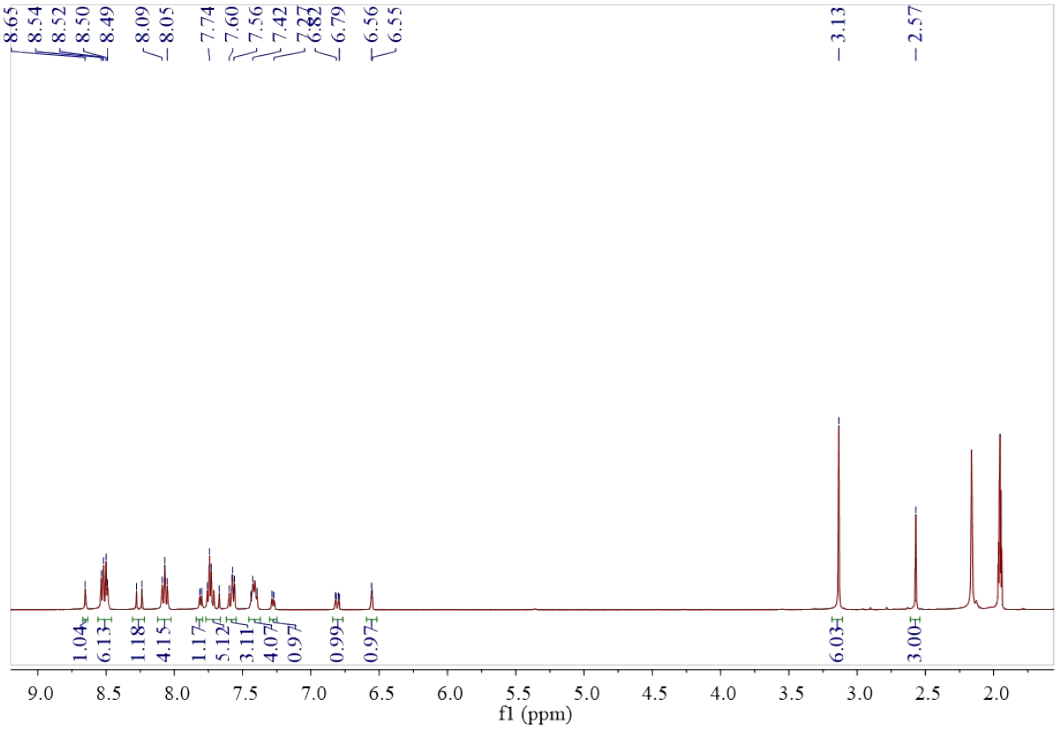


Fig. S4. ^{1}H NMR spectrum (600 MHz, CD_3CN) of **Ru-COU**.

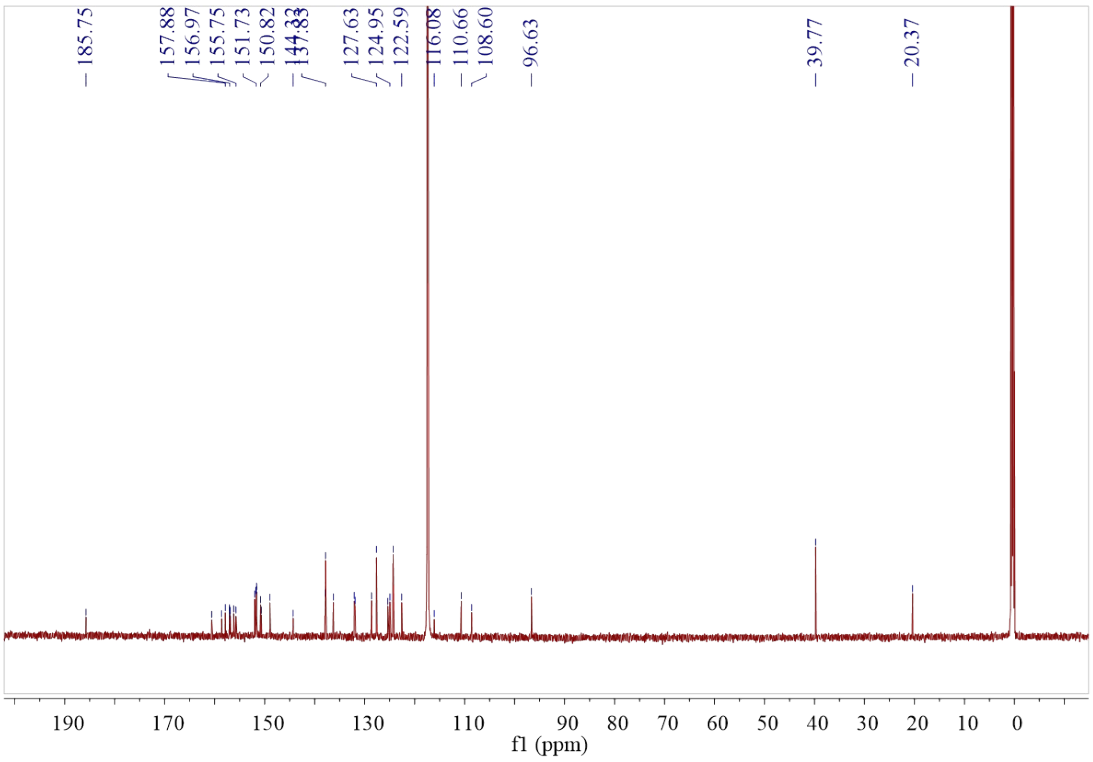


Fig. S5. ^{13}C NMR spectrum (150 MHz, CD_3CN) of **Ru-COU**.

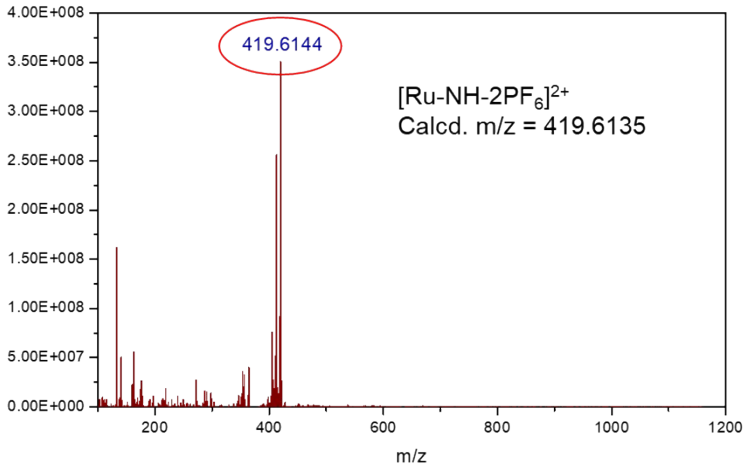


Fig. S6. HRMS analysis (positive mode) of the product of **Ru-COU** reacted with hydrazine hydrate, i.e., the formation of **Ru-NH**.

3. Detection of hydrazine hydrate in drugs and cells

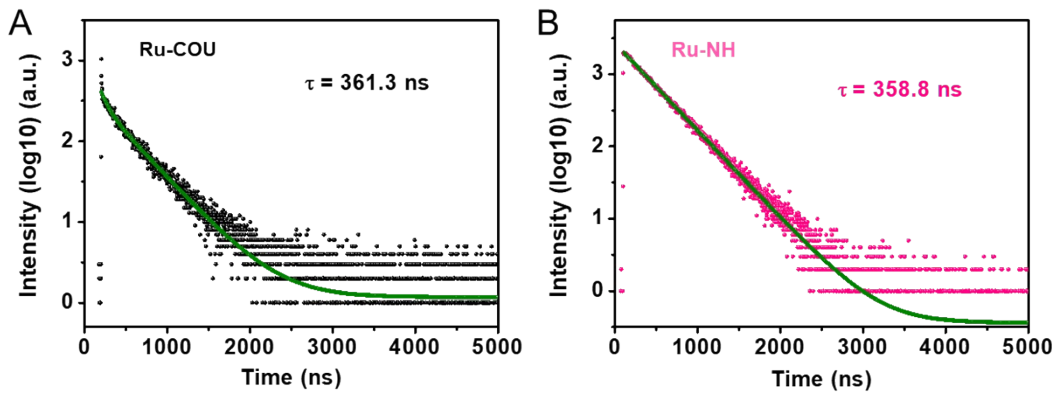


Fig. S7. Emission decay curves of **Ru-COU** before (A) and after (B) reacting with hydrazine hydrate in 10 mM PBS buffer at pH 7.4.

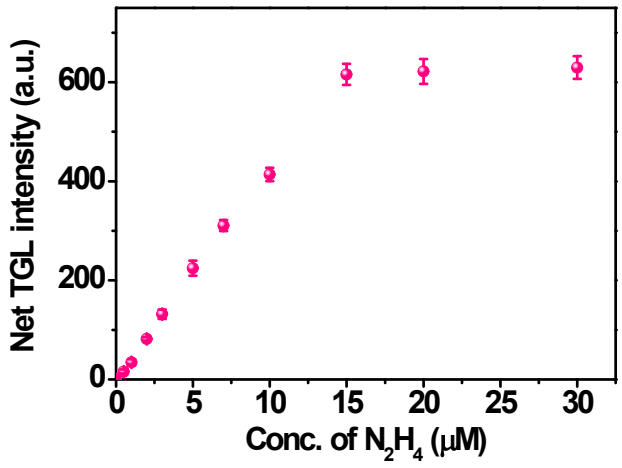


Fig. S8. Correlation of the net TGL intensity at 625 nm to the concentration of hydrazine hydrate (0-30 μM). The net TGL intensity was obtained by subtracting the blank signal (average of replicates, 9.25 a.u.) from the measured TGL intensity.

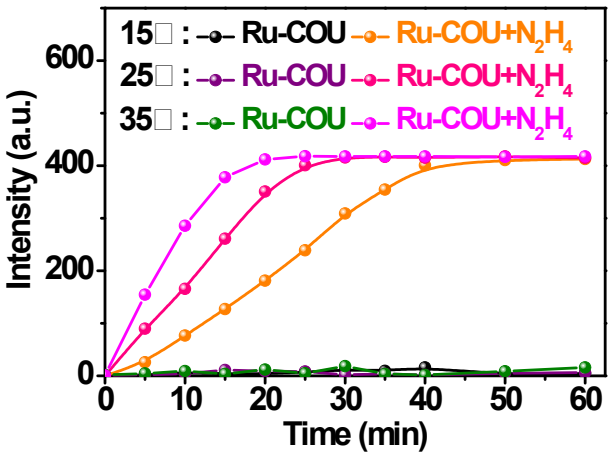


Fig. S9. TGL response kinetics of **Ru-COU** (10 μM) with hydrazine hydrate (10 μM) in 10 mM PBS buffer (pH 7.4) at different temperatures (15, 25, 35 °C).

Table S1. Quantification of hydrazine hydrate in drugs via TGL based on Fig. 1D

Samples	Drug concentration	Matrix	Found (μM)	CV (%, n = 3)
Riboflavin	100 μM	PBS (10 mM, pH 7.4, 10% DMSO)	0	2.31
Hydroquinine	100 μM	PBS (10 mM, pH 7.4, 10% DMSO)	0.006	1.65
Doxycycline	100 μM	PBS (10 mM, pH 7.4, 10% DMSO)	0.001	2.16
Tetracycline	100 μM	PBS (10 mM, pH 7.4, 10% DMSO)	0	2.55
Fluorescein sodium	100 μM	PBS (10 mM, pH 7.4, 10% DMSO)	0	3.12
Chloroquine	100 μM	PBS (10 mM, pH 7.4, 10% DMSO)	0	1.03
Ofloxacin	100 μM	PBS (10 mM, pH 7.4, 10% DMSO)	0	3.68
Chlorpromazine	100 μM	PBS (10 mM, pH 7.4, 10% DMSO)	0	2.19
Promethazine	100 μM	PBS (10 mM, pH 7.4, 10% DMSO)	0	2.86
Minocycline	100 μM	PBS (10 mM, pH 7.4, 10% DMSO)	0	1.36
Adriamycin	10 μM	PBS (10 mM, pH 7.4, 10% DMSO)	0.058	2.54

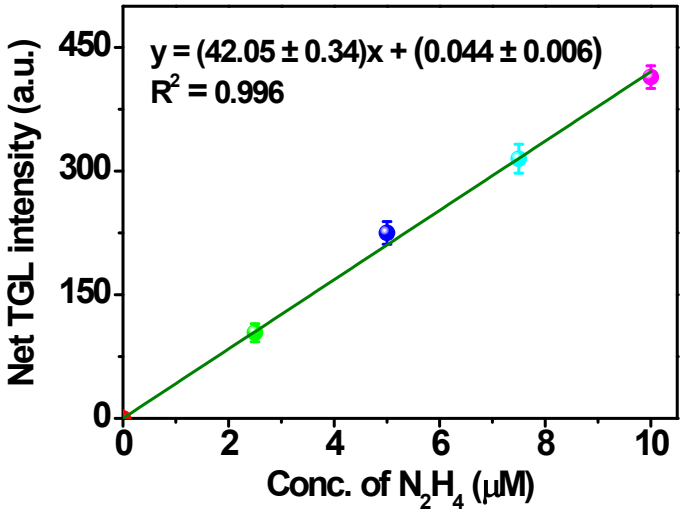


Fig. S10. Linear relationship between net TGL intensity and the spiked concentration of hydrazine hydrate in Fig. 3D.

Table S2. Quantification of hydrazine hydrate in drug, food and water samples via TGL based on Fig. 1D

Samples	Source	Dilution	Added (μM)	Found (μM, n =3)	CV (%, n = 3)	Recovery (%)
DOX	Doxorubicin hydrochloride (500 μM in PBS, Lunan Pharmaceutical Group Co., Ltd., Shandong, China)	50-fold in PBS (10 mM, pH 7.4, 10% DMSO)	-	0.058 ± 0.002	3.89	-
			2.5	2.53 ± 0.07	1.03	98.9
			5	5.0 ± 0.1	2.62	99.0
			7.5	7.6 ± 0.2	2.95	101.0
			10	10.2 ± 0.1	1.09	101.7
Wine	Changyu Cabernet Gernischt dry red wine (Yantai Changyu Pioneer Wine Company Ltd., Shandong, China)	5-fold in PBS (10 mM, pH 7.4, 10% DMSO)	-	-	-	-
			2.5	2.59 ± 0.03	1.61	103.6
			5	5.17 ± 0.09	1.57	103.4
			7.5	7.44 ± 0.06	0.89	99.2
			10	10.2 ± 0.3	2.82	101.7
Orange juice	Huiyuan 100% orange juice (Beijing Huiyuan Beverage & Food Group Co., Ltd., Beijing, China)	5-fold in PBS (10 mM, pH 7.4, 10% DMSO)	-	-	-	-
			2.5	2.5 ± 0.1	2.36	98.4
			5	5.1 ± 0.1	1.97	101.2
			7.5	7.5 ± 0.2	2.68	100.5
			10	10.3 ± 0.3	2.90	103.3
River water	Licun River (Licang District, Qingdao, China)	5-fold in PBS (10 mM, pH 7.4, 10% DMSO)	-	-	-	-
			2.5	2.6 ± 0.2	2.93	104.4
			5	4.9 ± 0.1	2.88	98.6
			7.5	7.55 ± 0.09	1.12	100.7
			10	9.9 ± 0.3	2.57	99.3
Lake water	Zhongxin Lake (Licang District, Qingdao, China)	5-fold in PBS (10 mM, pH 7.4, 10% DMSO)	-	-	-	-
			2.5	2.58 ± 0.03	0.71	103.2
			5	4.96 ± 0.09	1.91	99.2
			7.5	7.5 ± 0.1	2.01	100.3
			10	10.1 ± 0.3	2.71	100.8

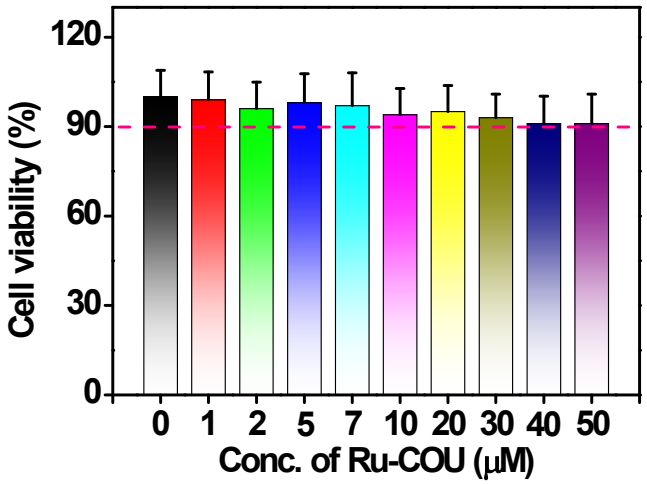


Fig. S11. Viabilities of 4T1 cells after incubated with different concentrations of **Ru-COU** for 24 h.

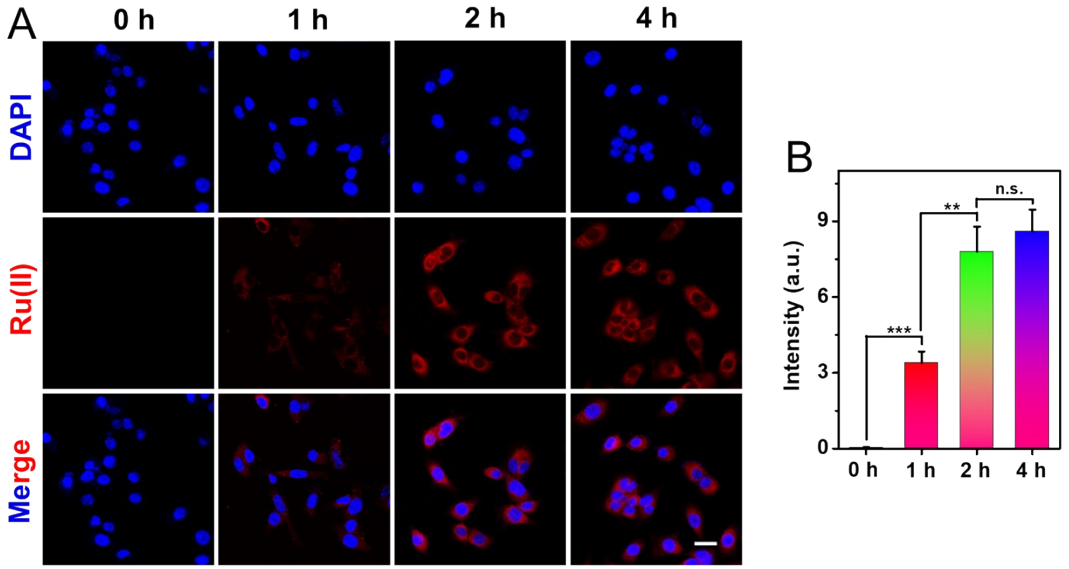


Fig. S12. Imaging hydrazine hydrate in 4T1 cells. (A) 4T1 cells were pretreated with hydrazine hydrate (50 μM, 30 min), followed by incubation with **Ru-COU** (10 μM) for varying durations, and then stained with DAPI. (B) Luminescence intensity in Ru(II) channel of (A). Ru(II) channel: λ_{ex} = 488 nm, λ_{em} = 570-660 nm. Scale bars: 20 μm. Data are presented as mean ± SD of three independent experiments. Statistical significance was determined by unpaired two-tailed Student's t-test. $^{**}p < 0.01$, $^{***}p < 0.001$, n.s. = not significant ($p \geq 0.05$).

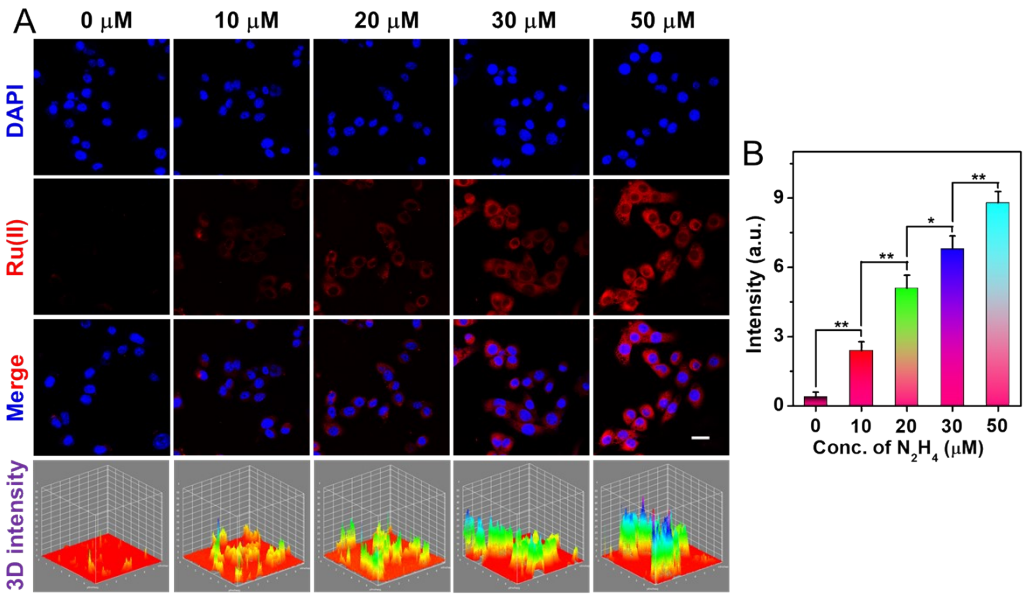


Fig. S13. (A) 4T1 cells were pretreated with different concentrations of hydrazine hydrate, followed by incubation with **Ru-COU** (10 μM, 2h), and then stained with DAPI. (B) Luminescence intensity in Ru(II) channel from (A). Ru(II) channel: λ_{ex} = 488 nm, λ_{em} = 570-660 nm. Scale bars: 20 μm. Data are presented as mean ± SD of three independent experiments. Statistical significance was determined by unpaired two-tailed Student's t-test. *$p < 0.05$ and **$p < 0.01$.

4. References

[1] Liu, C.; Zhang, R.; Zhang, W.; Liu, J.; Wang, Y.-L.; Du, Z.; Song, B.; Xu, Z. P.; Yuan, J. *J. Am. Chem. Soc.* **2019**, *141*, 8462-8472.

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[4] Liu, C.; Qin, M.; Jiang, L.; Shan, J.; Sun, Y. *Inorg. Chem.*, **2024**, *63*, 21627-21636.